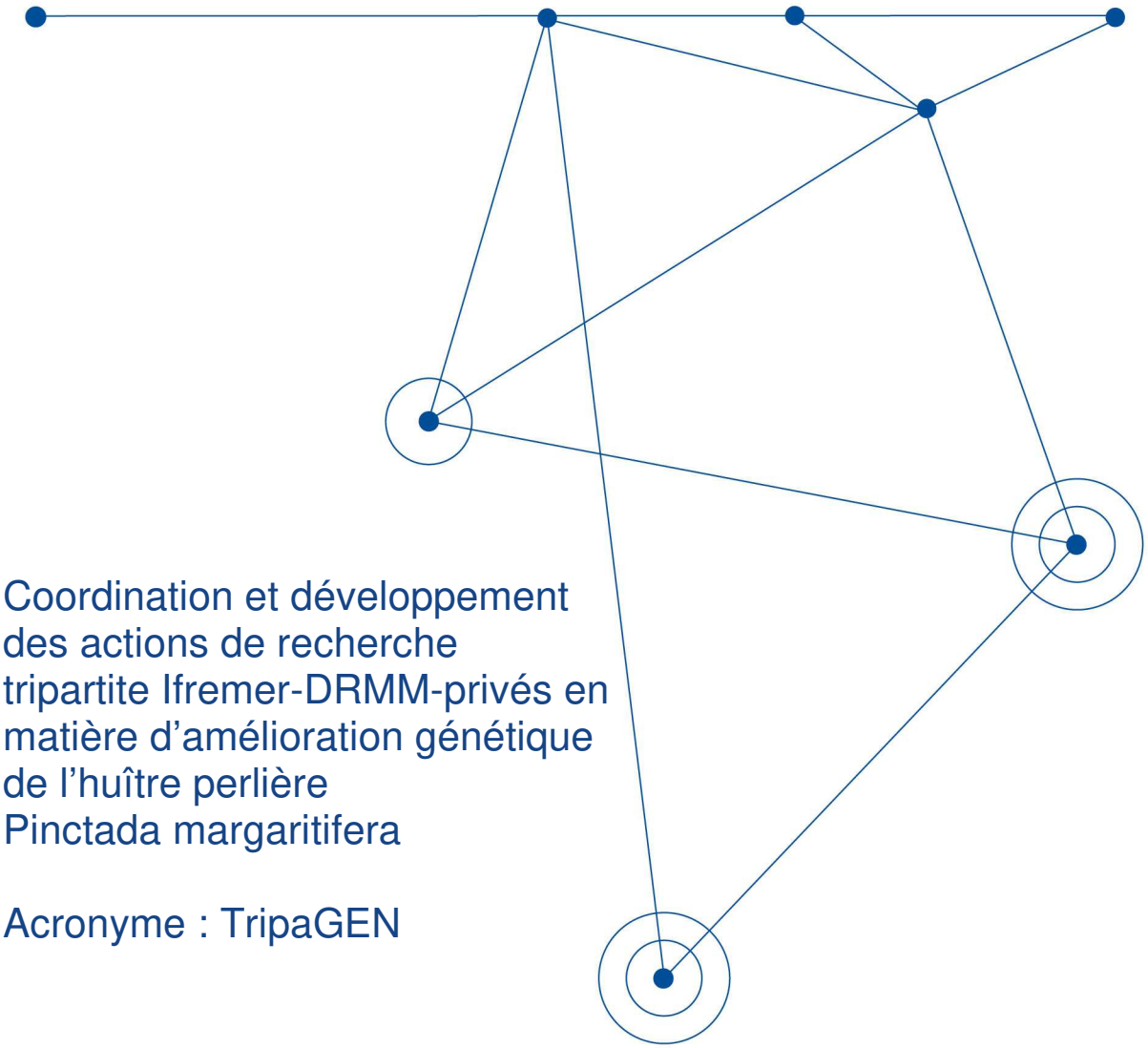
CONVENTION TRIPAGEN
RAPPORT INTERMEDIAIRE #2
N°/MDA/DRMM DU 11/2016



Coordination et développement des actions de recherche tripartite Ifremer-DRMM-privés en matière d'amélioration génétique de l'huître perlière Pinctada margaritifera

Acronyme : TripaGEN

RBE/ RMPF

Auteur KY Chin-Long

Novembre - 2018

RBE/ RMPF
Auteur : KY Chin-Long
Date : Novembre 2018

CONVENTION TRIPAGEN

RAPPORT INTERMEDIAIRE #2

N°9532/MEI/DRMM DU 06/12/2016

Coordination et développement des actions de recherche tripartite Ifremer-DRMM-privés en matière d'amélioration génétique de l'huître perlière *Pinctada margaritifera*
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Résumé/ Abstract : Les trois objectifs de la présente convention TripaGEN (Coordination et développement des actions de recherche tripartite Ifremer-DRMM-privés en matière d'amélioration génétique de l'huître perlière *Pinctada margaritifera*) sont : 1) La coordination des projets de recherche en matière d'amélioration génétique dans le cadre de l'ensemble des conventions de recherche Ifremer-DRMM-Privés (à compter de 2016 : AmeliGEN, ColoGEN, RikiGEN-2, et à compter de fin 2017 : MappyGEN) ; 2) La poursuite d'actions de recherche engagées en amélioration génétique dans le cadre du Marché négocié en perliculture 2013-2014 par le développement d'un outil d'aide à la sélection des huîtres donneuses de greffons et l'étude des relations greffe/sur-greffe ; 3) La participation au financement des deux thèses de recherche suivantes : "Influence de paramètres environnementaux sur les processus de minéralisation des perles de culture produites par l'huître perlière *Pinctada margaritifera*" et "Déterminisme génétique de caractères perlicoles d'intérêts chez l'huître perlière *Pinctada margaritifera* : du phénotype aux gènes".	
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Sommaire

1. Introduction

L'année 2018 est marquée par les publications des résultats acquis en lien aux thèses "affinage" et "génétique", qui se sont achevées en 2017, pour lesquelles TripaGEN fournissait le budget de fonctionnement (action D de TripaGEN). Les données en lien à l'action B (position du greffon) et C (relation greffe-surgreffe) ont aussi été valorisées. L'ensemble de ces publications figurent en annexe de ce rapport. L'année 2018 a aussi été marquée sur l'application de l'outil d'aide à la quantification de la couleur (subjective) des coquilles et perles (travaux de PL Stenger).

Le tableau 1 ci dessous illustre selon un découpage action par action, l'état d'avancement du projet sur les quatre années de son financement.

		2016				2017				2018				2019				avancée
A	Coordination																	en cours
B	Relation couleurs coquille - perle																	en cours
	Plasticité couleur coquille																	en cours
	Position du greffon																	réalisé
C	Relation greffe/ surgreffe																	réalisé
D	Thèse affinage (O. Latchere)																	réalisé
	Thèse génétique (C. Blay)																	réalisé

Tableau 1. Découpage des actions de recherche réalisées et en cours de la convention TripaGEN.

2. Coordination des projets

La coordination des projets de recherche en matière d'amélioration génétique et notamment pour le déploiement des opérations sur le terrain s'est poursuivi pour le bon fonctionnement de l'ensemble des conventions de recherche Ifremer-DRMM-Privés (AmeliGEN, ColoGEN, RikiGEN-2 et MappyGEN).

3. Outils d'aide à la sélection

3.1 Plasticité phénotypique de la couleur des coquilles et des perles

A l'aide de la chaîne méthodologique mise au point pour la qualification de la couleur des coquilles dans le cadre de la thèse de PL Stenger (rapport intermédiaire 2017), cette section présente les méthodologies, les résultats obtenus, dans le cadre du matériel biologique fourni dans le cadre d'une greffe expérimentale sur laquelle nous avons évalué la couleur des donneuses et la couleur des perles produites par ces donneuses.

Matériels et méthodes

Une greffe expérimentale a été réalisée en 2013 avec des donneuses de deux phénotypes de couleurs de *P. margaritifera* (rouge et vert) élevées à 4 et 30 m de profondeur. Visuellement, des différences de couleur ont été observées chez les donneuses vertes et rouges entre les deux groupes de profondeur. Ces quatre groupes ont été utilisés comme donneuses dans une greffe expérimentale. Après la récolte, des différences de couleur ont été de nouveau observées visuellement sur les différents groupes de perles. Nous avons décidé d'analyser ces variations de couleur en utilisant un espace colorimétrique approprié de manière automatisée.

Conditionnement animal et greffe expérimentale

Deux phénotypes de *P. margaritifera*, avec coloration de coquille interne verte ou rouge, ont été sélectionnés comme donneuses pour une greffe expérimentale (Figure 1). Les individus verts provenaient de l'île de Mangareva (archipel des Gambier, Polynésie française) et le phénotype rouge de l'atoll de Takaroa (archipel des Tuamotu, Polynésie française). Les huîtres rouges ont été transférées par avion à Mangareva un mois avant la greffe pour permettre l'acclimatation. Après un mois, les futurs donneuses de chaque phénotype ont été séparés en deux groupes : un groupe a ensuite été élevé à 4 m de profondeur (N = 9 pour le phénotype vert ; N = 5 pour le phénotype rouge) et l'autre à 30 m de profondeur (N = 6 pour le phénotype vert) ; N = 7 pour le phénotype rouge) pendant un mois supplémentaire afin d'obtenir une variation de leur couleur de coquille interne (donneuses finales).
Après le deuxième mois, les huîtres ont été collectées et utilisées dans une greffe expérimentale. Les huîtres receveuses utilisées avaient environ deux ans, et ont été collectées sous forme de naissain dans le lagon de l'île de Mangareva (archipel des Gambier, Polynésie française). Des techniques de capture passive pour les naissains avec des collecteurs commerciaux ont été utilisées.
La greffe expérimentale utilisant ces donneuses a été réalisée à la ferme perlière de la société Regahiga (archipel des Gambier, Polynésie française) en décembre 2013. La greffe expérimentale a utilisé 30 huîtres receveuses (poids du nucléus : 0,24 g; diamètre du noyau: 0,56 mm) (Figure 1). Les huîtres receveuses et donneuses mesuraient 12 cm de hauteur du bas vers le haut de leurs coquilles. Les coquilles d'huîtres donneuses ont été conservées pour analyse chromatique.
Toutes les huîtres receveuses ont été étiquetées individuellement (avec des étiquettes en plastique numérotées et codées par couleur) afin de maintenir la traçabilité entre l'identité des donneuses et des perles récoltées correspondantes. Toutes les huîtres receveuses greffées ont ensuite été

cultivées à 4 m de profondeur. Conformément aux pratiques aquacoles habituelles, et les huîtres ont été régulièrement nettoyées afin d'éliminer l'encrassement biologique (épibiontes), qui peuvent nuire à la croissance saine de l'huître et à la production de perles.
Après 42 jours, la rétention du nucléus a été vérifiée. Enfin, les perles ont été récoltées 20 mois plus tard et nettoyées par ultrasons à l'eau savonneuse avec un nettoyant de laboratoire (LEO 801, capacité 2 L, 80 W, 46 kHz) ; elles ont ensuite été rincées à l'eau distillée et évaluées pour l'analyse chromatique.

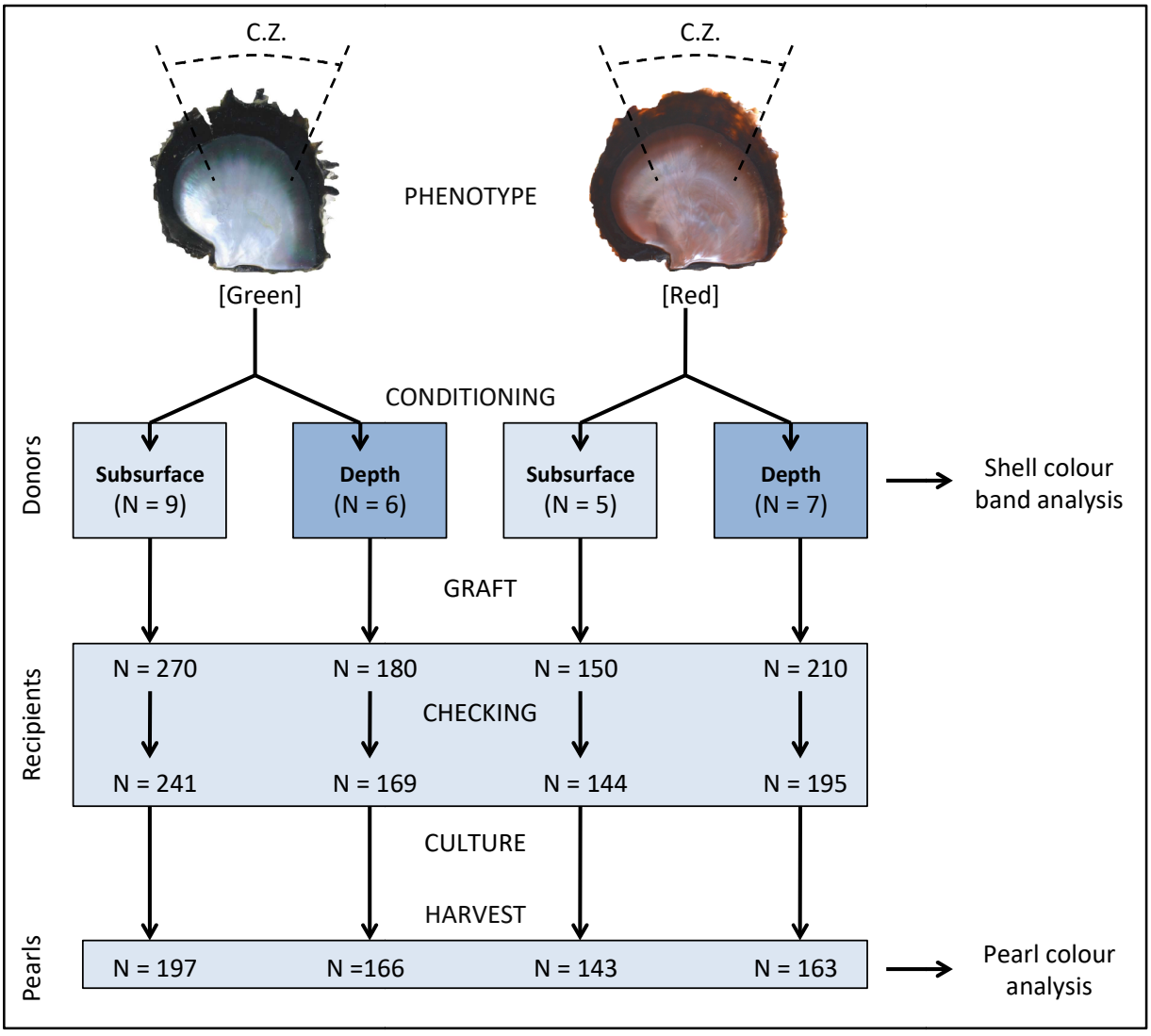


Figure 1. Procédure expérimentale de greffage des huîtres *Pinctada margaritifera* utilisant deux phénotypes d'huîtres donneuses : le vert et le rouge. Chaque phénotype a été conditionné pendant un mois avant l'opération de greffe, soit un élevage sous la surface (4 m) et une culture en profondeur (30 m). La zone commerciale (C.Z.), indiquée par les lignes pointillées, est la section du manteau de la donneuse à partir de laquelle les greffes sont habituellement coupées. Trente greffes ont été réalisées à partir de chaque donneuse. Une vérification de la rétention du noyau a été effectuée 42 jours après la greffe et la récolte de perles a eu lieu 20 mois après la greffe. Les nombres entre parenthèses correspondent aux fréquences des donneuses, des receveuses ou de perles.

Le choix de l'espace colorimétrique

Selon Vezhnevets *et al*. (2003), lors de l'élaboration d'une étude utilisant la couleur comme caractéristique principale, on se trouve généralement confronté à trois problèmes principaux : le choix de l'espace colorimétrique qui doit être pertinent pour le projet; les moyens d'obtenir et de modéliser une distribution des couleurs pour le modèle biologique ; et enfin, le choix de la manière de traiter la segmentation de la couleur qui permet d'obtenir une caractérisation et une quantification valides et reproductibles de la couleur.
Pour réduire la subjectivité de ce trait, différents espaces colorimétriques peuvent être utilisés, pour passer de données qualitatives à des données quantitatives. Parmi les espaces colorimétriques, l'espace HSL (Hue Saturation Lightness) commence à être utilisé en sciences biologiques car il peut apporter une solution en décrivant les composantes colorimétriques séparément.
HSV est un espace colorimétrique similaire à HSL et les deux sont utilisés comme moyen pour représenter la variation de couleur.
HSL et HSV ont deux géométries cylindriques, avec la teinte (H) décrivant le spectre de couleurs sur un disque chromatique. Toutefois, la saturation (S) est calculée différemment entre ces deux espaces (avec une conversion possible entre les deux valeurs) et la clarté (L) et la valeur (V) considèrent différents aspects de la couleur : Fairchild (2013) décrit la valeur (V) comme étant une perception de la quantité de lumière et la luminosité (L) comme une perception de la quantité de blanc. V et L sont tous deux donnés en pourcentages.
L'intuitivité de ces espaces à deux valeurs de couleur et à la discrimination explicite entre les propriétés S et L ou S et V ont rendu ces approches populaires dans les études sur la segmentation des couleurs. Cependant, ces espaces colorimétriques ne sont pas sans défauts. L'espace HSL, par exemple, a une interaction maladroite entre la luminosité et la saturation pendant le traitement de l'image. En effet, pour une valeur de luminosité maximale, la saturation donne toujours des données en blanc, alors que ce problème n'apparaît pas avec l'espace couleur HSV, ce qui donne une valeur plus proche de la vision humaine.
Nous avons donc choisi l'espace HSV pour analyser les variations chromatiques des coquilles internes d'huîtres et des perles.

Analyse chromatique des coquilles et des perles

Les coquilles des huîtres donneuses et des perles récoltées ont été nettoyées, conservées et protégées de la lumière. Les perles ont été mises dans des boîtes et classées par leurs donneuses respectives. Les donneuses et les perles en boîte ont été photographiées avec un Canon® PowerShot G9, avec une résolution maximale de 12,1 mégapixels et avec les mêmes paramètres pour chaque image. Les images ont été prises dans un Packshot Creator ™ (version 3.0.3.8) pour éviter les ombres sombres et la réflexion de la lumière. Les images des coquilles internes des donneuses ont été coupées pour extraire la zone périphérique colorée, qui a été collée sur un fond blanc. De même, les photographies de perles ont été découpées de manière à ne conserver que la sphère colorée et celles-ci ont été collées sur un fond blanc.
Nous avons sélectionné un côté de la perle (au hasard) à photographier et l'utilisons pour représenter la couleur de la perle.
Le logiciel gratuit GNU Image Manipulation Program (version 2.8.22) a été utilisé pour sélectionner la zone de couleur (sélection au lasso, copier, coller comme une image, exporter au format .jpeg).
Le logiciel R v 3.2.3 (base R du calcul statistique) a été utilisé pour développer un package d'analyse d'images, baptisé ImaginR, et pour l'exécuter.

Pour le reste de l'analyse, le répertoire de travail est défini et le dossier contenant toutes les images est placé à l'intérieur. Après avoir chargé le package ImaginR (V2), la seule fonction nécessaire pour exécuter l'analyse est appelée OutPutResult. En tapant simplement OutPutResult, R reconnaît le dossier d'images et effectuera automatiquement l'analyse. ImaginR importera toutes les images avec l'extension jpeg et listera les noms de fichiers dans un objet R. Ainsi, le nom de l'image (qui correspond à un échantillon) est stocké dans cet objet. L'analyse sera donc faite image par image. L'image est importée via la fonction load.image du package ImaginR. Ensuite, chaque pixel reçoit un codage RGB, qui sera converti en code de triplet hexadécimal (toutes les conversions sont réalisées avec le package R grDevices) et comparé à une base de données de triplet hexadécimal blanc. Les valeurs de triplet hexagonal blanches des images sont donc supprimées afin de supprimer toute information dérivée de l'arrière-plan de l'image. Les pixels restants sont reconvertis en une matrice RGB et une moyenne est calculée pour chaque chenal (R, G et B). La couleur moyenne de la zone chromatique de chaque échantillon est alors obtenue. Le code de triplet hexadécimal est également calculé à partir de cette moyenne. Le RGB moyen est converti en code HSV avec la fonction rgb2hsv du package grDevices. Ainsi, pour une image, la teinte (H), la saturation (S) et la valeur (V) fournissent une synthèse de l'état de la couleur. Le paramètre teinte (H) peut ensuite être utilisé dans un cadre d'apprentissage automatique et comparé à une base de données de référence pour classifier l'échantillon selon un phénotype connu. La base de données de référence a été construite en utilisant les deux valves de cinq individus de chacun des phénotypes contrastés majeurs d'intérêt. Ces huîtres perlières de la base de données ImaginR sont issues d'un programme de sélection de couleurs par la SCA Regahiga Pearls (île de Mangareva, archipel des Gambier, Polynésie française). Enfin, le package ImaginR donnera la teinte (H), la saturation (S), la valeur (V), le code de triplet hexagonal moyen et le phénotype de couleur interprété de chaque échantillon («vert», «rouge» ou «autre»). Cette tâche est mise en boucle sur tous les échantillons / images du dossier et le package produit un fichier tabulaire final résumant toutes les informations détaillées ci-dessus, ainsi que le nom de l'échantillon. Le fichier texte est enregistré au format .csv.

Pour les analyses statistiques ultérieures, le regroupement des données de perles récoltées a été réalisé de deux manières : i) toutes les perles ont été regroupées en fonction de la couleur du phénotype de la coquille interne du donneur, caractérisées avec ImaginR (le trait n'étant que partiellement hérité génétiquement, avec des phénotypes de couleur différents ont été regroupés dans la même catégorie) ; ii) seules les perles vertes des donneurs verts et les perles rouges des donneurs rouges ont été analysées.

Analyses statistiques

Pour l'étude expérimentale, nous avons effectué plusieurs comparaisons par paires afin de répondre à deux questions biologiques principales : (i) Y at-il une différence significative entre la couleur des coquilles de l'huître donneuse cultivée à 4 m de profondeur et à 30 m de profondeur ; et (ii) Pour chaque phénotype, existe-t-il une différence de couleur entre les perles provenant de donneuses cultivés à 4 m et 30 m? Un test Shapiro (package R Stats v3.5.0) a été utilisé pour vérifier la distribution normale des données. Pour tester la présence d'une différence de valeur et de saturation entre les groupes, nous avons utilisé un test de Wilcoxon (package R Stats v3.5.0 basé sur Hollander and Wolfe 1973 et Patrick Royston 1995) et un intervalle de confiance fondé sur Bauer (1972).

Des tests du Chi2 ont été effectués (package R Stats v3.5.0) sur le nombre de perles vertes (ou rouges) obtenues à partir de donneuses d'huîtres vertes (ou rouges) pour la profondeur d'élevage

des donneuses divisées par le nombre total de perles par la profondeur d'élevage, afin de déterminer lequel des phénotypes de couleur avait produit le plus grand nombre de perles de la même couleur.

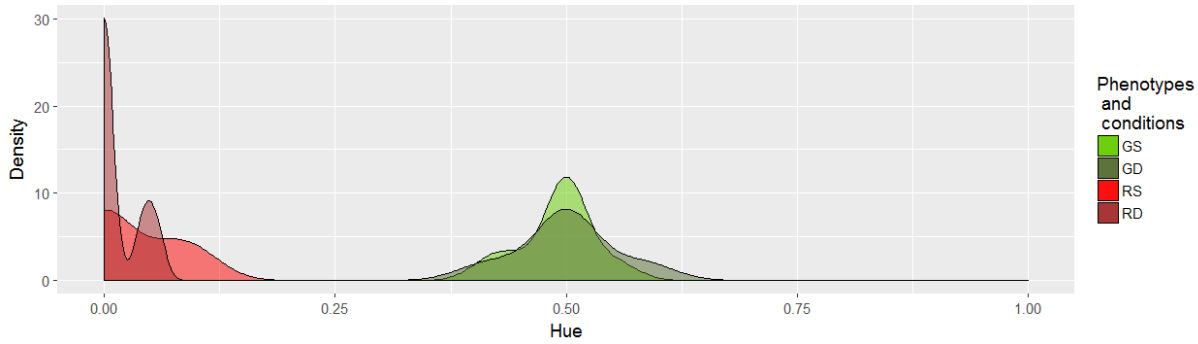
Résultats

Les couleurs de 669 perles issues de deux phénotypes de couleur (donneuses vertes et rouges) et cultivées à deux profondeurs (4 ou 30 m) ont été analysées afin de créer une base de référence pour mesurer la stabilité de la couleur. Le taux moyen de rétention du nucléus lors de la greffe expérimentale était de 93,0% (N = 749) 42 jours après la greffe. Après 20 mois de culture, les perles ont été récoltées avec succès chez 89,9% des individus initialement greffés (N = 669). La différence (3,1%) correspond au rejet du noyau après le 42e jour et à la mortalité des huîtres. Les nombres de perles récoltées par classe de couleur de donneuse et par groupe de profondeur sont les suivants : 363 perles pour le phénotype vert (197 pour le groupe à 4 m et 166 pour le groupe à 30 m) et 306 perles pour le phénotype rouge (143 groupe à 4 m et 163 pour le groupe à 30 m).

Valeurs de teinte (H – hue) pour les couleurs de coquilles internes des huîtres et les perles de culture

Les distributions de teintes pour les phénotypes de coquille et les conditions d'élevage ont révélé quatre teintes dominantes chez les donneuses vertes élevées à 4 m de profondeur (GS – green surface) (0,500 ; 0,555 ; 0,444 ; 0,4166), trois pour les donneuses vertes élevées à 30 m (GD – green depth) (0,500 ; 0,583 ; 0,416), trois pour les donneuses rouges élevées à 4 m (RS – red surface) (0,000 ; 0,066 ; 0,100) et trois pour les donneuses rouges élevées à 30 m (RD – red depth) (0,000; 0,055 ; 0,041) (Fig.2a).
Au total, 138 teintes différentes ont été trouvées parmi les 363 perles issues des donneuses vertes et 185 teintes pour les 306 perles issues de donneuses rouges.
Certaines perles de donneuses vertes élevées à 4 m (PGDS – Pearls from Green Donors in Surface) et 30 m (PGDD - Pearls from Green Donors in Depth) partagent les mêmes teintes, car l'indice de diversité (rapport entre le nombre de teintes sur le nombre de perles) pour le total des PGD (0,38) est inférieur à celui des Indices de diversité PGDS (0,43) et PGDD (0,59). L'indice de diversité de teinte est supérieur pour le phénotype rouge (0,60 au total) par rapport au vert, même si le phénotype rouge des donneuses partage également des teintes entre des perles de donneuse conditionnés à 4 m (PRDS – Pearls from Red Donors in Surface) (0,72) et des perles de donneurs conditionnés à 30 m (PRDD – Pearls from Red donors in depth) (0,69).
Statistiquement, nous avons observé plus de perles vertes de donneuses vertes lorsque les donneuses avaient été élevés à 4 m (GPGS : 67%, test Chi2 $p < 0,001$) que de perles rouges de donneuses rouges conservés à la même profondeur (RPRS : 58,7%, test Chi2 < 0,001).
Cependant, il y avait moins de perles vertes provenant de donneuses verts cultivées à 30 m (GPGD - 39,6%) que de perles rouges provenant de donneuses rouges élevés à la même profondeur (RPRD - 72,4%). En additionnant les GPGS aux GPGD (53,3%) et les RPRS aux RPRD (65,55%), il apparaît que, dans l'ensemble, les donneuses rouges ont donné plus de perles rouges que les donneuses vertes ont donné des perles vertes (test de Chi2 $p < 0,005$).

a) Coquilles



b) Perles cultivées

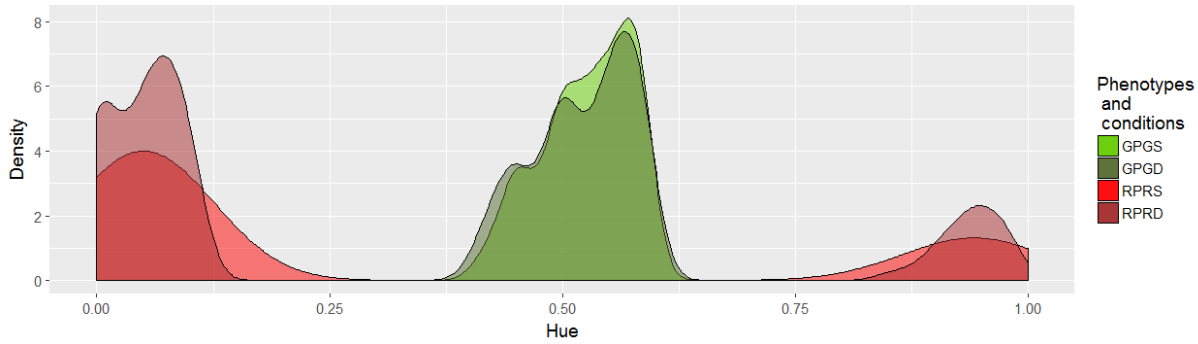


Figure 2. Répartition de la densité de teinte de *P. margaritifera* pour : (a) coquilles d'huîtres provenant de : GS (donneuses vertes élevées à 4 m ; N = 9), GD (donneuses vertes élevées à 30 m ; N = 6); RS (donneuses rouges élevées à 4 m; N = 5) et RD (donneuses rouges élevées à 30 m ; N = 7), et (b) perles de culture associées aux donneuses (a), avec GPGS (les perles vertes provenant des donneuses vertes élevées à 4 m; N = 132), les GPGD (perles vertes de donneuses vertes élevées à 30 m ; N = 66), RPRS (perles rouges de donneuses rouges élevées à 4 m ; N = 84) et RPRD (perles rouges de donneuses rouges élevées à 30 m ; N = 118). Les distributions vertes claire (ou rouge claires) et verte foncée (ou rouge foncée) correspondent au conditionnement des donneuses lors de la culture sous la surface (4 m) ou profonde (30 m) respectivement, avant les opérations de greffe.

L'analyse de la saturation (S) pour les couleurs de coquilles internes des huîtres et les perles de culture

La distribution de la couleur des coquilles interne des huîtres des donneuses s'est déplacée vers une saturation faible pour le groupe de profondeur (30 m) par rapport au groupe des 4 m (Fig. 3a).
Lorsque toutes les perles de culture sont considérées, les échantillons des donneuses vertes (p < 0,05) et rouges (p < 0,05) présente un décalage significatif de la saturation vers des valeurs plus faible de luminosité de la couleur après un conditionnement en profondeur (30 m) par rapport au conditionnement en profondeur (4 m) (Fig. 3b).

Lorsque seules les perles rouges des donneuses rouges et les perles vertes des donneuses vertes ont été prises en compte, la distribution de la saturation a également été significativement décalée vers une saturation plus faible avec une profondeur plus grande pour les RPRD uniquement (p < 0,005 et p = 0,2364, respectivement).
Ces résultats sont similaires lorsque toutes les perles de culture ont été prises en compte, mais les différences entre les profondeurs sont 140,8 fois plus fortes pour le phénotype rouge (Fig. 3c).
En termes de saturation, la couleur de la coquille interne de l'huître est donc devenue moins intense et moins brillante avec la profondeur.

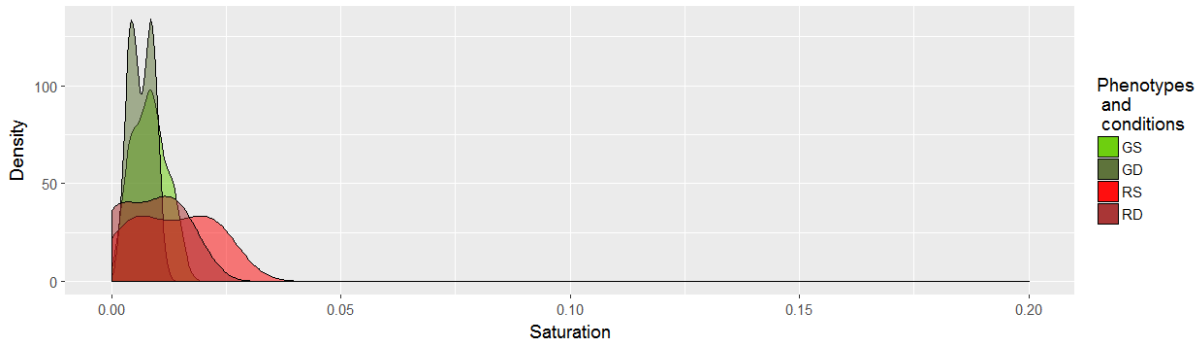
Foncitude (V - value) de la coquille intérieure des huîtres donneuses et des perles de culture

En ce qui concerne la couleur interne des coquilles d'huîtres, la valeur V des distributions ont été décalées vers des niveaux plus élevés de foncitude dans les échantillons conditionnés à 30 m de profondeur par rapport à ceux conditionnés à 4 m de profondeur (Fig. 4a).

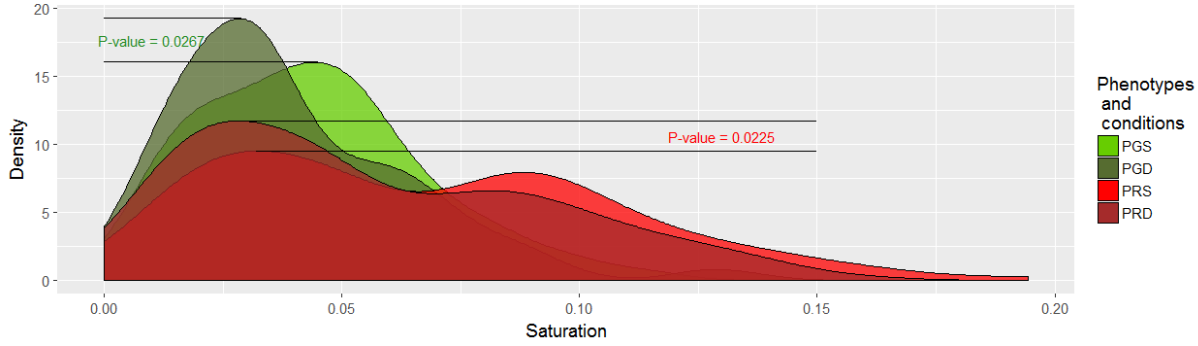
Lorsque toutes les perles de culture ont été prises en compte, les échantillons provenant de donneuses vertes (p < 0,001) et rouges (p = 0,014) ont présenté un passage significatif à des valeurs plus élevées de V après un conditionnement plus profond (30 m) par rapport à un conditionnement en surface (4 m). (Fig. 4b)

Lorsque les perles rouges des donneuses rouges (p < 0,005) et les perles vertes des donneuses vertes (p = 0,3682) sont considérées séparément, la distribution des valeurs de V passe de manière significative à une forte foncitude avec la profondeur, mais cette différence n'est pas significative pour les perles vertes (Fig. 4c). Les différences des valeurs V entre les profondeurs sont 5,07 fois plus importantes pour le phénotype rouge considéré seul. En termes de noirceur, la couleur de la coquille interne de l'huître est devenue plus grise et terne à une plus grande profondeur de conditionnement.

a) Coquilles



b) Toutes les perles de culture



c) Seules les perles rouges et vertes des donneuses rouges et vertes respectivement

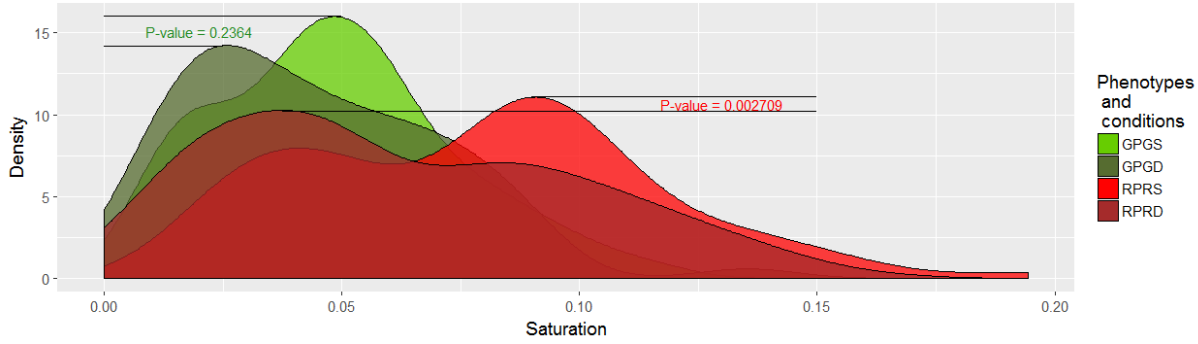
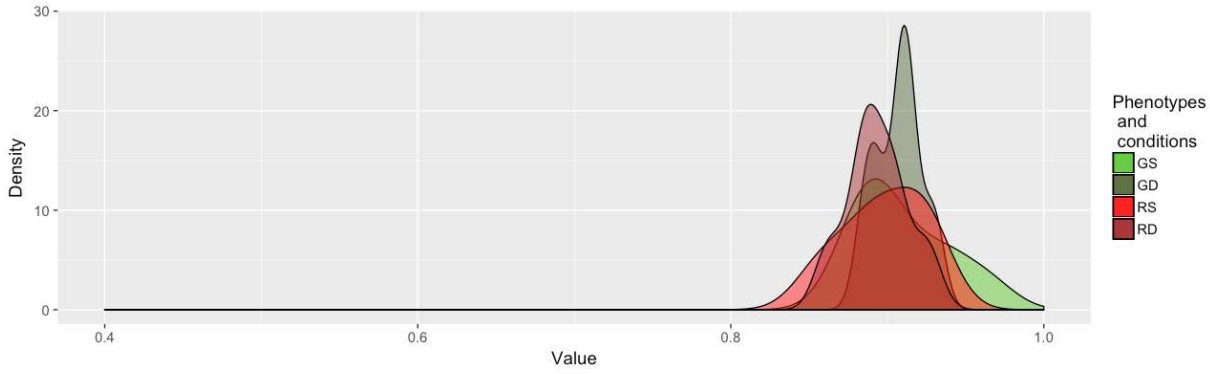
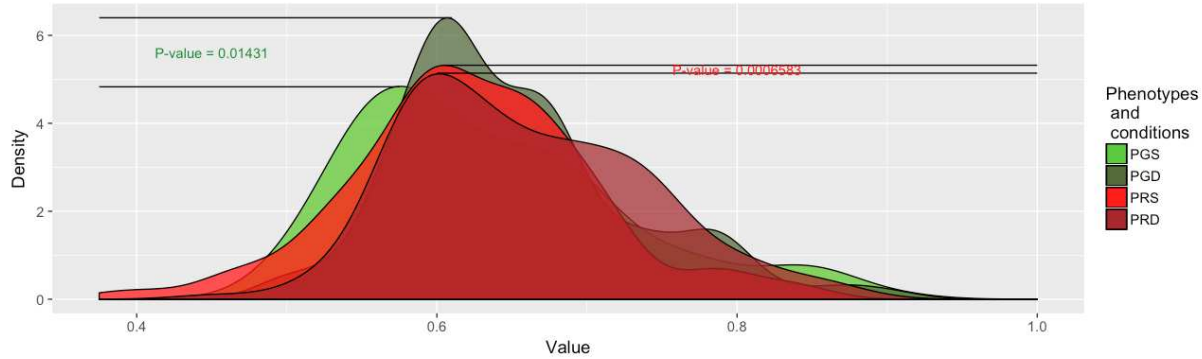


Figure 3. Diagramme de densité de saturation de *P. margaritifera* pour : (a) coquilles d'huîtres provenant de : GS (donneuses vertes élevées à 4 m ; N = 9), GD (donneuses vertes élevées à 30 m de profondeur; N = 6), SR (donneuses rouges élevées à 4 m ; N = 5), et RD (donneuses rouges élevées à 30 m ; N = 7), (b) perles de culture multicolores associées aux donneuses (a), avec PGDS (les perles provenant des donneuses vertes élevées à 4 m ; N = 197) et PGDD (perles de donneuses vertes élevées à 30 m ; N = 166); PRDS (perles provenant des donneuses rouges élevées à 4 m ; N = 143) et PRDD (perles provenant des donneuses rouges élevées à 30 m ; N = 163), (c) perles de culture qui présentent la même teinte de couleur que leurs donneuses correspondantes, GPGS (perles vertes provenant de donneuses vertes élevées à 4 m ; N = 132), GPGD (perles vertes provenant de donneuses vertes élevées à 30 m ; N = 66), RPRS (perles rouges issues de donneuses rouges élevées à 4 m ; N = 84) et RPRD (perles rouges provenant des donneuses rouges élevées à 30 m ; N = 118). Les distributions vertes claire (ou rouge claires) et verte foncée (ou rouge foncée) correspondent au conditionnement des donneuses lors de la culture sous la surface (4 m) ou profonde (30 m) respectivement, avant les opérations de greffe.

a) coquilles



b) perles de culture



c) seules les perles rouges et vertes des donneuses rouges et vertes respectivement

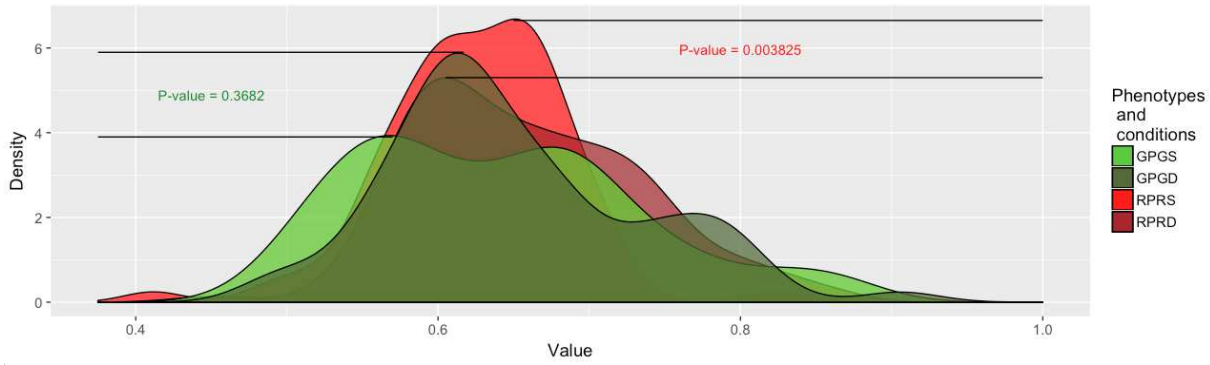


Figure 4. Distribution de la densité de valeur V (foncitude) de *P. margaritifera* pour : (a) coquille d'huître provenant de GS (donneuses vertes élevées à 4 m; N = 9), GD (donneuses vertes élevées à 30 m; N = 6), SR (donneuses rouges élevées à 4 m ; N = 5) et RD (donneuses rouges élevées à 30 m ; N = 7), (b) perles de culture pouvant avoir différentes couleurs: PGDS (perles provenant de donneuses vertes élevées à 4 m ; N = 197), PGDD (perles provenant de donneuses vertes élevées à 30 m ; N = 166), PRDS (perles provenant de donneuses rouges élevées à 4 m ; N = 143) et PPDD (les perles provenant des donneuses rouges élevées à 30 m ; N = 163), et (c) perles de culture de même couleur que leurs donneuses, GPGS (perles vertes provenant des donneuses vertes élevées à 4 m ; N = 132), les GPGD (perles vertes de donneuses vertes élevées à 30 m ; N = 66), RPRS (perles rouges provenant des donneuses rouges élevées à 4 m ; N = 84) et RPRD (perles rouges provenant des donneuses rouges élevées à 30 m ; N = 118). Les distributions vertes claire (ou rouge claires) et verte foncée (ou rouge foncée) correspondent au conditionnement des donneuses lors de la culture sous la surface (4 m) ou profonde (30 m) respectivement, avant les opérations de greffe.

3.2 Position du greffon

Des expérimentations ont été initiées dans le cadre de l'étude de l'influence du greffon et de sa position (sur le manteau de l'huître donneuse de greffon sélectionnées), sur les paramètres de qualité des perles et notamment l'expression de la couleur et de la taille. Les résultats ont été présentés dans le rapport intermédiaire #1 (2017) et valorisé en 2018 dans la publication (figurant en annexe de ce présent rapport) :

Ky Chin-Long, Quillien Virgile, Broustal Floriane, Soyez Claude, Devaux Dominique (2018). Phenome of pearl quality traits in the mollusc transplant model *Pinctada margaritifera*. Scientific Reports, 8, 2122 (1-11).

4. Etude des relations entre greffe et surgreffe

L'ensemble des résultats de cette action a été présenté dans le rapport intermédiaire #1 (2017) et ont fait l'objet de 2 publications scientifiques (en annexe du présent rapport) :

Demmer Jonathan, Cabral Philippe, Ky Chin-Long (2016). Comparison of harvested rate and nacre deposition parameters between cultured pearls issued from initial graft and second nucleus insertion in *P. margaritifera*. Aquaculture Research, 47(10), 3297-3306.

Ky Chin-Long, Demmer Jonathan, Blay Carole, Lo Cedrik (2017). Age-dependence of cultured pearl grade and colour in the black-lipped pearl oyster *Pinctada margaritifera*. Aquaculture Research, 48(3), 955-968.

5. Financement de thèses de recherche

Les deux thèses de recherche présentées ci-dessous sont achevées à ce jour. Les manuscrits associés ont été transmis à la DRMM. Il s'agissait des thèses de :

Carole BLAY
« Déterminisme génétique de caractères perlicoles d'intérêts chez l'huître perlière *Pinctada margaritifera* : du phénotype aux gènes ».
La thèse a été réalisée sur la période d'avril 2014 à juin 2017, sous les codirections du Docteur Chin-Long KY (Ifremer) et du Docteur Serge PLANES (Cnrs). La bourse doctorale a été cofinancée par l'Ifremer (50%), le Criobe et la DRMM. Le contrat doctoral a été géré par le Criobe-Cnrs. Cette thèse a été soutenue le 05/09/2017 à Perpignan et a fait l'objet d'une restitution à la DRMM le 25/09/2017 à Tahiti.

Oïhana LATCHERE
« Influence de paramètres environnementaux sur les processus de minéralisation des perles de culture produites par l'huître perlière *Pinctada margaritifera* ».
La thèse a été réalisée sur la période novembre 2013 à décembre 2017, sous la codirection du Professeur Nabila GAERTNER-MAZOUNI (Université de la Polynésie française) et du Docteur Denis SAULNIER (Ifremer). La bourse doctorale a été cofinancée par l'Ifremer et l'Université de la Polynésie française (UPF). Le contrat doctoral a été géré par l'UPF. Cette thèse a été soutenue le 7 décembre 2017.

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Annexe

Action B

Ky Chin-Long, Quillien Virgile, Broustal Floriane, Soyez Claude, Devaux Dominique (2018). Phenome of pearl quality traits in the mollusc transplant model *Pinctada margaritifera*. Scientific Reports, 8, 2122 (1-11).

Action C

Demmer Jonathan, Cabral Philippe, Ky Chin-Long (2016). Comparison of harvested rate and nacre deposition parameters between cultured pearls issued from initial graft and second nucleus insertion in *P. margaritifera*. Aquaculture Research, 47(10), 3297-3306.

Ky Chin-Long, Demmer Jonathan, Blay Carole, Lo Cedrik (2017). Age-dependence of cultured pearl grade and colour in the black-lipped pearl oyster *Pinctada margaritifera*. Aquaculture Research, 48(3), 955-968.

Action D :

Latchere Oihana, Fievet Julie, Lo Cedrik, Schneider Denis, Dieu Stephanie, Cabral Philippe, Belliard Corinne, Ky Chin-Long, Gueguen Yannick, Saulnier Denis (2016). Effect of electrolysis treatment on the biomineralization capacities of pearl oyster *Pinctada margaritifera* juveniles. Estuarine Coastal And Shelf Science, 182(Part.B), 235-242.

Latchere Oihana, Le Moullac Gilles, Gaertner-Mazouni Nabila, Fievet Julie, Magre Kevin, Saulnier Denis (2017). Influence of preoperative food and temperature conditions on pearl biogenesis in *Pinctada margaritifera*. Aquaculture, 479, 176-187.

Latchere Oihana, Mehn Vincent, Gaertner-Mazouni Nabila, Le Moullac Gilles, Fievet Julie, Belliard Corinne, Cabral Philippe, Saulnier Denis (2018). Influence of water temperature and food on the last stages of cultured pearl mineralization from the black-lip pearl oyster *Pinctada margaritifera*. Plos One, 13(3), e0193863 (19p.).

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Phenome of pearl quality traits in the mollusc transplant model *Pinctada margaritifera*

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The bivalve *Pinctada margaritifera* exhibits three main transplant phenotypes derived from the donor (from which a mantle graft tissue, the *saibo*, is excised), the recipient (into which the *saibo* is implanted with a nucleus, leading to the formation of a pearl sac "chimera") and the cultured pearls themselves. This first phenome study on the species derived from a large experimental graft. Transplant phenotype was assessed at three scales: 1) macro, pearl size, colour, grade, 2) micro, pearl surface microstructure, and 3) molecular, biomineralisation gene expression level in saibo and pearl sac tissues. From donor to pearl, the phenome revealed fine variations of quality traits dependent on the position on the mantle where the *saibo* was cut, whose variation could overlap with inter-individual donor phenotype differences. A single donor phenotype could therefore produce multiple pearl phenotypes at the scale of the *saibo* position, mirroring its original activity at the mantle position level and the colour and shape of the shell. This phenome study provides essential information on phenotypic trait architecture enabling us to explore and explain the main biological functions and pave the way for a phenomic project on *P. margaritifera* that could benefit the pearl industry.

The phenotype is the result of the interplay of genetics with developmental, environmental and stochastic influences, where the intensity, frequency, order and interaction of these influences all affect the outcome. In the era of next generation sequencing with continued decreases in cost and increasing availability of high-throughput genotyping platforms, genomic data acquisition and associated bioinformatics treatments have become common even for non-model organisms. The complexity of plant and animal genomes, constructed from a pool of four nucleic acids and organized in a one-dimensional sequence, pales in comparison to their corresponding phenome. Phenome serves an unknown number of functions, many of which show enormous inter-individual variation that is at best only partially understood and for which the dimensionality remains unknown. This is not only due to the recent advances in genomics but also the complex multidimensional nature of phenotypes[1]. The vast number of phenotypic states of a genotype can be viewed as its phenotypic space, which is often referred to as its phenome. In practice, the phenome is a theoretical entity which can never be fully characterized[2]. Phenomics, operationally defined as the systematic study of phenotypes, is critically important to provide essential information for advances in genetic improvement for many cultured plant and animal species in the post-genomic era.

Species from the *Pinctada* genus are regularly used for the production of valuable free round cultured pearls, the only gems produced by a living organism[3]. The production of cultured pearls is both unique and biologically complex compared to that of other aquaculture industries. These are nucleated pearls produced in the gonad of a recipient pearl oyster following surgical implantation of a spherical shell-based bead (the nucleus) together with a piece of mantle (the *saibo*) cut from a particular section (located between posterior and anterior zone, without considering the junction of the mantle with the oyster gills) of a selected donor oyster. The mantle is clearly a metabolically and transcriptionally active tissue, indispensable for mollusc shell formation, with prominent transcriptional activity of biomineralisation genes[4]. The biomineralisation process is responsible for both pearl and shell formations. A few weeks after the graft operation, the pearl sac develops as a result of the epithelial cells of the mantle epidermis growing around the nucleus to completely cover it[5]. The pearl formation process then starts, by the deposition of successive biomineral layers onto the nucleus[6]. *P. margaritifera* is an ideal model animal for the study of biomineralisation because of the intriguing microstructure of: 1) its shells, which consist of outer calcitic prismatic layers and inner aragonitic nacreous layers, and 2) the pearls, which display mostly aragonitic

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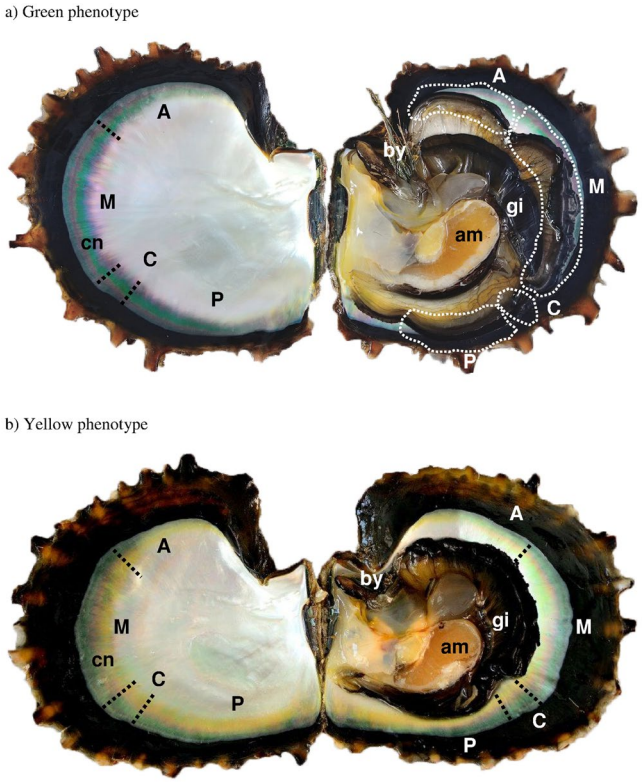

Figure 1. Donor *Pinctada margaritifera* of green (**a**) and yellow (**b**) phenotypes, each with two shell valves showing, on the right, the four sections of the entire dilated mantle tissue in (**a**), and contracted mantle tissue in (**b**): posterior position (P), connection with the gill (C), middle corresponding to the zone usually used for commercial *saibo* production (M) and anterior (A). In each case, on the left, the correspondence is shown with the zones of contact with the inner shell zone that exhibits the colourful band characteristic of donor oysters. The dotted lines indicate the areas of mantle tissue excised for *saibo* production from the four positions A, M, C and P. General anatomy: am, adductor muscle; gi, gills; by, byssus; cn, coloured nacreous zone.

structures, similar to the inner layer of the shell in both appearance and structure, through the chimeric pearl sac activity. Cultured pearl value is based on four main quality traits: size (nacre weight and thickness), shape, colour (including darkness level) and grade (the combination of lustre and surface quality)[3,7].

Phenotype transmission in the *Pinctada* transplant model has been mostly studied from an applied point of view, in relation to its economic importance in the pearl industry. Indeed, much research effort has been focused on size and colour determination in relation, to the donor oyster, the recipient oyster, their interplay, and their interaction with the environment[8–11]. In *P. margaritifera*, pearl size is known to be mostly driven by recipient oyster growth performance, and the donor oyster to be responsible for pearl qualitative trait determination, including colour[12]. The genotype of the donor therefore persists within the recipient in the form of the pearl sac. This chimeric organ displays more complex interactions, particularly when spatial (geographic origin of the population, depth of culture practices), temporal (age of hatchery-produced or wild collected spat), and environmental (grow-out site culture, season of graft, experimental temperature or pH variation) factors are introduced into the equation of the pearl quality trait determination[3]. Previous studies have all considered the transplant model at the individual scale: *i.e.*, at the level of individual pearl oysters[13–15]. The associated phenotypic variations recorded were then systematically related to inter-individual/ family variation among donors or recipients.

The objective of the present study was to explore for the first time the phenome of cultured pearl quality traits at the scale of the *saibo*, *i.e.*, at an intra-individual donor scale. For this, two fixed hatchery-produced pearl oyster phenotypes of *P. margaritifera*, exhibiting predominately green or yellow inner shell coloration (twelve of each) were used as donors. The entire mantle was taken for *saibo* production, including all the following four sections: posterior, connection (with gills), middle (used in commercial production) and anterior (Fig. 1). Experimental grafts (N = 1798) with traceability between donor line and *saibo* mantle position has been designed and performed in a single culture site. These grafts provided the biomineralised materials for the phenome study: the *saibo* tissue, the chimeric pearl sac tissue, and the associated pearls. Phenotypic data were collected at the macroscopic level (pearl quality traits), microscopic level (pearl surface ultrastructure) and molecular scale (expression level of a panel of biomineralisation genes representative of the nacreous aragonite and/or prismatic calcite synthesis in both *saibo* and pearl sac, derived from each of the mantle sections). This first phenome study, initiated with a set of easy-to-use tools, will provide: 1) basic knowledge to help us to understand phenotypic transmission, range of variation in an animal transplant model, and 2) useful information for the improvement of pearl quality for the industry.

Results

Experimental graft. The nucleus retention rate was 75.1% (N = 1350) at 45 days post-grafting (the remainder, 24.9%, N = 448, correspond to nucleus rejection, oyster mortalities and/or predation). Between the two phenotypes used as donors (green and yellow), no significant difference was detected for overall retention rate, even at the mantle section scale (*i.e.*, pairwise comparison between oysters grafted with the same mantle section between the two phenotypes). By contrast, comparison between the mantle sections revealed that the middle position had significantly a higher retention rate than the posterior (+7.0%; $p = 0.0315$) and connection positions (+10.7%; $p = 0.0315$) (Fig. 2A).

Cultured pearl quality traits. The overall mean cultured pearl nacre weight was 0.67 g (±0.32 g). A significant difference ($p = 0.031$) was detected between the two donor phenotypes, with grafts from the yellow donor leading to heavier nacre (+6.2%), in comparison with the green phenotype: 0.65 (±0.31 g) *vs.* 0.69 (±0.33 g). This was due, at the mantle position scale, to the anterior position on the yellow phenotype, which led to nacre 18.8% heavier ($p = 0.019$) than the green phenotype (Fig. 2B). Differences between the mantle positions were observed within the donor phenotypes. For the green phenotype, the middle section was 17.3% heavier than the anterior one (0.68 ± 0.31 g *vs.* 0.58 ± 0.30 g; $p = 0.001$). For the yellow phenotype, the two extremities of the mantle gave the most disparate results: the posterior position was 20.6% significantly ($p < 0.001$), heavier than the anterior one (0.76 ± 0.39 g *vs.* 0.63 ± 0.27 g).

The overall distribution of the cultured pearls among the colour categories was as follows: 53.1% (N = 632) for dark, 5.8% (N = 70) for green, 5.4% (N = 64) for light dark and 35.7% (N = 425) for light. The anterior section was significantly different from the three others and showed the highest rate of light pearls whatever the donor phenotype considered. For the yellow phenotype, 73.5% light pearls were found with anterior section grafts compared with an average of 30.0% for the other sections (Fig. 2C). The same tendency was observed in the green phenotype, with 66.9% of light pearls from the anterior section grafts and an average of 15.3% for the three other sections. The posterior and middle sections differentiated the two donor phenotypes, whereas the connection and anterior sections showed no difference between the colour rates. The yellow phenotype posterior position grafts produced 18.1% more light pearls than those cut from the same position on green phenotype donors ($p = 0.001$), which showed a significantly higher proportion of green pearls (+8.9%; $p = 0.014$). The middle position also had a significantly higher proportion of light pearls for the yellow phenotype (+21.0%; $p < 0.001$), while the green phenotype showed more dark pearls for this position (+15.7%; $p < 0.001$) (Fig. 2C).

For the cultured pearl quality grade, no significant differences between positions were detected for the green phenotype (Fig. 2D). For the yellow phenotype, the anterior position was different, with only 10.5% top quality pearls (A–C grade) compared with 22.9% on average for the three other positions ($p = 0.002$). Comparison between the two phenotypes showed that: 1) the green phenotype produced significantly ($p < 0.001$) more pearls of grades A–C, with +23.1% and +20.3% for the posterior and middle positions, respectively, 2) the yellow phenotype produced significantly more *rebut* (reject) grade peals, with +9.5% ($p = 0.012$) and +12.1% ($p < 0.001$) for the posterior and the middle positions, respectively, and also more D2 grade pearls with + 16.0% ($p < 0.001$) and +9.8% ($p = 0.041$) for the posterior and middle positions, respectively (Fig. 2D). A significant difference was also observed between the two phenotypes when comparing the results of anterior position grafts, with the yellow donors producing more *rebuts* (+12.1%, $p < 0.001$), D2 (+13.3%, $p < 0.001$) and D1 (+7.1%, $p < 0.001$) pearls and less grade D pearls (−10.5%, $p = 0.01$), than the green phenotype (Fig. 2D).

For cultured pearl shapes (Fig. 2E), no significant differences were detected within donor phenotype groups among the four different mantle positions from which *saibo* was cut. By contrast, significant differences were observed between the two donor colour lines. For posterior and anterior positions, yellow phenotype donors produced significantly more baroque pearls than green phenotype donors with +16.2% ($p = 0.01$) and +16.6% ($p = 0.006$), respectively. For these two positions pearl circles were, on the contrary, more frequent in pearls from green phenotype donors than yellow ones, with +13.4% ($p = 0.034$) for posterior position *saibo* and +12.5% ($p = 0.038$) for the anterior position. For the middle position, round pearls were significantly more frequent with the green phenotype (+8.9%, $p = 0.02$).

Cultured pearl surface ultrastructure observation. The mineralised portion of the nacre was observed on the cultured pearl surface by scanning electron microscopy and was seen to consist of aragonite tablets organized into growth fronts (Fig. 3). Observation of the corresponding microscopic patterns showed clear differences in the distance between the parallel growth fronts on the pearl surface, between the different mantle sections. The growth fronts of pearls produced with middle position *saibo* were significantly larger (35.1 ± 4.5 µm; Fig. 3C,G), than those of the connection zone (30.8 ± 6.4 µm; $p = 0.045$; Fig. 3B,F) and the posterior and anterior sections, which showed similar growth front distances (mean of both: 26.0 ± 3.5 µm; $p < 0.0001$; Fig. 3A,E,D,H).

Biomineralisation gene expression levels in *saibo*. Concerning the genes involved in the nacreous layer produced from the mantle, all four corresponding genes (*MRNP34*, *MSI60*, *Pearlin* and *Pif177*) showed the same expression profile, which distinguished the yellow and the green donor phenotypes, whatever the mantle position considered (Fig. 4). The *saibo* originating from the green donor phenotype systematically showed overexpression of these four genes in comparison to the *saibo* from the yellow variant (Fig. 4A–D). For example, in *saibo* from the green phenotype the *Pif177* gene was overexpressed by 4.90 ($p = 0.01$), 3.98 ($p < 0.001$) and 1.96 ($p = 0.023$) times for the posterior, middle and anterior positions respectively. Comparison among mantle sections within the green phenotype revealed systematic overexpression of genes in the posterior section compared with the middle and anterior sections, except for *MRNP34*.

For the four genes involved in prismatic layer formation (*Aspein*, *Shem9*, *KRMP7* and *Prism14*), near identical expression profiles were observed among donor phenotypes and mantle positions (Fig. 4). The same tendencies

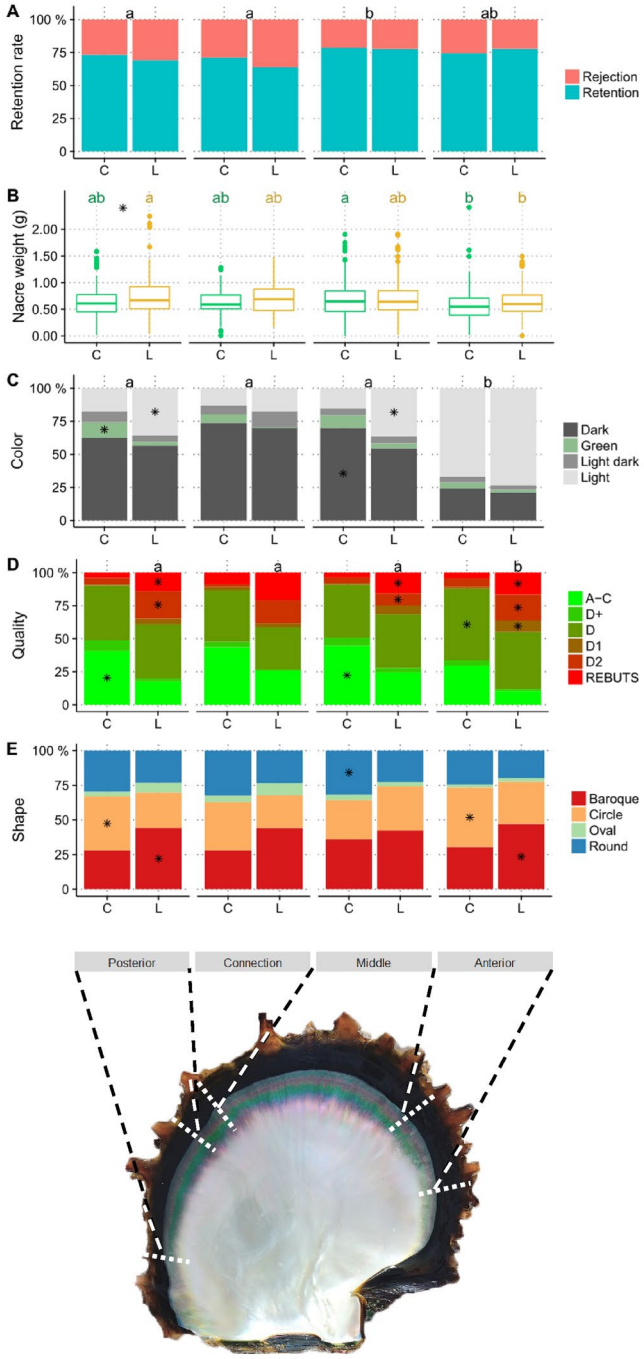

Figure 2. Graft and cultured pearl quality trait variation in *P. margaritifera* according to both mantle graft position (posterior, connection, middle and anterior) and the two phenotypes of donor (green C and yellow L). The five variables are the: (**A**) experimental graft retention rate; (**B**) cultured pearl nacre weight in g (boxplot); (**C**) pearl colour category percentages (dark, green, light dark and light), (**D**) pearl grade rate (A–C, D^+, D, D1, D2 grade and *rebuts*) and (**E**) pearl shape rate (baroque, circle, oval or round). Letters indicate significance between the mantle graft positions within each variable. Asterisks indicate within each mantle graft position the significant differences between the proportions of the categories of each cultured pearl trait, between the C (green) and L (yellow) phenotypes. The shell valve corresponded to a green C phenotype donor oyster.

were observed between the green and yellow donor phenotypes for all prismatic genes, except for the *KRMP7* gene expression in the middle section, where there was significant overexpression (×4) in the yellow phenotype compared with the green one (Fig. 4H). Expression of *Shem9* showed significant differences between: 1) phenotypes for the anterior position only ($p = 0.048$), with nearly twice the expression level in the green phenotype compared with the yellow, and 2) mantle section for both phenotypes only for the anterior position which was

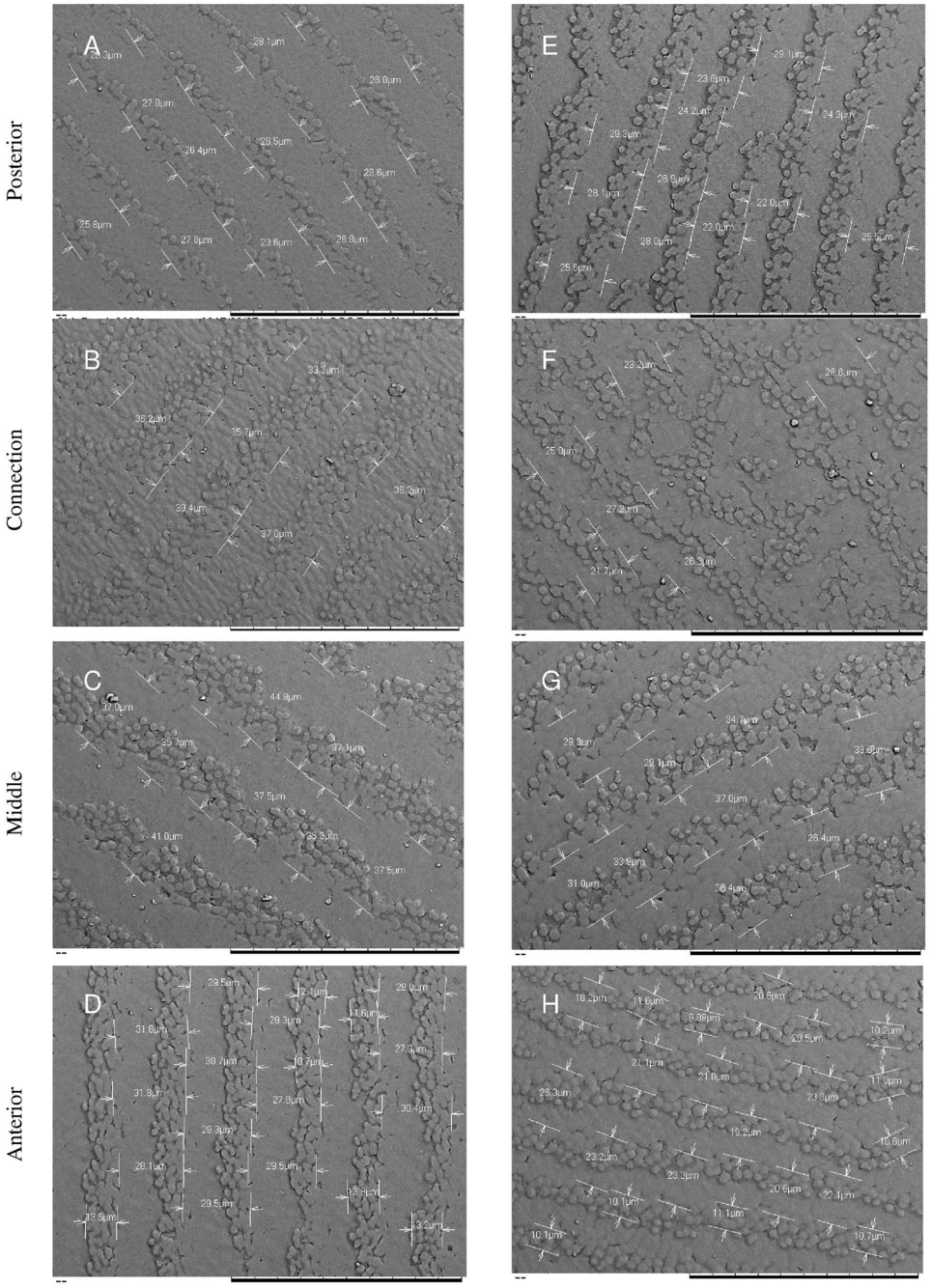

Figure 3. *P. margaritifera* cultured pearl surface, assessed by electronic microscopy (magnification: x1000) according to mantle graft position (posterior, connection, middle or anterior) based on grafts with tissue from two donor oysters. The first column (**A**,**B**,**C** and **D**) corresponds to sample of pearl surfaces from grafts made with the same yellow phenotype donor, whereas the second column (**E**,**F**,**G** and **H**) corresponded to pearl surface from another donor, which had the green phenotype. Cultured pearl grades were all D grade for those pearls produced with the yellow phenotype donor (first column) and (**A**–**C**) grade for pearls produced with the green phenotype donor (second column). The black bars at the bottom of each picture correspond to 100 µm. Distances between the fronts of aragonites were expressed in µm and illustrated with white bars and arrows.

different from the two others (Fig. 4G). For *Aspein* gene expression, no differences were observed between the donor phenotypes for any of the mantle positions.

Expression levels of the *Nacrein A1* gene (involved in both nacreous and prismatic formation), were not significantly different between mantle positions for the yellow donor phenotype (Fig. 4E). By contrast, the middle section of the green phenotype was significantly different from the anterior and posterior positions. Inter-phenotype comparison revealed significant differences for the posterior and middle sections, with respectively overexpression factors of 1.77 for the green and 3.02 for the yellow phenotype.

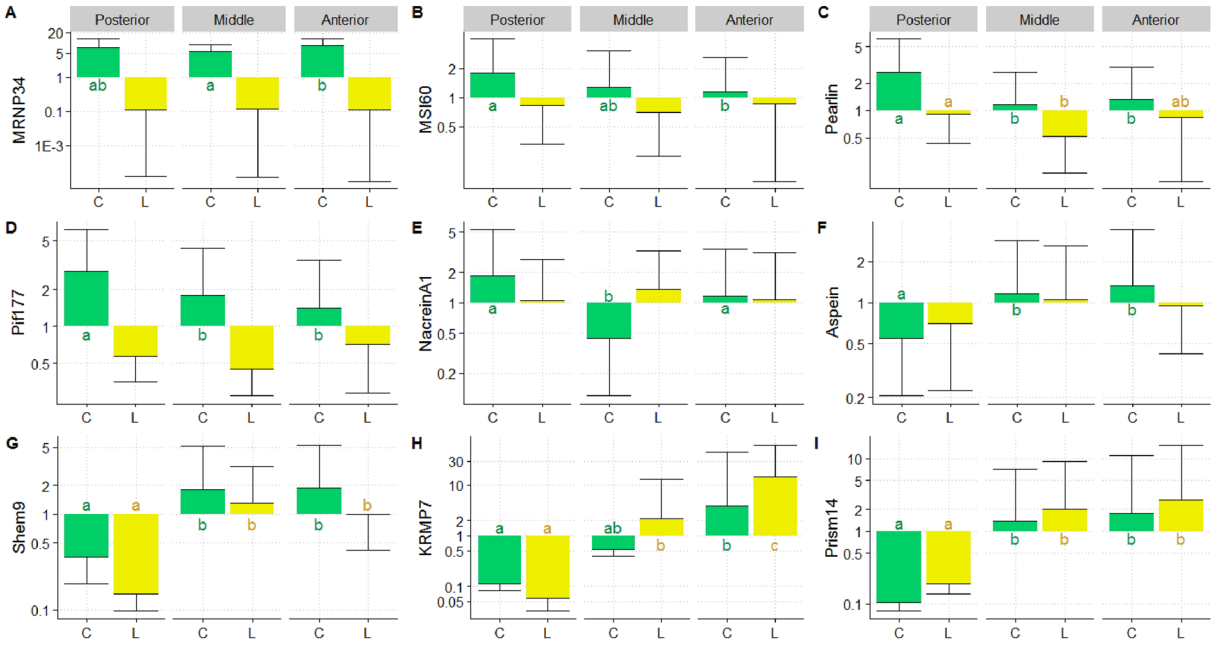

Figure 4. Relative expression of nine biomineralization genes in the mantle graft tissue (mixed of 3 *saibo* per position) of *P. margaritifera* obtained from the green C (Posterior N = 6; Middle N = 10; Anterior N = 11) and yellow L (Posterior N = 6; Middle N = 12; Anterior N = 6) donor phenotypes. Y axes are in the logarithmic scale. Error bars correspond to standard deviations. Letters indicate significance between mantle graft positions within each phenotype.

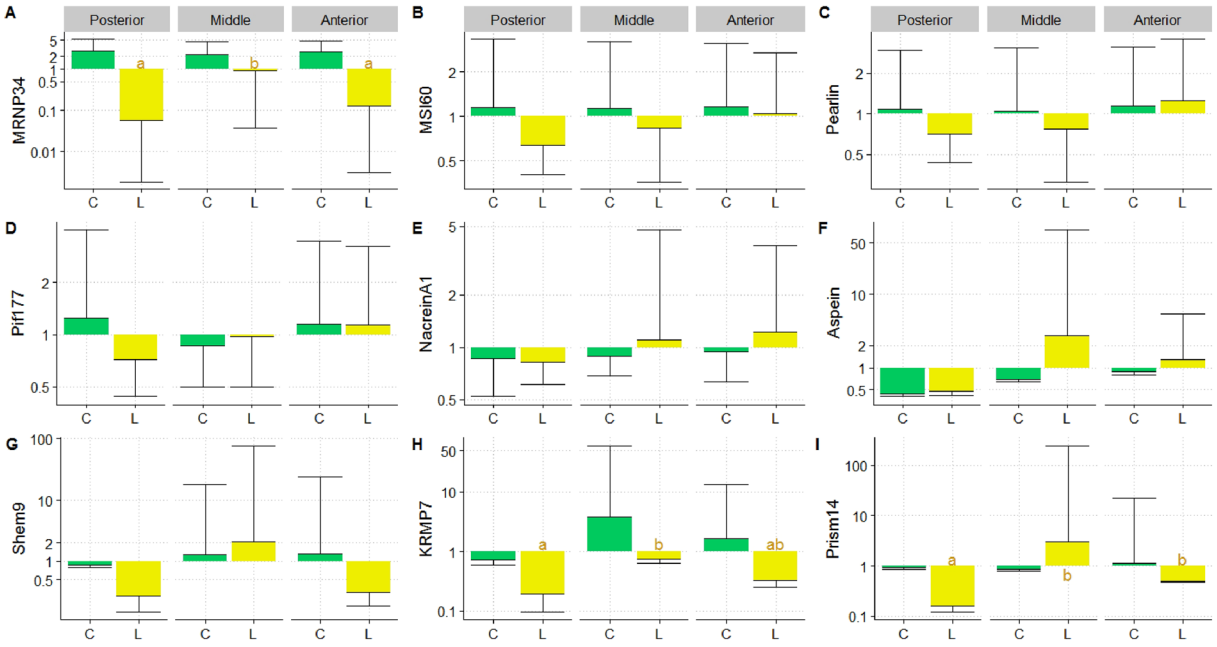

Figure 5. Relative expression of nine biomineralization genes in the pearl sac of *P. margaritifera* originated from the green C (Posterior N = 13; Middle N = 20; Anterior N = 32) and yellow L (Posterior N = 13; Middle N = 20; Anterior N = 16) donor phenotypes. Y axes are in the logarithmic scale. Error bars correspond to standard deviations. Letters indicate significance between the mantle graft positions within each phenotype.

Biomineralisation gene expression levels in the pearl sac. For the genes involved in the nacreous layer produced by the pearl sac, the expression of *MSI60*, *Pearlin* and *Pif177* were not different between either phenotypes or mantle positions (Fig. 5B–D). For *MNRP34* gene expression, differences were detected only for the yellow phenotype, where the posterior ($p = 0.002$) and anterior positions ($p = 0.006$) were overexpressed 47 and 20 times, respectively, in comparison to the middle (Fig. 5A).

For the genes implicated in cultured pearl prismatic layer formation, the expression of the *Aspein* gene showed no significant difference between phenotypes or positions (Fig. 5F). *Shem9* was expressed 4.7 times more in pearl sacs from anterior position *saibo* from green donor phenotypes than in those from the same position in the yellow donor phenotype ($p = 0.04$) (Fig. 5G). For *KRMP7* gene expression, the two phenotypes were highly different for all mantle sections: in pearl sacs from the green phenotype donors, *KRMP7* was overexpressed 5.4 times for the anterior position, 4.5 times for the middle section and 3.8 for the posterior position, compared with the yellow phenotype (Fig. 5H). For *Prism14* gene expression, only the posterior position showed a significant difference between phenotypes, with the green donor phenotype showing significantly higher expression (5.9 times, $p = 0.014$). Within phenotype, no significant differences were revealed between mantle positions (Fig. 5I).

Concerning the gene involved in both nacreous and prismatic layer formation, *Nacrein A1* showed no significant differences between donor phenotypes or mantle positions (Fig. 5F). For the two phenotypes, no significant differences were detected between positions.

Discussion

Phenotypic variations were assessed in the present study in a particular transplanted animal model, *P. margaritifera*, which combines a complex three-way interaction between the donor oyster, recipient oyster and the final cultured pearl product. The associated phenome, in relation to biomineralising and biomineralised tissues (graft tissue, pearl sac tissue, pearl samples) was assessed through a set of easy-to-use tools (visual and microscopic observation, RT-PCR) applied at three observation levels: macro-, micro- and molecular.

This initial cultured pearl quality phenome mirrored, to a certain extent, *P. margaritifera* shell morphology and characteristics. Oyster shell and cultured pearls are respectively formed in two distinct biomineralised tissues: the mantle and the pearl sac, which are derived from mantle tissue from the donor[16]. 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[17,18]. The pearl sac consists of mucous cells containing large acidophilic granules and epidermal cells[19] that secrete proteins resulting in cultured pearl formation, a highly controlled biomineralisation process similar to the development of the inner shell regulated by the mantle[20]. Similarly to other bivalves, the shell of *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[21–23]. Shell formation is a highly controlled process involving multiple matrix proteins[24–26]. In the *Pinctada* genus, the anterior zone of the shell is characterised by two growth-related features. First, the byssus location gives an anteriorly oblique shell conformation, with specifically subtriangular anterior auricles. Second, the concentric growth lines are closer in the anterior zone, than in the posterio-ventral part of the shell corresponding to the other mantle sections[3].

At macro-scale observation, and for pearl size, results revealed that the anterior shell-growth potential was conveyed by the *saibo* from the donor into the chimera formed in the recipient and thereby impacted nacre weight and thickness of pearls produced, giving smaller pearls compared to those grown from *saibo* from the other mantle sections, whatever the donor phenotype considered. This intra-phenotype variation from the donor, at the level of mantle position, was sometimes greater than variation observed at an inter-phenotype level. This result is consistent with earlier findings where donor effect for pearl size was shown using the same middle mantle section compared among wild[27] or hatchery-produced oysters[28]. The present study pointed the existence of a mantle effect within a same donor phenotype, which must be considered for pearl size determination to avoid any artefacts for genetic selection program aiming to improve this trait.

Colour transmission to the pearls is also dependent on the section of the mantle used at the scale of the individual donor. Here again, intra-individual variation could be greater than inter-individual variation. This was the case for example for the attractive green pearl colour, whose rate was significantly lower for the posterior section of yellow donor phenotypes compared with overall results from green donors, but similar to results from the green anterior section. Differences in pearl colour distribution also mirrored, to a certain extent, the inner shell colour profile of the donor oyster. Indeed, when looking at the interior nacreous surface of the shell, the anterior zone corresponds to the least colourful part of the donor oyster, compared with the parts adjacent to the other three sections, which show the largest and strongest intensity of the characteristic coloured band, particularly in the middle section of the mantle (Fig. 1). It is commonly known that donor tissue influences the colour of the resulting pearls and is mostly dependent on the species of *Pinctada* used[11,29]. Among pearl oysters of the genus, *P. margaritifera* is a good model for phenotypic colour variation studies, as it displays the largest range of pearl colours, reflected by the large diversity of inner shell colour phenotypes, in comparison to its two competing species *P. maxima* and *P. fucata*[3]. The green and yellow inner shell colour phenotypes have been studied recently and are known to also depend on rearing/culture site[30], with a colour "signature" at the archipelago scale in French Polynesia[7].

At the micro-scale observation level, assessment of the pearl surface growth fronts by electron microscopy showed the smallest distance growth front for the middle mantle section, where maximum growth was observed and is characteristic of the *Pinctada* shell shape. Such fronts have been already observed at the growth surface of bivalve nacre[31,32] and in cultured pearls from *P. margaritifera*[33], without looking for any direct connection between the pearl surface and the shell zone adjacent to their position of origin. The dynamics of nacre assembly therefore vary according to shell zone, and thus impact pearl quality depending on where the *saibo* was excised from the mantle. Change in structural assembly was related to variation in optical and mechanical properties, and was consequently connected to pearl colour, a character known to arise from light interference within the nanocomposite structure of the aragonite tablets[34]. This contributes to explaining the relation between microstructure and pearl colour expression.

At the molecular phenotype level, gene expression analysis, based on a panel of genes encoding proteins implicated in the shell biomineralisation process, indicated for the first time significant differences among donor phenotypes. The green and yellow donor groups revealed clearly different patterns of expression in the *saibo*

tissue, particularly for the genes related to aragonite formation (*MRNP34*, *MSI60*, *Pearlin* and *Pif177*). The proteins corresponding to *Pif177* and *MSI60* genes regulate growth, nucleation and the organization of the aragonite crystal[25,35,36]. Pearlin is the protein equivalent to the N14 protein previously identified in *P. maxima*[37] and seems to be specifically involved in the formation of the nacreous layer and promotion of aragonite crystal nucleation[38]. Whatever the mantle section, the *saibo* from the green donor phenotype showed systematic overexpression of these four aragonite-related genes compared with the yellow variant. These results could correspond to the higher proportion of good quality pearls (A–C and D+ grades), or difference among pearl colour distribution observed using the green phenotype compared to the yellow one. Pearl grade and colour was mainly attributed to aragonite tablets nature and assembly, which composed nacre produced from the internal regions of the mantle when this was used as *saibo*. The marginal area of the mantle produced the outer shell layer, constructed from densely packed calcite prisms, was associated with low pearl grade[39,40]. This marginal mantle area was excluded from the *saibo* cutting process. The different pattern of aragonite gene expression discriminating the two donor phenotype groups could not be attributed to any *saibo* cutting artefacts. Indeed, calcite gene expression pattern was equivalent for both donor phenotype group and followed the same tendency, such as for *Prism14* gene expression.

From an aquaculture point of view, such a phenome study could be of benefit to the pearl industry. At the scale of the donor phenotype, the present study detected specific donor-expression level of biomineralisation genes, with regard to the diversity of the shell colouration[29]. An establishment of systematic donor-specific phenomes could then be an interesting tool for the prediction of more appropriate colour lines to be selected and/or propagated for specific pearl colour and/or quality production. At the scale of the mantle graft phenotype, the results clearly show that mantle tissue from the posterior and connection sections can be successfully used as *saibo* for pearl production, as the resulting pearl quality traits were comparable to those produced with the middle zone, which is the part commonly used on commercial pearl farms, as was the nucleus retention rate. Indeed, by using these sections, more *saibo* could be obtained from the same number of donors. As the supply of pearl oysters for producers in French Polynesia has always been wild collection, finding colourful donor oysters was always a prerequisite, and sometimes limiting, step before the grafting process. Frequency of colourful donor oysters has been studied recently[29], revealing that such individuals are rare and dependent on spat collection site. It is therefore crucial to find enough donor oysters to supply grafters, who need from 400 to 700 *saibo* per day.

Our exploration of phenotype variation at the level of *saibo* position was the first conducted in the *Pinctada* genus. A single donor oyster individual exhibiting a particular shell colour phenotype could therefore produce multiple pearl phenotypes at the scale of the *saibo* unit, mirroring its original activity at the mantle position level. This intra-phenotypic variation could overlap with the inter-donor phenotype variation. Our results suggest that systematic study of multiple phenotypes across multiple biological function and scales would be important in future phenome studies in *P. margaritifera*. Increased sample sizes could potentially succeed in revealing robust genetic associations with pearl quality trait improvement that would benefit the pearl industry.

Materials and Methods

Animals. First generation hatchery-produced *P. margaritifera* were used as donor oysters for this study. These oyster families were issued from multi-parental crosses using highly coloured broodstock with green "C" (Fig. 1a) and yellow "L" (Fig. 1b) phenotypes (inner shell coloration), carried out at the Regahiga Pearl Farm and Hatchery company, located on Mangareva Island (Gambier archipelago, French Polynesia). To discern the inner shell colour for this set, the grafter used a speculum to gently pry open the oyster valves. Broodstock breeding, larval rearing and culture of this family were done as described previously[41]. At the age of 30 months post-hatching, 24 donor pearl oysters from the green (N = 12) and yellow (N = 12) phenotypes, were randomly selected from a sets of healthy animals with a mean (±SD) dorso-ventral measurement of 113.9 ± 8.7 mm. The entire list of the shell length corresponding to each donor can be found in Supplementary Table S1.

Wild *P. margaritifera* were collected as spat in the Mangareva Island lagoon (Gambier Archipelago, French Polynesia) to serve as recipients. Passive techniques were employed for catching spat using commercial collectors. Oysters were reared from the juvenile to adult stage as previously described[42]. The oysters were used in the grafting procedure once they were almost 20 months old, with a mean (±SD) dorso-ventral measurement of 76.30 ± 6.5 mm.

Experimental graft. The grafting operation was conducted in October 2014, with all grafts performed by an expert from the Regahiga Pearl Farm and Hatchery company as previously[43]. The nuclei used for this purpose were made from the shells of freshwater mussels (1.8 BU size, equivalent to 5.45 mm diameter, 0.26 g weight; Imai Seikaku Co. Ltd., Japan). The thickness and hardness of the nacreous layers of these beads offer a specific gravity and thermal conductivity that make them particularly suitable for use as pearl nuclei[44]. The epithelial cells required for grafting *saibo* were excised from the entire mantle of the selected donor pearl oysters and include all the following sections: posterior, connection, middle and anterior. A total of 1798 grafts were performed over five days (865 grafts using the green phenotype and 933 grafts using the yellow phenotype). Supplementary Table S1 gives the number of grafts performed for each donor oyster and per mantle section. All the grafted oysters were checked for nucleus retention/rejection and mortality 45 days after the grafting operation, as previously described[42]. The oysters that had retained their nuclei were drilled and fixed onto chaplets (within chaplets, oysters were attached in pairs to a rope with a monofilament fishing line), which constituted the rearing system. All recipient oysters were individually labelled (attribution of a plastic label with a number) in such a way as to maintain the traceability between graft position and corresponding harvested pearls. Furthermore, the pearl oysters were regularly cleaned in order to remove biofouling (epibiota), which can hinder healthy oyster growth and pearl production.

Pearl quality trait measurements. After approximately 20 months, the cultured pearls were harvested. The pearls were cleaned by ultrasonication in soapy water (hand washing) with a LEO 801 laboratory cleaner (2-L capacity, 80 W, 46 kHz); they were then rinsed in distilled water. Pearl size was assessed by measuring nacre thickness and nacre weight as previously described[28]. Shape, colour and grade were evaluated for all pearls by the same professional expert from the Poe O Rikitea association, according to Tahitian pearl auction classification categories[7].

Electron Microscopy. The structure of the pearl surface was observed by scanning electron microscopy (SEM) with a Hitachi Analytical Table Top SEM TM3030 at the electron microscopy facility of Université de la Polynésie Française, Tahiti. Before observation, the pearls were sawn at the point of their largest diameter so as to be positioned flat on the observation plate. Subsequently, the surfaces were metallized (Quorum Technologies, Q150R ES model) with a thin layer of gold (15 nm). This step eliminates any electromagnetic load that might interfere with observation. Observations were based on pictures taken at the pearl surface and particularly of the space between two layers of aragonite deposition (magnification 1000x, accelerating voltage 15 kV). The gap between layers of aragonite deposition was measured with post-acquisition image tools: we measured the gap between a finished deposition layer and the next or previous one (Fig. 3). Several locations on the surface of the pearl were thus measured for a statistical analysis of the averages.

Gene expression analysis. Two donors among the twelve per phenotype were randomly sampled. For each donor (N = 4), all *saibo* pieces prepared from 1 valve were preserved in RNAlater (Qiagen) (50 mg/mL). The *saibo* obtained from the other valve were used in the graft processes and the corresponding pearl sacs were all sampled (at the same time as the pearls) and likewise preserved in RNAlater (Qiagen). For the pearl sac sampling, the gonads were first cut from the recipient. The gonad tissue was then removed with a surgical blade to leave only a thin (<0.5 mm) layer of tissue surrounding the pearl. At this point, only the pearl sac and the pearl remained. Next, an incision was made in the pearl sac and the pearl removed. The pearl sac was transferred into a 5.0-ml tube with RNAlater® until RNA extraction[45]. The samples were stored at −80 °C until RNA extraction. A total of 170 *saibo* and 120 pearl sacs were sampled.

Expression levels were measured by RT-PCR for a panel of nine genes involved in biomineralisation: four aragonite-related genes (*MRNP34*, *MSI60*, *Pearlin* and *Pif177*), four calcite-related genes (*Aspein*, *Shematrin 9*, *Prismalin-14* and *KRMP7*) and one involved in the formation of both nacreous aragonite and prismatic calcite (*Nacrein A1*). Two housekeeping genes were also measured, chosen based on their ubiquitous and constitutive expression pattern in *P. margaritifera* tissue: SAGE (SAGES: AGCCTAGTGTGGGGGTTGG/ SAGER: ACAGCGATGTACCCATTTCC) (called REF[36]) and GAPDH (GAPDHS: AGGCTTGATGACCACTGTCC/ GAPDHR: AGCCATTCCCGTCAACTTC)[46]. Primer sequences of the nine biomineralisation genes can be found in Supplementary Table S2.

After removing the RNAlater by pipetting and absorption, total cellular RNA was extracted from the individual graft tissue (pooled into 55 samples with respect to the different mantle positions studied, with 3–4 *saibo* caming from the same individual per extraction) or pearl sac samples (N = 120), using TRIzol® reagent (Life Technologies) according to the manufacturer's recommendations. *Saibo* from connection zone were not used for this part of the study as only 3 to 6 pooled sample could be obtained. 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 analysed 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[47] as follows: $\text{Relative expression}_{(\text{target gene, sample x})} = 2^{-(\Delta\text{Ct sample, sample x} - \Delta\text{Ct calibrator, sample x})} = 2^{-\Delta\Delta\text{Ct}}$. 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[48]. 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%.

Statistical analysis. All analyses were performed using R© version 3.2.3 software (R foundation for Statistical Computing). The significance threshold was set at $p \leq 0.05$ and the tests were two-sided. All measures are given as means and variability as standard deviations.

Pearl quality and retention differences according to *saibo* positions were tested, for qualitative parameters, using χ^2 tests and Fisher's exact tests when an expected value <5 was found. When significant differences were detected, pairwise comparisons were made for proportions. For quantitative categories, normality and homoscedasticity were tested using Shapiro–Wilk test and Bartlett's tests, respectively. Due to non-normality, Kruskal–Wallis tests were used to test differences between *saibo* positions. When differences were detected, post-hoc analyses were performed with Dunn tests and Bonferroni correction. Kruskal-Wallis tests were also used to test difference of gene expression levels between the phenotypes.

Data availability statement. The authors declare that all datas are available.

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Author Contributions

C.L.K. designed the experiment and wrote the manuscript. V.Q. realized the molecular gene expression analysis. C.S. performed the electron microscopy observations. F.B. analyzed the data. D.D. supported the logistical and technical organization for the realization of the experimental grafts. All authors read and approved the final manuscript.

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Comparison of harvested rate and nacre deposition parameters between cultured pearls issued from initial graft and second nucleus insertion in *P. margaritifera*

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Abstract

Cultured pearls produced with *Pinctada margaritifera*, using the surgreffe method (implantation of a second nucleus following pearl harvest) were studied for the first time to: (1) examine family effect on nacre thickness, nacre weight and nacre deposition speed and (2) compare variation in these three traits with that obtained from the cultured pearls previously harvested after the corresponding initial grafts. A surgreffe experiment using 783 recipient oysters was realized in Rangiroa atoll (French Polynesia). After 24 months of culture, 389 cultured pearls were harvested. Significant donor family effect was found for the harvested pearl rate from surgreffe ($P = 0.046$). Highly significant donor family effect was recorded for nacre thickness ($P = 0.004$). Very highly significant donor family effects were recorded for nacre weight and nacre deposition speed ($P < 0.0001$). Comparison between surgreffe and initial graft showed: (1) no significant effect for the average cultured pearl rate harvested ($P = 0.052$) and average cultured pearl nacre deposition speed ($P = 0.622$) and (2) very highly significant differences ($P < 0.0001$) for the average cultured pearl nacre thickness and nacre weight. This study highlighted three major implications for pearl industry management: (1) donor family effect was maintained from initial graft to surgreffe, for nacre thickness, weight and deposition speed, (2) the persistence of the pearl sac metabolic activity over three years of culture and (3) the relation between harvested pearl rate and the size of the nucleus inserted in the pearl sac.

Keywords: *Pinctada margaritifera*, surgreffe, family effect, cultured pearl, nacre weight, nacre thickness

Introduction

The mollusc, *Pinctada margaritifera* (Linnaeus, 1758) var. *cumingi* (Reeve), also known as the black-lip pearl oyster, is encountered throughout the Indo-Pacific region and is particularly abundant in the lagoons of French Polynesia. The aquaculture of *P. margaritifera* is devoted to the production of its valuable cultured black pearls. Cultured pearls are the most important export commodity of French Polynesian foreign trade. The improvement of pearl quality production is therefore a major challenge for pearl farmers (Ky, Blay, Sham-Koua, Lo & Cabral 2014). In fact, slowdown of the world economy and overproduction have caused difficulties for this industrial sector (Le Pennec & Buestel 2010; Wane 2013). From an environmental point of view, production of high quality pearls could reduce the pressure on lagoons by reducing the maritime culture area and the number of pearl oysters per hectare, and limit (1) pollution due to overproduction and (2) impoverishment of food resources. From an economic point of view, this could also reduce pearl farm costs (Pouvreau, Bacher & Héral 2000). Cultured pearl production relies on a surgical operation, consisting of 'grafting': inserting a round

nacreous bead (nucleus) together with a small piece of mantle tissue (i.e. the graft of around 4 mm^2) dissected from the mantle of a sacrificed pearl oyster, the donor, into the gonad of another oyster, the recipient. During the first few weeks after the grafting operation, a pearl sac is formed by cellular multiplication of the epithelial cells of the implanted mantle (Machii 1968 and Inoue, Ishibashi, Ishikawa, Atsumi, Aoki & Komaru 2011), in which cultured pearl formation occurs by secretion and deposit of successive nacre layers around the nucleus (Taylor & Strack 2008).

Among cultured pearl quality traits, size remains one of the most important criteria that determine pearl value and cost. Indeed, more a pearl is heavy and more the cultured pearl price will be high, with all other quality traits equal (luster, surface defects and colour) (Matlins 1996; Strack 2006). Ever since the cultured pearl market has existed, size improvement has been one of the main goals of producers. One way to produce bigger pearls is a common operation, known as a surgreffe, which allows larger nuclei to be inserted. Surgreffe operations can only be performed on pearl oysters that have already yielded a pearl. They are performed directly after harvest of a good quality pearl and consist of inserting a new nucleus of the same size as the harvested pearl into the pearl sac. Consequently, the future cultured pearl issued from a surgreffe will be formed by the well-established pearl sac, with no need for further graft tissue insertion into the same recipient (Sarikaya, Liu & Aksay 1995). These surgreffe operations can be realized successively three to four times (maximum) and could lead to harvested pearls up to 20 mm in diameter. Commonly, the first surgreffe takes place at the harvest of the pearl from the initial graft, and the second surgreffe takes place at the harvest of the pearl from the first surgreffe, etc. As pearl culture following a surgreffe takes around 1–2 years, harvest from a third surgreffe would correspond to a recipient of 7–10 years old.

Another way to improve pearl size is though "pearl oyster" genetic selection through the selection of potential donors with high biomineralization capacity (Tayale, Gueguen, Treguier, Le Grand, Cochennec-Laureau, Montagnani & Ky 2012; Ky, Blay, Sham-Koua, Vanaa, Lo & Cabral 2013; and Blay, Sham-Koua, Vonau, Tetumu, Cabral & Ky 2013). Indeed, donor influence on quantitative pearl quality traits was definitively demonstrated when a donor was found to have a significant influence on pearl growth using reciprocal xenografting between two pearl oysters species, *P. maxima* and *P. margaritifera* (McGinty, Evans, Taylor & Jerry 2010). The combination of successive surgreffe operations after an initial graft that has shown high biomineralization capacity would be of great interest for increasing pearl size in a short culture time. No data existed on first surgreffe effect, or on its comparison with the corresponding graft effect, on cultured pearl quantitative traits (weight and thickness).

The aim of this study was therefore to evaluate if the donor families effects was maintained at the surgreffe level, on three quantitative cultured pearl traits, that described pearl size: nacre thickness, nacre weight and nacre deposition speed. In other words, could family-based genetic selection goes beyond initial graft? To ensure such goal, an analysis was made of pearls issued from surgreffe of the recipient oysters that had initially been grafted using specific donor oyster families and whose first harvested pearls had already been examined by Ky *et al.* (2013) following culture in a single site. This comparison between initial graft and surgreffe, with real traceability avoiding the influences of genetic and environmental variation, highlight also the relation between harvested pearl rate and nucleus size to be inserted in the pearl sac for both surgreffe and graft.

Materials and methods

Experimental animals and surgreffe procedure

Ten bi-parental families of *P. margaritifera*, produced in the Ifremer hatchery facilities in Vairao (Tahiti, French Polynesia), were used as donors in a graft experiment (Ky *et al.* 2013). A pool of corresponding recipient oysters were first grafted in this experiment (Ky *et al.* 2013), then used in the present surgreffe trial by the insertion of a second nucleus (Table 1). In order to minimize environmental effects, the surgreffe experiment was done: (1) in a single grow-out site on Rangiroa atoll (Tuamotu Archipelago, French Polynesia), (2) on the same pearl farm (Gauguin's Pearl Farm) and with the same professional grafter so as to minimize grafter effects (the same grafter who made the initial grafts described in Ky *et al.* 2013), and (3) with the same nucleus size and brand: 3.5 BU nucleus (10.48 mm diameter and 1.84 g weight – Nucleus Hyakusyo Co., Osaka, Japan). This nucleus size was chosen because it is the average size and the most

Table 1 Data summary on graft and surgreffe operations and corresponding pearl harvest rates according to *Pinctada margaritifera* donor families (named by letters A–J). Three times periods were described: (1) t_0, with the number of oysters used as donors, (2) t_{18}, with number (N) and rate (%) of harvested pearls 18 months after graft, and (3) t_{42}, with number (N) and rate (%) of harvested pearls 42 months after graft (24 months after surgreffe)

	Graft			Surgreffe		
	t_0	$t_{18\ \text{months post graft}}$ Harvested pearls		$t_{18\ \text{months post graft}}$	$t_{42\ \text{months post graft}}$ Harvested pearls	
Family	Grafted oysters	*N*	%	Surgreffed oysters	*N*	%
A	150	100	66.7	96	53	55.2
B	150	60	40.0	49	29	59.2
C	150	77	51.3	67	36	53.7
D	150	103	68.7	95	43	45.3
E	150	87	58.0	79	35	44.3
F	151	81	53.6	75	36	48.0
G	150	79	52.7	73	36	49.3
H	150	85	56.7	83	45	54.2
I	146	92	63.0	76	36	47.4
J	147	110	74.8	90	40	44.4
Total	1494	874	58.5	783	389	50.1

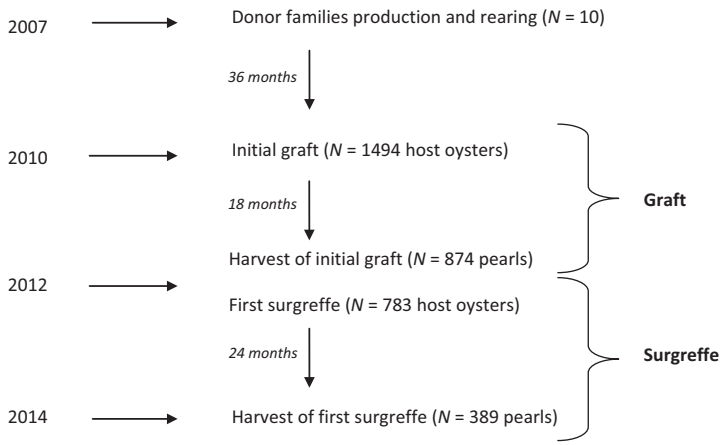

Figure 1 Experimental design of the graft and the surgreffe.

frequently used for the first surgreffe operation on this farm. Each surgreffed nacre was labelled with the same number as the corresponding harvested pearl from graft procedure. This method allowed us to have a real traceability between harvested pearl from graft and harvested pearl from surgreffe (Fig. 1). Table 1 shows that, 1494 of the pearl oysters initially grafted, 711 were not used for the surgreffe. 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, which was performed over three days in April 2012 (Table 1). After the surgreffe operations, the recipient pearl oysters were put in groups of 10 on chaplets, which were protected by a plastic mesh to avoid predation. During the culture time, the pearl oysters were cleaned every 6 months using high pressure seawater Kärcher®.

Measurement of cultured pearl traits

After 24 months of culture, the pearls were harvested and placed into a compartmented box that allowed traceability between samples 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).

Harvested pearl rate from the first surgreffe was calculated after 24 months of culture, where as harvested pearl rate following initial grafting had been previously evaluated at 18 months post grafting in Ky *et al.* (2013).

Nacre thickness was evaluated using the formula: nacre thickness = [(cultured pearl diameter) – (nucleus diameter)] / 2. Cultured pearl diameter was measured by scanning the pearl on an Epson® (DSSC EPSON, Parc technologique EUROPARC, Créteil Cedex, France) perfection V750 Pro, and analysing the images using Adobe Photoshop® (Adobe Systems France, Paris, France) CS3 and Image J (Bethesda, MD, USA). For harvested pearls with an irregular or asymmetrical shape, called baroque pearls (because of the presence of nacre bulges), we took into account the thickest diameter.

Nacre weight was evaluated using the formula: nacre weight = (cultured pearl weight) – (nucleus weight). Cultured pearls were weighed using a digital balance Mettler Toledo Excellence Plus (0.1 mg precision).

Nacre deposition speed was calculated by dividing nacre weight by the number of months of culture following the formula: nacre deposition speed = (nacre weight) / (number of month of culture). There had been 24 months of culture for the surgreffe and 18 months for the initial graft, see Ky *et al.* (2013).

Statistical analysis

Surgreffe family effect was analysed by a chi-square test. For the three quantitative nacre variables, a Shapiro–Wilk test was used. When normalization was not possible, a Kruskall–Wallis test was performed followed by Dunn's multiple comparison procedure (Siegel & Castellan 1988; Winer, Brown & Michels 1991). If data followed a normal law, an ANOVA followed by a Tukey multiple comparison test was used.

Means comparisons between surgreffe and graft for harvested pearl was performed using Mann–Whitney *U*-test. The same test, Mann–Whitney *U*, was used to compare nacre thickness, nacre weight and nacre deposition speed at an inter-family scale between surgreffe and graft.

All tests were performed using XLSTAT (version 2009.4.02; Paris, France) and *P*-values lower than 0.05 were considered significant (Dagnelie 2007).

Results

Cultured pearl harvest rate

From the 783 'pearl oysters' on which a surgreffe was performed, an average rate of 50.1% ($N = 389$) cultured pearls were harvested per family, with a minimum value of 44.3% (Family E, $N = 35$ nuclei retained out of 79 surgreffe operations) and a maximum of 59.2% (Family B, $N = 29$ nuclei retained out of 49 surgreffe operations). A significant donor family effect was found for the harvested pearl rate following surgreffe, $P = 0.046$. Classifying families from the best to worst harvest rate following surgreffe gave the order: B, A, H, C, G, F, I, D, J, E (Table 1).

The mean pearl harvest rate did not differ significantly between surgreffe and graft ($P = 0.052$).Within the families A, C, E, F, G and H there was also no significant difference between surgreffe and graft (Fig. 1), but significant differences were found for the other four families: B ($P = 0.029$), I ($P = 0.036$), D and J (both $P < 0.0001$). For three of these four families, harvested pearl rate was lower following surgreffe than after the initial graft (−23.4%, −15.6% and −30.4% on average for families D, I and J, respectively), while family B showed a better result following surgreffe (+19.2% on average; Fig. 2).

Nacre thickness

The average nacre thickness following surgreffe was 0.88 mm, with a minimum of 0.74 mm (family A) and a maximum of 0.99 mm (family G). A highly significant family effect was recorded for nacre thickness following surgreffe ($P = 0.004$). Families could be ranked from the thickest to the thinnest nacre as follows: G, F, H, B, E, C, D, J, I and A (Fig. 3 and Table 2).

Comparison between the surgreffe and graft results revealed a highly significant difference for average pearl thickness ($P < 0.0001$), which was lower following surgreffe than after the initial graft. The families each showed a significant difference for the nacre thickness in average formed

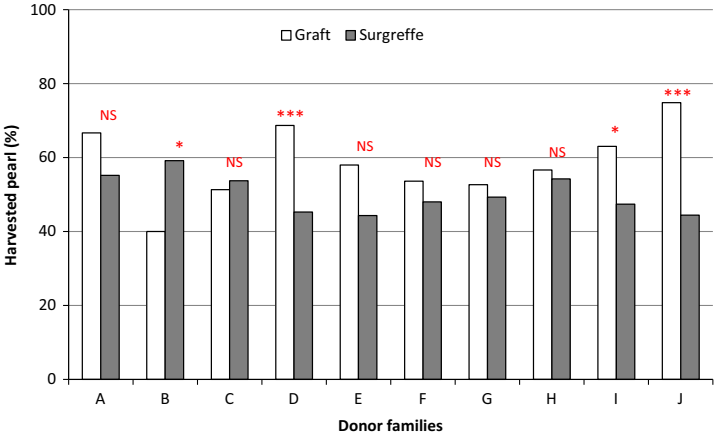

Figure 2 Harvested pearl rate (in %) from graft and surgreffe operations for the ten donor families (named A–J). Significance of differences between graft and surgreffe is noted as follows: NS, non-significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

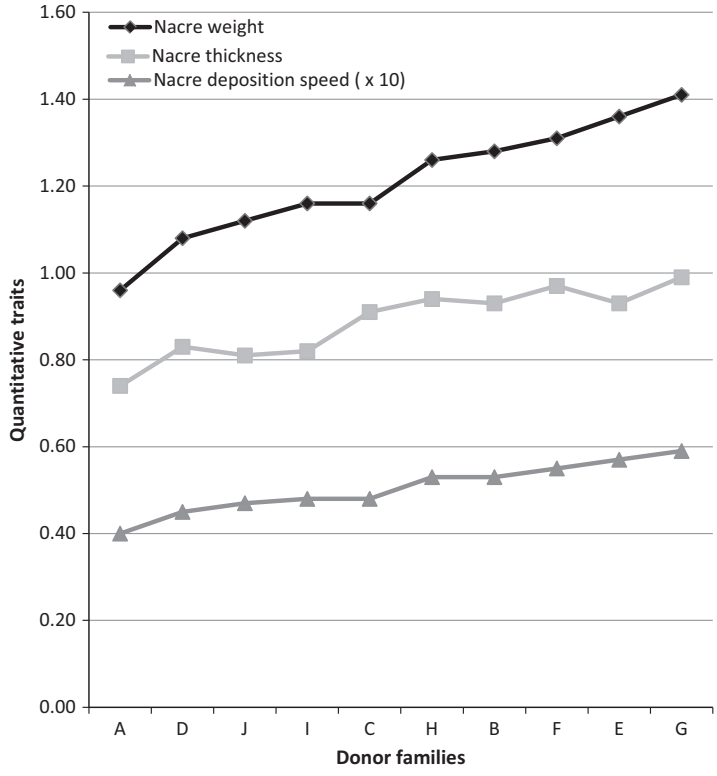

Figure 3 Quantitative traits for surgreffe operations for the ten donor families (named A–J) ranked from the heaviest to lightest with: (1) Nacre weight in black (in g); (2) Nacre thickness in light grey (in mm) and (3) Nacre deposition speed in dark grey (in g month^{-1} × 10).

after these two operations. Indeed, the average thickness per family was (1) significantly finer for families C ($P = 0.003$), E ($P = 0.004$), F ($P = 0.002$), G ($P = 0.009$), H ($P = 0.001$), and J ($P = 0.004$), and (2) very highly significantly finer for A, B, D and I ($P < 0.0001$; Fig. 4).

Nacre weight

The average nacre weight following surgreffe was 1.21 g, with a minimum value of 0.96 g (family A) and a maximum value of 1.41 g (family G). A very highly significant family effect was recorded for nacre weight from surgreffe ($P < 0.0001$). Families could be ranked from the heaviest to the lightest as follows: G, E, F, B, H, C, I, J, D and A (Fig. 3 and Table 2).

Comparison between surgreffe and graft experiments revealed a highly significant difference for average pearl weight, with a greater weight after surgreffe ($P < 0.0001$). All families also individually showed significant differences in the average

Table 2 Data summary of surgreffed families of *Pinctada margaritifera* donor oysters (named by letters A–J). Three variables are described: (1) nacre thickness (in mm), (2) nacre weight (in g) and (3) nacre deposition speed (in g month^{-1}) with Mean, Min (minimum), Max (maximum) and SD (standard deviation)

Donor progenies	A	B	C	D	E	F	G	H	I	J
Nacre thickness										
Mean	0.74	0.93	0.91	0.83	0.93	0.97	0.99	0.94	0.82	0.81
Min	0.21	0.44	0.43	0.45	0.45	0.39	0.42	0.37	0.37	0.34
Max	1.46	1.49	1.54	1.71	1.49	1.56	2.09	2.5	1.62	2.09
SD	0.31	0.3	0.26	0.26	0.29	0.32	0.39	0.37	0.29	0.39
Nacre weight										
Mean	0.96	1.28	1.16	1.08	1.36	1.31	1.41	1.26	1.16	1.12
Min	0.17	0.56	0.52	0.43	0.51	0.42	0.44	0.38	0.5	0.38
Max	2.52	2.1	2.07	2.51	2.35	2.44	3.41	3.68	3.35	2.29
SD	0.49	0.43	0.36	0.49	0.49	0.47	0.66	0.64	0.58	0.47
Nacre deposition speed										
Mean	0.04	0.053	0.048	0.045	0.057	0.055	0.059	0.053	0.048	0.047
Min	0.007	0.023	0.022	0.018	0.021	0.018	0.018	0.016	0.021	0.016
Max	0.105	0.087	0.086	0.105	0.098	0.102	0.142	0.153	0.14	0.095
SD	0.02	0.017	0.015	0.02	0.02	0.019	0.027	0.027	0.024	0.019

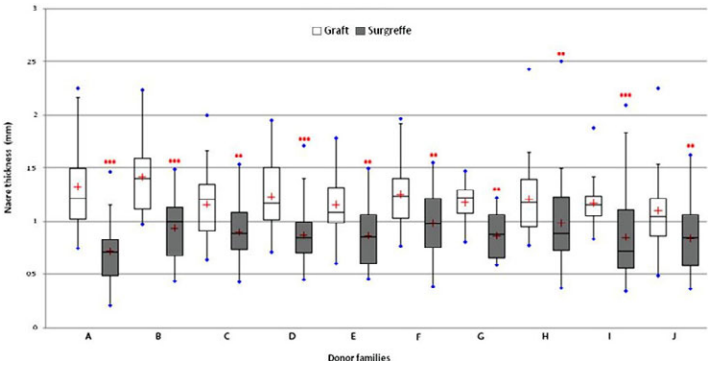

Figure 4 Nacre thickness (in mm) of the cultured pearls from graft and surgreffe for each of the *Pinctada margaritifera* donor families (A–J). Box-plots in white represent data from graft and box-plots in grey represent data from surgreffe. Each box-plot has the following six elements: (1) mean ('+' cross in the box-plot); (2) median (solid bar in the box-plot); (3) 25th–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). Significance of differences between graft and surgreffe is noted as follows: NS, non-significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

pearl weight between surgreffe and graft experiments, except for family A ($P = 0.717$). Indeed, the average weight per family was: (1) significantly higher following surgreffe in B ($P = 0.024$), D ($P = 0.021$), and G ($P = 0.038$), (2) highly significantly higher for E ($P = 0.003$) and I ($P = 0.006$), and (3) very highly significantly higher for C, F, H and J ($P < 0.0001$; Fig. 5).

Nacre deposition speed

The average nacre deposition speed following surgreffe was 0.050 g month^{-1}, with a minimum of 0.040 g month^{-1} (family A) and a maximum of 0.059 g month^{-1} (family G). A very highly significant family effect was recorded for nacre deposition speed from surgreffe ($P < 0.0001$). Families could be ranked from the fastest to the slowest as follows: G, E, F, B, H, C, I, J, D and A (Fig. 3 and Table 2).

There was no significant difference between surgreffe and graft results for average nacre deposition speed ($P = 0.622$). Furthermore, no individual families showed a significant difference in nacre deposition between surgreffe and graft except family A ($P = 0.001$).

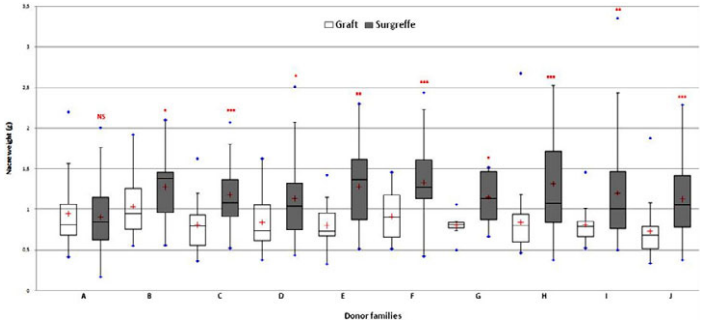

Figure 5 Nacre weight (in g) of the cultured pearls from graft and surgreffe for each of the *Pinctada margaritifera* donor families (A–J). Box-plots in white represent data from graft and box-plots in grey represent data from surgreffe. Each box-plot has the following six elements: (1) mean ('+' cross in the box-plot); (2) median (solid bar in the box-plot); (3) 25th–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). Significance of differences between graft and surgreffe is noted as follow: NS, non-significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Discussion

This study on cultured pearls harvested following first surgreffe on *P. margaritifera* is the continuation of the previous graft experiment realized by Ky *et al.* (2013) in which the same recipients were used. Indeed, the pearl sacs in these recipients, used in the present study, came from the 10 donor oyster families used for grafts in this previous study. This provided us with the first opportunity to compare nacre thickness, weight, and deposition speed between harvested pearls from an initial graft and the corresponding first surgreffe with real traceability.

Cultured pearl harvest rate

The pearl harvest rate (success rate) is a parameter of major importance for pearl farm incomes. Our results showed that donor families significantly influence the percentage of pearls harvested following surgreffe. These results are consistent with those found after initial grafting with wild (Tayale *et al.* 2012) or farmed donor oysters (Ky *et al.* 2013; same donors as the present study). Indeed, harvested pearl rate is strongly related to nucleus retention rate. Pearl harvest rate results from nucleus retention rate, mortalities and predation during the culture period. Predation seems to be constant between graft and surgreffe periods, as no notable differences existed between the culture periods. Nevertheless, predation might be expected to affect the older oysters less after surgreffe because, by this time, they are thicker with a more resistant shell than those at the younger stage corresponding to the culture period following the first graft. The similarity in rates could be due to the fact that post-surgreffe culture time lasted an additional six months compared with the culture period that followed the initial graft (24 vs. 18 months). Mortalities, mainly due to post-surgical trauma after harvest and insertion of the new nucleus, need to be evaluated more closely in surgreffe studies, as no data are available on nucleus retention rate following surgreffe. Other studies have demonstrated that nucleus retention rate is linked to a number of causes such as inflammatory reaction, presence of numerous tissue lesions (Cochennec-Laureau, Montagnani, Saulnier, Fougerouse, Levy & Lo 2010), and that it may depend on grafter skill and the season in which the grafting operation is performed (Ky *et al.* 2014).

Comparison between first surgreffe and initial graft shows no significant difference for the average pearl harvest rate. Nevertheless, farmers currently say that harvested pearl rate is higher from surgreffe than from initial graft (farmers pers. comm.). This is consistent with the fact that the pearl sac is already formed at surgreffe, with no need to introduce another 'saibo'. When comparing performance within families, our results (except for B family) showed the opposite trend. This could be explained by the need for correspondence between the size of the harvested pearl from the initial graft and the size of the second nucleus

inserted at surgreffe. As already stated, the common process during the surgreffe operation is that the grafting technician inserts a nucleus in the pearl sac that is larger than the one used in the initial graft operation but the same size as the harvested pearl. In our experiment, however, the surgreffe nucleus size was standardized (3.5 BU – 10.48 mm), which meant that no size adjustment was made to match the size of the harvested pearl. Family B produced medium-sized cultured pearls (average diameter was 1.28 mm) and consequently showed a better harvested pearl rate. By contrast, families D, I and J produced the smallest pearl size on average (average diameter was 0.88 mm), and then the standardized surgreffe nucleus was too big and consequently, the harvested pearl rate decrease from graft to first surgreffe. For the case where the second nucleus inserted is too small, as in families G, F and H (average diameter 0.96 mm), this had no negative consequences on harvested pearl rate from surgreffe. This therefore suggested that size correspondence was of prime importance for increasing harvested pearl rate at first surgreffe. The principle could also be taken into account at the scale of the initial graft, where correspondence between nucleus size and the gonad size may impact nucleus retention rate.

Maintenance of donor family effect

Donor family effect was maintained between initial graft and first surgreffe, for nacre thickness, weight and deposition speed, underlining the continuity of the donor effects first reported by Ky *et al.* (2013) on nacre thickness and nacre weight. At first surgreffe, the average differences between extreme progenies (family A and G) were 25% and 32% for nacre thickness and weight respectively. This difference was correlated with the results obtained by Ky *et al.* (2013) following the first graft, where these differences were 14% and 21% for nacre thickness and weight respectively. Our results therefore highlight the persistence of the donor effect for these quantitative traits long after the graft operation. It was well known that the ability to produce nacre depends directly on the biomineralization process, which takes place in the pearl sac, and that this metabolic activity originates from 'saibo' transplantation (Wada 1972; Joubert, Piquemal, Marie, Manchon, Pierrat, Zanella-Cléon, Cochennec-Laureau, Gueguen & Montagnani 2010; Montagnani, Marie, Marin, Belliard, Riquet, Tayalé, Zanella-Cléon, Fleury, Gueguen, Piquemal & Cochennec-Laureau 2011). The biomineralization is the secretion of an organic cell-free matrix (proteins, glycoproteins, lipids and polysaccharides) by the external mantle epithelium (Rousseau, Plouguerné, Wan, Wan, Lopez & Fouchereau-Peron 2003). This process involves several genes, including those of the *pif*, *aspein* and *pearlin* families (Miyazaki, Nishida, Aoki & Samata 2010; Joubert 2011; Marie, Joubert, Tayalé, Zanella-Cléon, Belliard, Piquemal, Cochennec-Laureau, Marine, Gueguen & Montagnani 2012). The maintenance of donor family effect, therefore demonstrated that the pearl sac biomineralization process persisted through the graft culture period and the first surgreffe culture period.

Although a strong correlation existed between nacre thickness and weight ($R^2 = 0.88$ and $P < 0.0001$), the results showed that donor families were not ranked in the same order for average nacre thickness (G, F, H, B, E, C, D, J, I and A) and nacre weight (G, E, F, B, H, C, I, J, D and A) following surgreffe. This difference could be partially explained by another pearl quality trait: shape, commonly classified into four categories, round, oval, baroque and circled. In fact, among these families, two groups could be clearly distinguished: (1) G, F, B, H and E families, which gave thicker and heavier pearls, and (2) C, I, J, D and A families which produced the finest and lightest pearls. For the baroque shape in particular, which is very irregular (often with the presence of nacre bulges), the nacre thickness determination was an approximation, as it could vary for a single pearl seen from different orientation (in our study, the thickness estimation for such samples was made by calculating the largest diameter). By contrast, for a round or semi round pearl, the thickness measurement was more accurate. Therefore, if the baroque pearl rate was different among the different donor families, this could explain the difference observed in family order between nacre thickness and weight.

Nacre thickness, weight and deposition speed

The nacre thickness measured at first surgreffe was significantly lower than following the initial graft. This paradoxical result, could be explained by the size of the nucleus inserted at first surgreffe (3.5 BU), which was larger than the one

used for the initial graft (2.4 BU). This difference in nucleus size corresponded to a surface that was twice as large in the surgreffe (3.45 cm^2), than in the initial graft (1.72 cm^2). For a constant nacre deposition speed, the biomineralization process must therefore have deposited nacre over a larger surface following the surgreffe, than following the initial graft. Consequently, to obtain cultured pearls with the minimum nacre thickness required for sale (more than 0.8 mm for Tahitian cultured pearls), a farmer must leave pearl oysters for a longer culture time on the same grow-out site following surgreffe than he/she would leave the same pearl oysters following the initial graft.

By contrast, the nacre weight measured after first surgreffe was significantly higher than for the initial graft. This difference could be easily explained by the fact that the culture time for oysters following surgreffe was 24 months, instead of 18 months for grafted oysters. This difference of six months therefore influenced nacre weight significantly, through the lengthened period of biomineralization. This was confirmed by the nacre deposition speed, which showed no significant difference between the culture periods following first surgreffe and graft. This means that, during the entire period of culture of both graft and surgreffe, which took three and a half years, the pearl sac metabolic activity was constant and depended on the family considered. Molecular tools (qPCR) could be used to study biomineralization gene expression during culture to gain knowledge about whether gene expression was affected by the biological age of the pearl sac/ recipient oysters. For example, the expression of Pif-177, a gene encoding protein implicated in part of the biomineralization activity, and especially in the aragonite nacreous layer, could be traced in a way to confirm (or not) its constant expression level throughout the culture time (Zhao, He, Huang & Wang 2014).

Conclusion

This study was the first on a *P. margaritifera* surgreffe and allowed the comparison with the corresponding initial graft operation. The results highlight several major implications for the pearl industry management such as: (1) the relation between harvested pearl rate and nucleus size to be inserted in the pearl sac, to increase harvested pearl rate with both surgreffe and graft, (2) the maintenance of donor family effect at first surgreffe scale for nacre thickness, weight and deposition speed, all of which point to the potential benefit of family-based genetic selection, in combination with surgreffe for increasing pearl size; (3) the persistence of the pearl sac metabolic activity over three years of culture.

Further studies should be conducted to analyse the gene expression (qPCR), coding for molluscan shell matrix components, implicated in the biomineralization process, in order to identify the most relevant gene that controlled the pearl sac activities for nacreous deposition, and thus the pearl formation. These genes could then be used as markers to assist genetic selection through the choice of appropriate broodstock and production of selected donor families with high biomineralization capabilities in the hatchery. Increasing pearl size must be considered through a multi-trait selection approach, which should integrate environmental effects, recipient oyster effects, and the correlation of nacre thickness, weight and deposition speed with other qualitative traits (cultured pearl lustre, colour, shape, grade etc…) to avoid any negative co-selection.

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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 surgreffe 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 surgreffe 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-surgreffe 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*, surgreffe, 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 mm^2) 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).

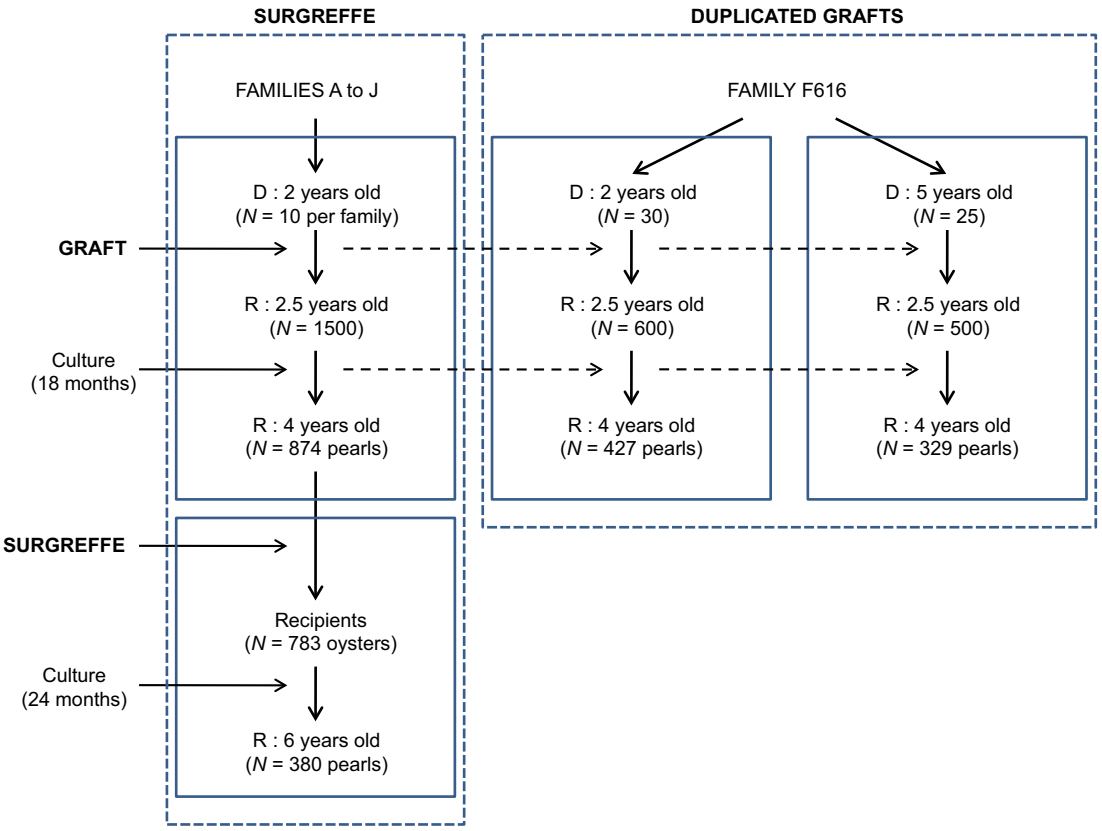

Figure 1 Experimental designs for the surgreffe method (following the experimental graft conducted by Ky *et al.* 2013) and the duplicated grafts. All operations were performed on Rangiroa atoll (Tuamotu Archipelago).

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).

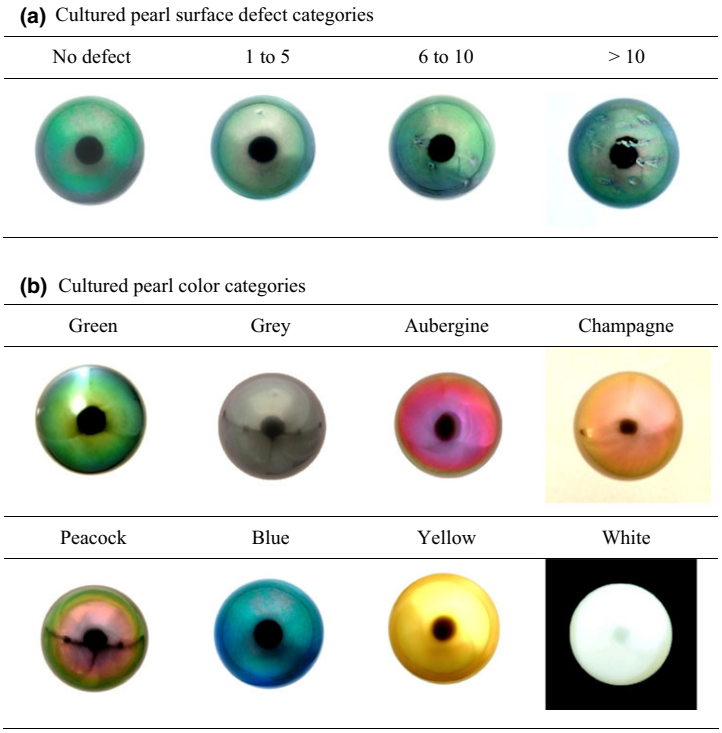

Figure 2 Cultured pearl surface defect (a) and visual colour (b) categories produced by *Pinctada margaritifera*. The picture samples indicate a round pearl shape.

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 (*rebuts*) (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 surgreffe 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 surgreffe experiment. These 295 paired samples made it possible to perform a comparison between graft and surgreffe 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 surgreffe experiment.

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

Results

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

For the 380 harvested pearls produced using the surgreffe 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 surgreffe and graft results ($N = 295$) revealed a highly significant difference ($P < 0.0001$) in average lustre pearl rates of 47.8% (surgreffe) versus 89.1% (initial graft) (Table 1). Thus, there was a −41.3% decrease

Table 1 Comparison between the graft and surgreffe 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	Surgreffe	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 surgreffe 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 surgreffe operations ($P < 0.0001$) are denoted by ***.

in the lustre pearls obtained from the surgreffe 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 surgreffe 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 surgreffe method, in comparison with the graft method (Table 1). In addition, the rate of pearls with no defects was seven times smaller. Using surgreffe, 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 surgreffe 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 surgreffe 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 surgreffe 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 surgreffe 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 surgreffe ($P = 0.484$). Considering the families separately, highly significant differences were found for surface defects between surgreffe and graft methods for all families. By contrast, a significant donor family effect was observed for the grade categories when surgreffe 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 surgreffe and graft methods across all grade categories for all the families (Fig. 3).

Surgreffe effect on cultured pearl darkness level and colour

Looking at the pearl darkness level obtained using surgreffe, 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

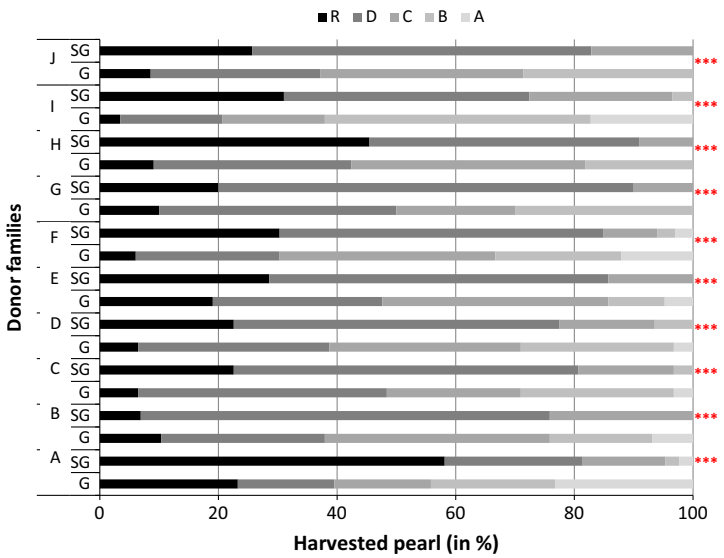

Figure 3 Comparison of culture pearl grade (A, B, C, D and R – reject) rate (in %) from graft (G) to surgreffe (SG), for each of the *Pinctada margaritifera* donor families (A–J). The difference between G and SG methods found to be very highly significant ($P < 0.0001$) are denoted by ***.

($N = 96$). Comparing the surgreffe 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 surgreffe method. In terms of the pearl colours obtained by surgreffe, 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 surgreffe 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 surgreffe 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 surgreffe 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 surgreffe 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 surgreffe ($P < 0.0001$). 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 surgreffe 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 surgreffe 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	Surgreffe	Significance
Darkness	High	27.80	18.98	*
		(82)	(56)	
	Moderate	48.14	54.58	NS
		(142)	(161)	
	Low	24.06	26.44	NS
		(71)	(78)	
Colour	Green	56.27	43.73	**
		(166)	(129)	
	Grey	23.39	30.85	*
		(69)	(91)	
	Aubergine	5.42	8.14	NS
		(16)	(24)	
	Champagne	4.75	7.80	NS
		(14)	(23)	
	Peacock	4.07	2.37	NS
		(12)	(7)	
	White	4.07	5.08	NS
		(12)	(15)	
	Yellow	2.03	2.03	NS
		(6)	(6)	

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').

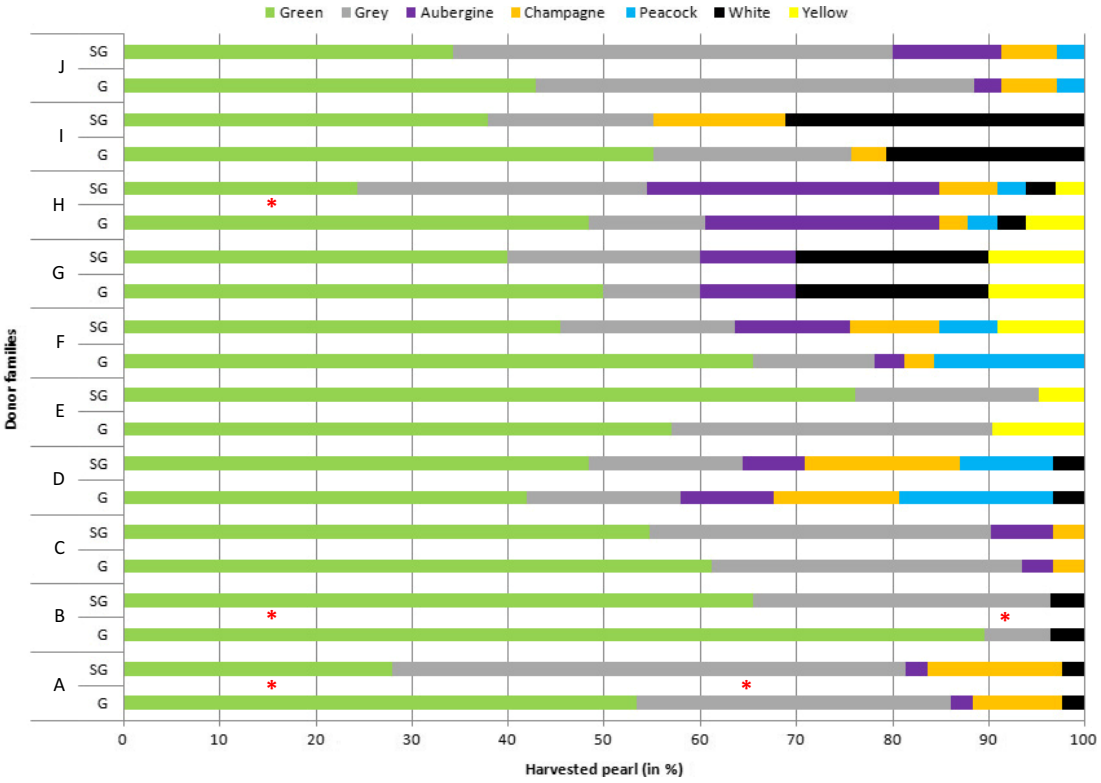

Figure 4 Comparison between the cultured pearl colour rates (green; grey; aubergine, champagne, peacock, white, and yellow) (in %) in the graft (G) and surgreffe (SG) experiments for each of the *Pinctada margaritifera* donor families (A–J). Significant differences between the graft and surgreffe methods 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	87.3	NS
		(388)	(288)	
	No	9.1	12.7	NS
		(39)	(41)	
Surface defects	0	34.2	16.1	***
		(146)	(53)	
	1 to 5	37.2	37.9	NS
		(159)	(124)	
	6 to 10	26.0	41.5	***
		(111)	(137)	
	>10	2.5	4.5	NS
		(11)	(15)	
Grade	A	0.7	0.6	NS
		(3)	(2)	
	B	3.3	9.1	***
		(14)	(30)	
	C	27.2	36.1	*
		(116)	(118)	
	D	35.8	25.5	*
		(153)	(94)	
	R	33.0	25.8	*
		(141)	(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').

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	8.2	NS
		(40)	(27)	
	Moderate	78.5	78.2	NS
		(335)	(257)	
	Low	12.2	13.6	NS
		(52)	(45)	
Colour	Green	42.6	11.8	***
		(182)	(39)	
	Grey	44.7	68.2	***
		(191)	(225)	
	Aubergine	0.2	7.3	***
		(1)	(24)	
	Blue	4,4	0.6	***
		(19)	(2)	
	Peacock	4.0	4.5	NS
		(17)	(15)	
	White	2.8	3.3	NS
		(12)	(11)	
	Yellow	1.2	4.2	**
		(5)	(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').

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 ($P = 0.007$), 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, Cochennec-Laureau, Gueguen & Montagnani 2010; Montagnani, Marie, Marin, Belliard, Riquet, Tayalé, Zanella-Cléon, Fleury, Gueguen & Piquemal 2011; Marie, Joubert, Tayalé, Zanella-Cléon, Belliard, Piquemal, Cochennec-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 surgreffe 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 surgreffe 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 surgreffe 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 surgreffe operation harvest), and the recipient oysters themselves (4 years old in the graft operation harvest and 6 years old in the surgreffe 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 *Laternulla 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 surgreffe 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-surgreffe 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-surgreffe 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-surgreffe experiment, where its rate was halved. This finding is supported by the similar pearl lustre rate found between the grafts realized before the surgreffe 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-surgreffe 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-surgreffe 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 *Pintada 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 surgreffe 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, Ressmann, 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-surgreffe 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-surgreffe 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 surgreffe operations, where younger and larger recipient oysters could be used for initial graft in order to move the graft-surgreffe sequence forward in relation to recipient age and thus increase the rates of lustre and colour in pearls produced by the surgreffe 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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Effect of electrolysis treatment on the biomineralization capacities of pearl oyster *Pinctada margaritifera* juveniles

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ABSTRACT

The present study investigated the effect of electrolysis on the biomineralization capacities of juveniles of the mollusk *Pinctada margaritifera* for the first time. Size-selected individuals from two groups, "Medium" and "Large", from a multi-parental family produced in a hatchery system were subjected to electrolysis under a low voltage current over a nine-week experimental period. The growth of the juveniles was individually monitored and assessed weekly by wet weight and shell height measurements. At the end of the experiment, mantle tissue was sampled for biomineralization-related gene expression analysis. Electrolysis significantly increased pearl oyster growth in terms of shell height and wet weight for Large juveniles from the 5th and the 2nd week, respectively, until the end of the experiment. However, differences were only significant for Medium individuals from the 7th week for shell height and from the 9th week for wet weight. Furthermore, transcriptional analysis of six known biomineralization genes coding for shell matrix proteins of calcitic prisms and/or nacreous shell structures revealed that five were significantly overexpressed in the mantle mineralizing tissue under electrolysis: three in common between the two size class groups and two that were expressed exclusively in one or the other group. Finally, we found no statistical difference of the shell thickness ratio between individuals undergoing electrolysis and control conditions. Taken together, our results indicate, for the first time in a calcifying marine organism, that electrolysis influences molecular mechanisms involved in biomineralization and may stimulate some parameters of pearl oyster growth rate.

1. Introduction

The cultured pearl industry, with around US$784 million worth of production in 2005 (Tisdell and Poirine, 2008), is of great economic importance for a number of countries in tropical and subtropical regions. In French Polynesia, the black-lip pearl oyster *Pinctada margaritifera* "Linnaeus 1758" is the top aquaculture species and the basis of the mass production of a unique gem built by a living organism. Not only is pearl culture the second highest economic resource of French Polynesia (65 million Euros export value in 2013, customs statistics, Wane, 2013), but it also represents an important source of employment (nearly 5000 people employed on 487 farms in 2013) (Ky et al., 2014). However, since the early 2000s, this industry has suffered a severe crisis, mainly due to overproduction and a slowdown of the world economy, leading to a dramatic fall in mean pearl value per gram. Pearl size and quality are among the most important factors that go into determining pearl value (Blay et al., 2014). Increasing cultured pearl quality, through cultural practices and/or genetic selection, is the biggest challenge for research and development.

Production of cultured pearls is achieved starting with a surgical operation called "grafting" carried out by skilled technicians. A small piece of mantle tissue is removed from a donor oyster to be inserted into the gonad of a recipient oyster, along with a spherical

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nucleus made of mollusk shell or synthetic material (Kishore and Southgate, 2014; Taylor and Strack, 2008; Cochennec-Laureau et al., 2010). *P. margaritifera* recipient oysters are used for graft operations when their shell height has reached 11 cm, at approximately two years of age (Gervis and Sims, 1992). An additional 18- to 24-month period is required to produce a pearl with a sufficiently thick layer of nacre (0.8 mm) for harvest. In French Polynesia, *P. margaritifera* shell growth increments are highly variable, with higher growth rates in island lagoons and the open ocean compared with the atoll lagoons where they are usually reared (Pouvreau and Prasil, 2001). Improving pearl oyster growth and reducing the length of the culture time needed to reach a suitable size for graft operations would contribute significantly to increase the cost-effectiveness of the industry. Moreover, recipient pearl oyster shell increments are correlated with the pearl nacre deposition rate (Coeroli and Mizuno, 1985; Le Pabic et al., 2016). Thus, producing larger pearl oysters would potentially lead to the formation of thicker nacre layers.

P. margaritifera shell growth relies on the formation of a mineral phase composed of layers of calcium carbonate and an organic matrix containing mostly proteins, glycoproteins, lipids and polysaccharides (Joubert et al., 2010; Levi-Kalisman et al., 2001). This organic matrix, secreted by the epithelial cells of the external mantle, controls nucleation, orientation, growth, and the polymorphism of the calcium carbonate crystals formed as aragonite or calcite (Mann, 1988; Belcher et al., 1996). Shell matrix proteins play a major role in the shell biomineralization process. Some genes encoding matrix proteins have been identified and are known to be specifically involved in the formation of the nacreous layer and/or prismatic layer (Joubert et al., 2010; Montagnani et al., 2011; Marie et al., 2012). For example, the genes *Pif 177* and *MSI60* are involved in shell nacreous layer formation by regulating aragonite crystal growth (Suzuki et al., 2009; Sudo et al., 1997). Shematrin proteins are secreted into the prismatic layer where they are thought to establish a structure for calcitic prism formation (Yano et al., 2006). Prismalin 14 controls calcitic prism calcification (Suzuki et al., 2004), and Aspein is thought to play a key role in calcite precipitation (Isowa et al., 2012). In contrast, some proteins such as Nacrein are involved in both the aragonite and calcite mineralization processes (Miyamoto et al., 2013).

The mineral accretion method, based on the electrolysis of seawater, involves a low-voltage direct electrical current through two submerged electrodes to induce deposition of dissolved minerals on conductive substrates (Hilbertz, 1979). Seawater is split into hydrogen gas H_2 and hydroxide ion HO^-, leading to an increase of the pH in the vicinity of the cathode. Calcium ions Ca^{2+} from seawater combine with dissolved bicarbonate HCO_3^- to precipitate as aragonite $CaCO_3$ and magnesium ions Mg^+ with hydroxide ions to precipitate as brucite $Mg(OH)_2$. Several experiments have been conducted to study the effect of this mineral accretion method on survival and growth rate of marine calcifying organisms, such as corals and oysters (Borell et al., 2010; Piazza et al., 2009; Sabater and Yap, 2002, 2004; van Treeck and Schuhmacher, 1997). Results vary considerably, since some studies on the effect of the mineral accretion method report increased survival rate of coral transplants (van Treeck and Schuhmacher, 1997; Sabater and Yap, 2002) and enhanced coral growth rate (Sabater and Yap, 2004) whereas other studies show lower growth rates for juvenile oysters (Piazza et al., 2009) and no effect or a negative effect on coral survival (Borell et al., 2010).

Surprisingly, studies on the effect of electrolysis on mollusk and coral biomineralization have only focused on biometric analysis of calcifying tissues. Indeed, to our knowledge, no molecular approaches have yet been explored to characterize biomineralization processes under electrolysis treatment. With the advent of proteomic, transcriptomic, and genomic technologies, several biomineralization-related proteins, referred to as the biomineralization "toolkit" have been recently identified in the pearl oyster *P. margaritifera* (Marie et al., 2012).

This study is the first aiming to investigate the effect of electrolysis on the biomineralization capacities of the black-lipped pearl oyster *P. margaritifera*. Some growth parameters (shell thickness, height, animal weight) and the expression level of six biomineralization-related genes were measured in juvenile *P. margaritifera*.

2. Materials and methods

2.1. Biological material

A multi-parental family was produced in the Ifremer hatchery facilities in Vairao (Tahiti, French Polynesia) using a cross between three female and six male broodstock oysters. Artificial spawning, larval rearing, and oyster culture were conducted as described in Ky et al. (2013). Juveniles were reared in the same natural environment, in Aquapurse® plastic trays suspended on long lines located in Vairao lagoon (Tahiti). At 180 days post fertilization, oysters were categorized into two groups according to their shell size: 40 "Medium" size (mean shell height of 3.8 cm ± 0.4 and mean wet weight of 5.64 g ± 1.47) and 30 "Large" size (mean shell height of 5.1 cm ± 0.4 and mean wet weight of 12.44 g ± 3.42). All pearl oyster juveniles were transferred by airplane from Vairao lagoon to Bora Bora lagoon (GPS location, 16.528553 S, 151.768184 E, French Polynesia).

2.2. Experimental design

Two conditions were tested for an experimental period of nine weeks in the lagoon of Bora Bora using a total of 70 pearl oysters: electrolysis using low-voltage electric current and control conditions (no electrolysis). Twenty Medium and 15 Large juvenile pearl oysters were randomly selected and subjected to each condition. These pearl oysters were randomly hung on chaplets (ropes) in two Aquapurse® plastic trays to prevent predation from shellfish and fish (Fig. 1). Pearl oysters under electricity were placed on a steel structure subjected to a low-voltage current of 3.7 V, flowing between the positively charged anode and the negatively charged cathode. The electrolysis structure was switched on every other hour from 4 a.m. to 7 p.m. alternating with periods of an hour with no current. This structure was used two months prior to the oyster experiment so that mineral accretion occurred at the cathode where calcium carbonate and magnesium hydroxide were deposited. Both electrically charged structures and the identical uncharged control structures were fixed to pillars at 3.5 m depth set 20 m apart from one another.

Tagged juvenile pearl oysters were individually measured weekly for shell height and live weight. For each individual, absolute cumulative shell growth and wet weight gain were calculated by the formula $PR = (100 \times (V_W - V_{W0}))/V_{W0}$, where PR is the percent change, V_W the present value by week "W" and V_{W0} the initial value by week W0 when oysters were placed on the charged and uncharged (control) structures. After nine weeks of monitoring, all the pearl oyster juveniles were collected.

2.3. Mantle gene expression

For gene expression analysis, mantle tissue samples from four to five randomly chosen individuals were pooled for each of the tested conditions (electrolysis versus control), resulting in three and five pools per condition for Large and Medium oyster batches,

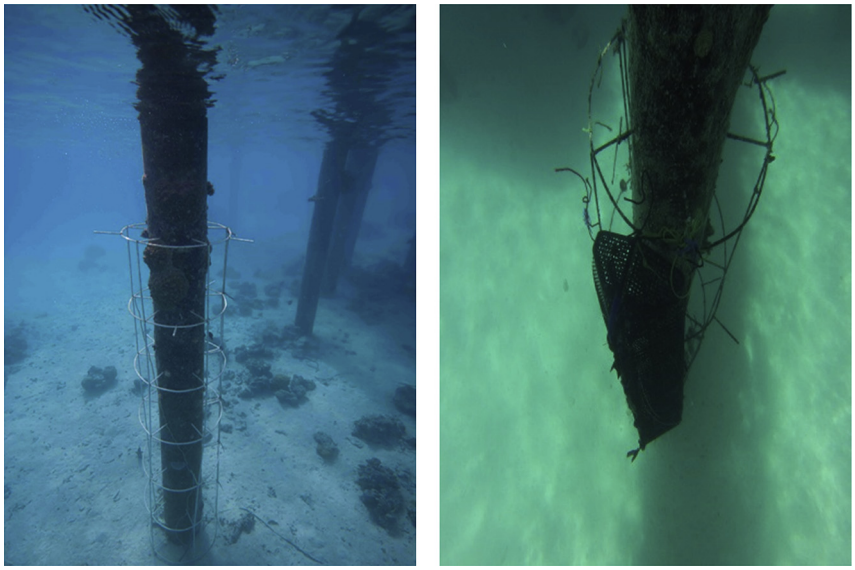

Fig. 1. Photograph of the empty culture structure (left), to which an Aquapurse plastic tray was fixed containing chaplets of Large and Medium pearl oysters (right).

respectively. Total cellular RNA was extracted 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 was treated with DNase (Ambion) to degrade any potential DNA contaminants. The expression levels of six biomineralization-related genes were analyzed by quantitative RT-PCR analysis using a set of forward and reverse primers (Table 1). Three other genes were used as housekeeping genes, including 18S rRNA (Larsen et al., 2005), REF1 (Joubert et al., 2014) and GAPDH (Lemer et al., 2015). First-strand cDNA was synthesized from 400 ng of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) and a combination of random hexamer and oligo(dT) primers, in a final reaction volume of 25 μl. Quantitative PCR (qPCR) amplifications were carried out on a Stratagene MX3000P, using Brilliant II SYBR Green QPCR Master Mix (Stratagene) with 400 nM of each primer and 10 μL of 1:100 diluted cDNA template. The PCR reactions consisted of a first step of 10 min at 95 °C followed by 40 cycles (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.

Expression levels were estimated by evaluating the fluorescence signal emitted by SYBR-Green®. This fluorescent marker binds to double-stranded DNA (dsDNA) and the fluorescence emitted is proportional to the dsDNA present in the reaction mix. Calculations are based on cycle threshold (Ct) values. The relative gene expression ratio of each biomineralization-related gene was calculated following the delta–delta method normalized with three reference genes (Livak and Schmittgen, 2001), which is defined as: ratio $= 2^{-[\Delta Ct\ sample - \Delta Ct\ control]} = 2^{-\Delta\Delta Ct}$. In this formula, the ΔCt control represents the mean of the ΔCt values obtained for each target gene in control pearl oysters.

2.4. Shell labeling and thickness ratio

One day before shipment to Bora Bora Island, the seventy oysters were immersed for 12 h in a 150 mg L^{-1} calcein (Sigma Aldrich) solution prepared with 0.1-μm filtered seawater. After the experimental period of nine weeks, shells were sawn along the dorso-ventral axis using a "SwapTop Trim Saw" machine (Inland, Middlesex, United Kingdom). Ventral sides of shell cross sections were observed by epifluorescence microscopy under a Leica DM400B UV microscope (I3 filter block and LAS V.8.0 software for size measurements). The shell thickness ratio was measured by dividing the thickness of the new nacre deposits formed during the nine-week experimental period by the total thickness of the shell cross section. A mean of two measurements was calculated for each cross section.

2.5. Statistical analysis

Normality of data distribution and homogeneity of variance were tested using the Shapiro-Wilk test and Bartlett test, respectively. Data analysis was performed at 5% alpha level using XLSTAT (version 1.01, 2014). As the assumptions for parametric tests were not met for shell height growth and wet weight gain data, even after an arcsine square root transformation, we used the Kruskal-

Table 1
Set of forward and reverse primers used in the gene expression analysis.

Gene	GenBank accession numbers	Forward primer (5′–3′)	Reverse primer (5′–3′)
PIF 177	HE610401	AGATTGAGGGCATAGCATGG	TGAGGCCGACTTTCTTGG
MSI60	SRX022139[a]	TCAAGAGCAATGGTGCTAGG	GCAGAGCCCTTCAATAGACC
Nacrein A1	HQ654770	CTCCATGCACAGACATGACC	GCCAGTAATACGGACCTTGG
Shematrin 9	ABO92761	TGGTGGCGTAAGTACAGGTG	GGAAACTAAGGCACGTCCAC
Prismalin 14	HE610393	CCGATACTTCCCTATCTACAATCG	CCTCCATAACCGAAAATTGG
Aspein	SRX022139 [a]	TGAAGGGGATAGCCATTCTTC	ACTCGGTTCGGAAACAACTG

[a] SRA accession number; EST library published in Joubert et al., 2010.

Wallis test to test for differences between treatments (electrolysis vs control). As the overall test was significant, a Dunn procedure with a Bonferroni correction was performed to determine which means were significantly different. Pearson's correlation coefficient was used to measure the correlation between shell height and wet weight for Medium and Large pearl oysters.

Shell thickness ratio was analyzed using the arcsine square root transformation. The data followed the conditions for application of parametric tests and the effect of the treatment (electrolysis or control) was tested using a one-way ANOVA.

The expression values of the six candidate genes did not meet the conditions for parametric tests. Kruskal-Wallis tests were therefore used to test for differences in gene expression between the treatments (electrolysis vs control). As the overall test was significant, a Dunn procedure with a Bonferroni correction was performed to determine which means were significantly different.

3. Results

3.1. Juvenile growth: shell height and oyster weight

Whatever the size group (Large or Medium) or type of treatment (electrolysis or control), no mortality of juveniles was observed during the nine weeks of the experiment. Pearson's correlation coefficient revealed a significant positive correlation between shell height and wet weight for Medium and Large individuals by each week ($\rho = 0.622$ with p-value < 0.0001 for Medium individuals and $\rho = 0.693$ with p-value < 0.0001 for Large individuals). However, we decided to study these two parameters separately for both size-class group and condition. Shell height growth rate was higher for Medium juveniles subjected to electrolysis in comparison to the control. Shell height growth increased from 1.2% (week 1) to 7.3% (week 8) in electrolysis conditions and from 0.9% (week 1) to 4.9% (week 8) in control conditions (Fig. 2a). The difference was only significant by the seventh week of the experiment. For the Large juveniles group, the growth rate increased from 0.5% (week 1) to 6.42% (week 8) under electrolysis conditions and from 0.5 to 3.6% under control conditions (Fig. 2b). The difference was significant from weeks 5 to 8.

Wet weight gain of Medium juveniles increased from 1.9 to 11.3% and from 2.1 to 8.4% under electrolysis and control conditions, respectively (Fig. 3a). The difference was significant by the ninth week. For Large individuals, the wet weight gain increased from 2.5 to 10.2% and from 1.2 to 3.7% under electrolysis and control conditions, respectively (Fig. 3b). The difference was significant from weeks 2 to 9.

3.2. Shell thickness ratio

Shell thickness ratio represents the thickness of aragonite deposited during the experiment divided by the total thickness of the shell cross section (Fig. 4). The mean shell thickness ratio and standard error (SE) varied from 24.8% ± 3.1 to 28.3% ± 3.6 for Medium juveniles and from 26.3% ± 7.2 to 28.0% ± 5.3 for Large juveniles under electrolysis and control conditions, respectively. There were no statistically significant differences between the electrolysis treatment and the control for either Medium or Large juveniles according to one-way ANOVA.

3.3. Mantle gene expression

For juvenile oysters belonging to the Medium group, all the six biomineralization-related targeted genes were strongly up-regulated by electrolysis in comparison to the control (without electrolysis), with expression ratios ranging from 17.02 to 90.09 for the *Pif 177* and *Shematrin 9* genes, respectively (Fig. 5a). Despite great variation in the expression levels of control oysters, the expression ratios of four genes were significantly higher after electrolysis treatment: *Pif 177* ($p = 0.016$), *Prismalin 14* ($p = 0.016$), *Shematrin* 9 ($p = 0.009$), and *Aspein* ($p = 0.009$). Similar results (but of lower amplitude) were obtained for the Large juveniles group, in which *Nacrein*, *Shematrin 9*, *Prismalin 14*, and *Aspein* were significantly upregulated by electrolysis with *p*-values of 0.017, 0.008, 0.001, and 0.003, respectively (Fig. 5b). Overall, the results revealed that *MSI60* was the only gene in the panel tested that was not significantly regulated by electrolysis.

4. Discussion

The effect of electrolysis on the pearl oyster *P. margaritifera* biomineralization process was evaluated in this study. Measurements of the growth rate (shell height, wet weight and shell thickness ratio) as well as the levels of expression of a panel of six biomineralization-related genes were assessed in the calcifying pearl oyster.

4.1. Electrolysis may increase some growth rate parameters in Pinctada margaritifera

P. margaritifera growth rate depends on a combination of genetic and environmental factors (Pouvreau and Prasil, 2001; Mavuti et al., 2005), making it highly variable among rearing sites. In our experiment, we used individuals issued from a limited number of parents to minimize genetic influence on the results. On the one hand, *P. margaritifera* growth rates (shell height and wet weight) were only significantly higher for Medium individuals subjected to electrolysis compared to the control at the 7th week and the 9th week, respectively. This group showed higher variability than Large individuals for the shell height growth measures regardless of the treatment. This variability could have potentially masked the electrolysis effect, preventing its detection. Experiment with a greater number of oysters is necessary to test this hypothesis. On the other hand, Large juvenile shell height and wet weight growth rates were significantly higher for individuals subjected to electrolysis compared with the control from the 5th and the 2nd week, respectively, until the end of the experiment.

Growth performance is of great interest for the reduction of bivalve mortality. Johnson and Smee (2012) found an inverse relationship between bivalve size and susceptibility to predation. Juvenile *P. margaritifera* pearl oysters are particularly vulnerable to predation, and the presence of predators could reduce shell growth rates (Pit and Southgate, 2003). However, we found no difference in shell thickness ratio between individuals subjected to electrical current and those in control conditions. According to Crossland (1911), shell growth of pearl oysters usually begins with a rapid increase in the shell height to reach a maximum size, which is then followed by shell thickness growth. Thus, the ratio of the shell thickness to the shell length increases with age for pearl oysters of the genus *Pinctada* (Hynd, 1955). As pearl oysters in our study were juveniles, they might well have invested their energy in shell length increment rather than shell thickness. Similar experiments should be repeated with older *P. margaritifera* individuals to test this hypothesis.

4.2. Electrolysis stimulates some biomineralization-related gene expression levels in Pinctada margaritifera

Despite the use of several pools of animals (n = 5), the same environmental rearing conditions and individuals issued from a limited number of parents to minimize genetic influence of

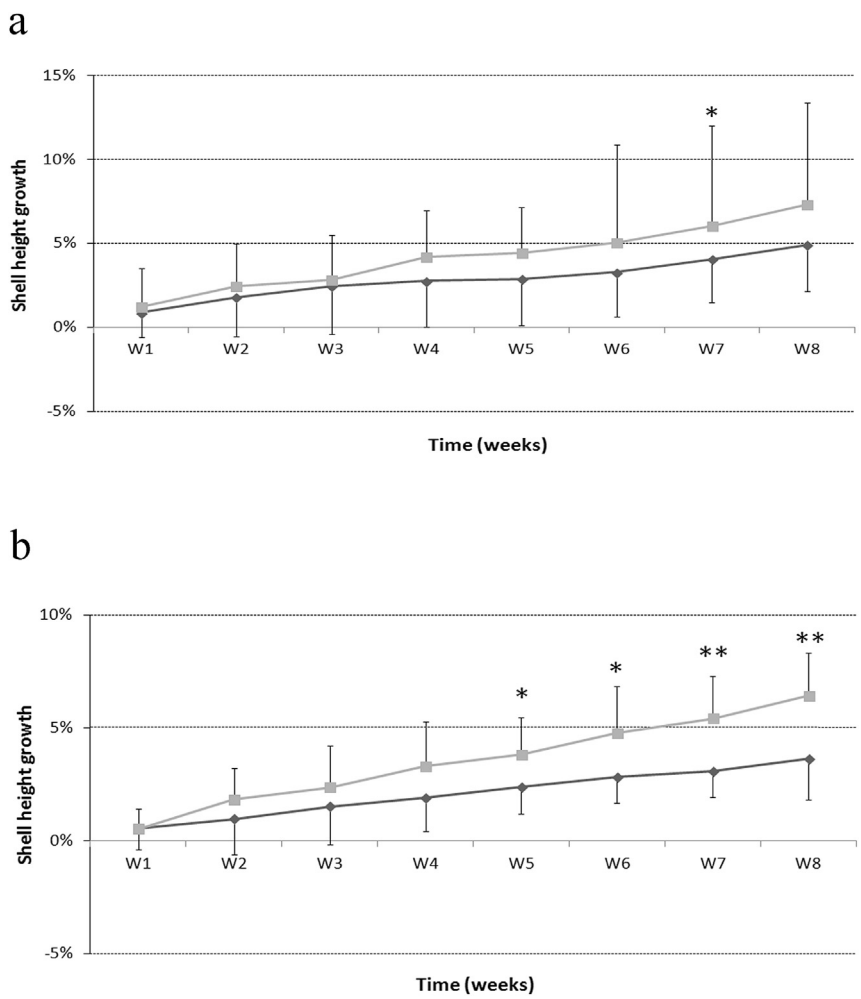

Fig. 2. Average shell height growth (expressed in %) for Medium (a) and Large (b) *P. margaritifera* juveniles (n = 20 per condition for Medium individuals and n = 15 for Large individuals). Shell heights were measured each week in treatments with (light grey, square symbol) and without (dark grey, diamond-shaped symbol) electrolysis. At the beginning of the experiment (W0), absolute average shell heights were 3.71 cm ± 0.44 and 3.92 cm ± 0.38 in Medium pearl oysters in control and electrolysis conditions, respectively. They were 5.05 cm ± 0.42 and 5.11 cm ± 0.32 in Large pearl oysters in control and electrolysis conditions, respectively. Cumulative shell height growth was calculated with the formula $PR = (100 \times (V_W - V_{W0}))/V_{W0}$, where PR is the percent change, V_W the present value by week "W" and V_{W0} the initial value by week W0. Error bars indicate standard deviations; statistical analysis is based on the Kruskal-Wallis test and Dunn's test with Bonferroni correction. Statistical significance is indicated by asterisks as follows: $^*p < 0.05$, $^{**}p < 0.01$. Week 9 data (W9) are missing due to a technical problem during measurement.

parentage, wide-ranging variations in gene expression levels were observed, mainly in Medium oysters. A high variability in gene expression has already been reported in *Pinctada margaritifera* (Lemer et al., 2015), both in pooled (n = 2 with 5 individuals per pool) and individual (n = 10) analyses, targeting genes potentially involved in the color of the nacreous layer of the pearl oyster, most of which are also involved in biomineralization of the nacreous and calcitic layers, such as *Pif 177* and *Shematrin 9*, respectively. In the present study, the transcript levels of the *Pif 177*, *Shematrin 9*, *Prismalin 14*, and *Aspein* genes were significantly higher for the electrolysis treatment than for the control conditions in Medium individuals.

Regarding the Large individuals, the relative expression levels of *Nacrein*, *Shematrin 9*, *Prismalin 14*, and *Aspein* genes were significantly higher for the electrolysis treatment than for the control. Two of the studied genes, *Pif 177* and *Shematrin 9*, have previously been found to be positively correlated with shell deposition rates in *P. margaritifera* (Joubert et al., 2014). Only *MSI60* gene expression levels were not statistically different between the two treatments for either of the two size-selected groups used in our study. Interestingly, Joubert et al. (2014) found a significant negative correlation between the expression level of this gene and shell deposition rate.

Our results suggest that some biomineralization-related genes could be up-regulated by electrolysis. Biomineralization is an energetically costly process, with the production of skeletal organic matrix, which is considered to be more demanding metabolically than the crystallization of calcium carbonate (Palmer, 1983). The cost of calcification was calculated as equivalent to 75% and 410% of the energy invested in somatic growth and reproduction, respectively, for the gastropod *Tegula funebralis* (Palmer, 1992). In our experiment, the higher abundance of biomineralization-related transcripts could result from extra energy transfer to the mantle for shell matrix protein synthesis. Concerning the present study, it would be of interest to identify *P. margaritifera* genes involved in the metabolism of ATP, such as the F1-β-subunit found in *P. fucata* (Liu et al., 2007), in order to further quantify their expression levels in the mantle and better understand the effect of electrolysis on biomineralization processes.

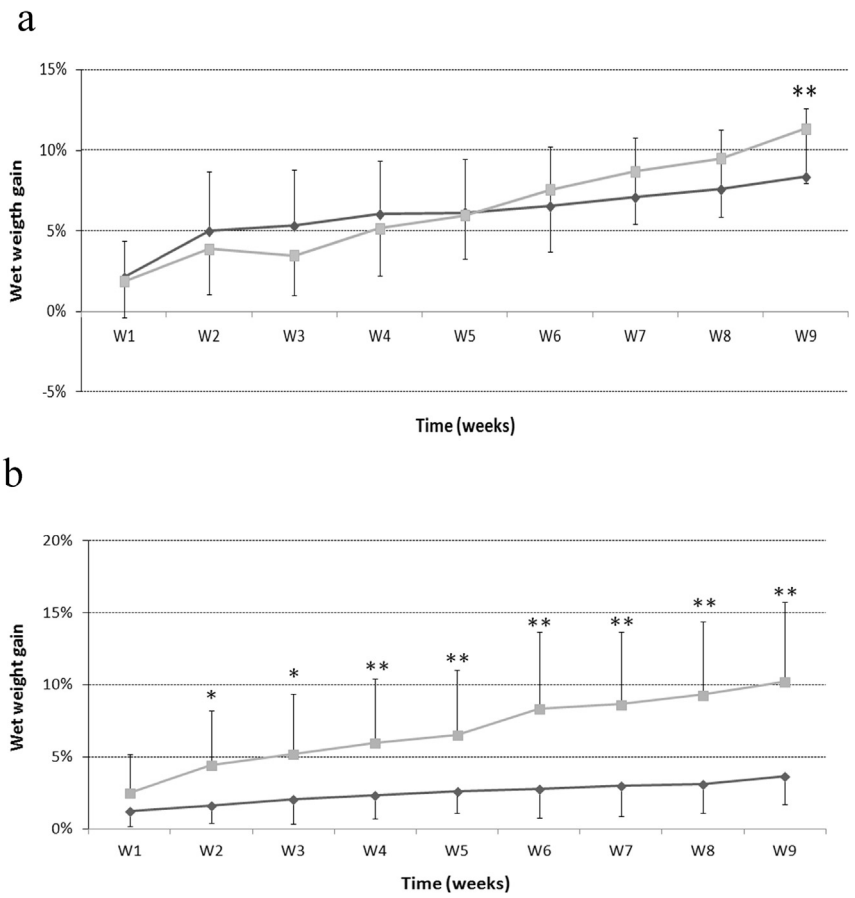

Fig. 3. Average wet weight gain (expressed in %) for Medium (a) and Large (b) *P. margaritifera* juveniles (n = 20 per condition for Medium individuals and n = 15 for Large individuals). Juveniles were weighed each week in treatments with (light grey, square symbol) and without (dark grey, diamond-shaped symbol) electrolysis. At the beginning of the experiment (W0), absolute average weights were 5.24 g ± 1.51 and 6.04 g ± 1.34 in Medium pearl oysters in control and electrolysis conditions, respectively. They were 13.02 g ± 3.68 and 11.87 g ± 3.15 in Large pearl oysters in control and electrolysis conditions, respectively. Cumulative wet weight gain was calculated with the formula $PR = (100 \times (V_W - V_{W0}))/V_{W0}$, where PR is the percent change, V_W the present value by week "W" and V_{W0} the initial value by week W0. Error bars indicate standard deviations; statistical analysis is based on the Kruskal-Wallis test and Dunn's test with Bonferroni correction. Statistical significance is indicated by asterisks as follows: $^*p < 0.05$, $^{**}p < 0.01$.

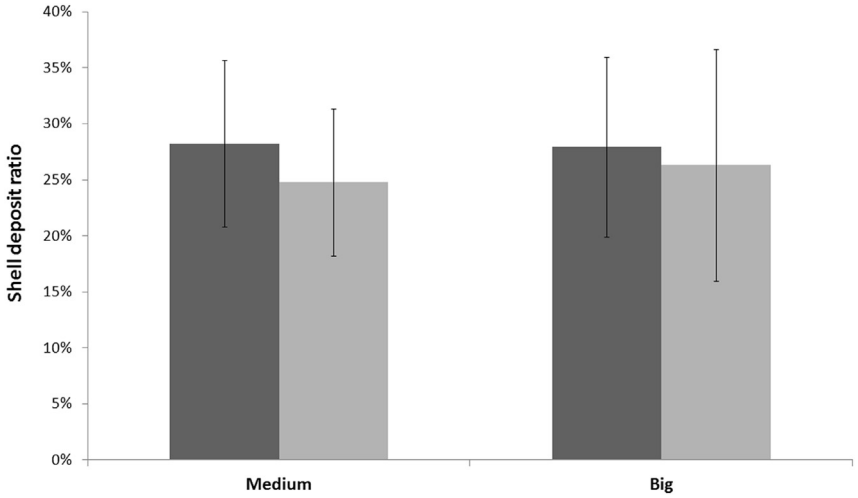

Fig. 4. Average shell thickness ratio for Medium and Large *P. margaritifera* juveniles after 9 weeks with (light grey) or without (dark grey) electrolysis. Shell deposit ratios were measured by dividing the thickness of the deposits formed during the experiment by the total thickness of the cross section of the shells and expressed as a percentage. A mean of two measurements was calculated for the cross section of each individual. Error bars indicate standard deviation. No statistically significant differences were found between group means using one-way ANOVA.

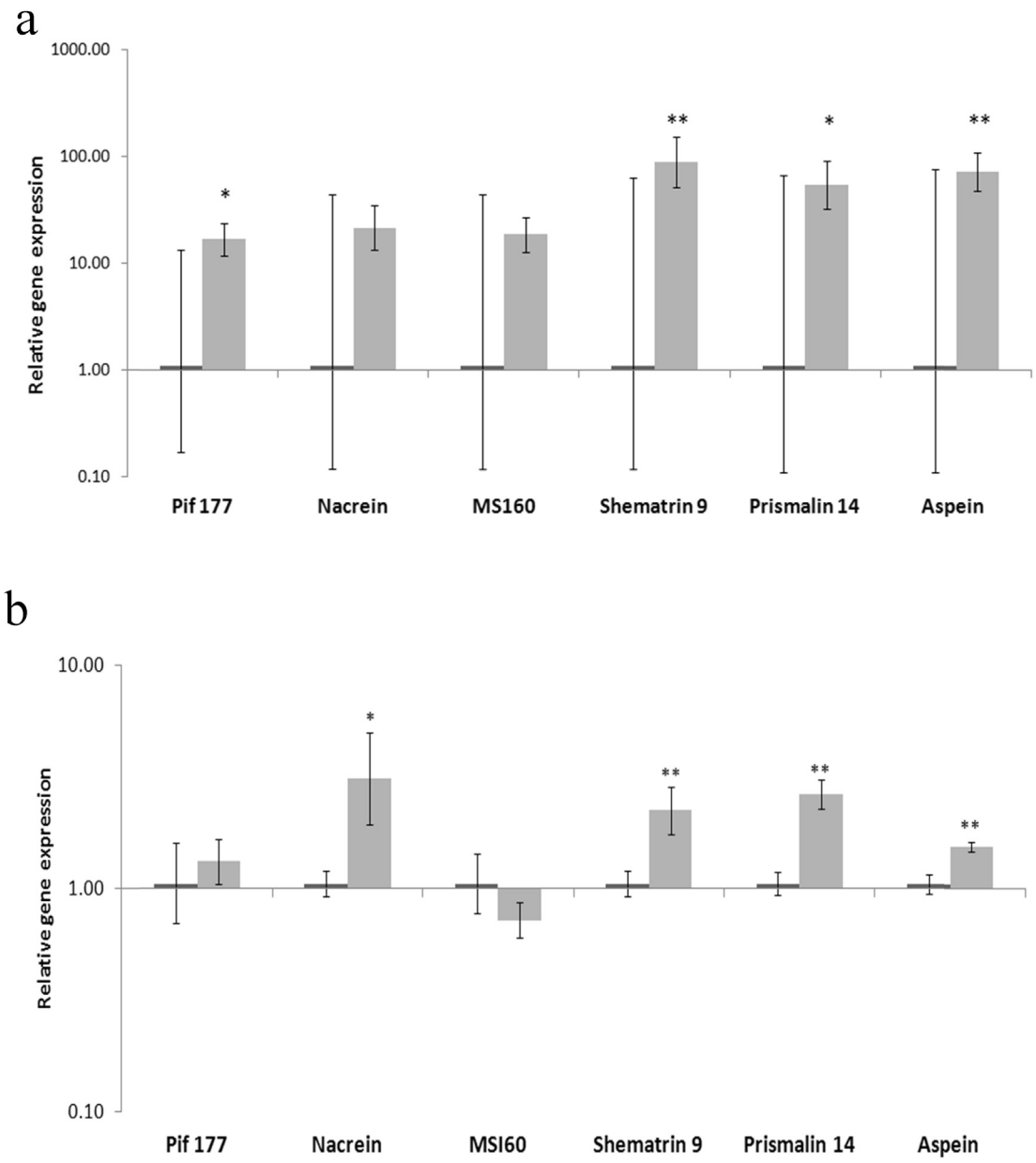

Fig. 5. Mean relative expression of genes coding for proteins involved in the formation of the nacreous layer (Pif 177, MS160), prismatic layer (Shematrin 9, Prismalin 14, Aspein) and both the prismatic and the nacreous layers (Nacrein), following 9 weeks of exposure of Medium (a) and Large (b) oysters to treatments with (light grey) and without (dark grey) electrolysis. The fold change means were calculated from five pools of four individuals (a) and from three pools of five individuals (b) for each treatment, respectively. Y axes are in the logarithmic scale. Error bars indicate standard deviations; statistical analysis is based on Kruskal-Wallis test and Dunn's test with Bonferroni correction. Statistical significance is indicated by asterisks as follows: $^{*}p < 0.05$, $^{**}p < 0.01$.

5. Conclusion

We show that electrolysis may enhance some growth rate parameters in *Pinctada margaritifera*. Our findings also indicate that some biomineralization-related genes are overexpressed under electrolysis compared with control conditions. However, we found no significant differences in shell thickness ratio between the treatments for either of the two size-class groups studied. Individuals in our study were juveniles and might have invested their energy in shell length increment rather than thickness growth. Stimulating pearl oyster growth to more rapidly reach a size suitable for the graft operation would significantly help to increase the cost-effectiveness of the pearl industry. Furthermore, these first results open the way for the evaluation of electrolysis effects on: 1) selected donor oyster lines with high potential for nacre deposition as pearl oyster aquaculture takes a long time (18–24 months), and 2) cultured pearl quality traits, especially nacre thickness, as size remains one of the most important traits for pearl value.

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Influence of preoperative food and temperature conditions on pearl biogenesis in *Pinctada margaritifera*

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ABSTRACT

Trophic conditions and water temperature strongly influence bivalve physiological processes and metabolism. In black-lip pearl oyster *Pinctada margaritifera*, these parameters have been shown to affect shell biomineralization. The present study investigated the effect of preoperative food level (i.e., microalgal concentration) and temperature on pearl biomineralization. Donor and recipient oysters were conditioned at different levels of food and temperature during the preoperative phase to evaluate the influence of these factors on 1) pearl retention rate (grafting success), 2) expression of genes involved in biomineralization in the mantle and pearl sac and 3) pearl quality traits. Our study confirmed the influence of both microalgal concentration and temperature on shell growth. Food level of donor oysters was decisive for pearl biomineralization, with donors that had been fed at a high microalgal concentration producing pearl sacs with significantly higher biomineralization capabilities and faster nacre establishment during early stages of pearl formation. However, food level showed no effects on quality traits of the pearls harvested 12 months postgrafting, while preoperative temperature only influenced the relative expression of two genes in pearl sacs at 12 months postgrafting. No significant effects of the preoperative conditioning of recipient oysters were detected in either experiment considering gene expression measurements and pearl quality traits. However, mortality was significantly lower in grafted recipient oysters fed at an intermediate trophic level. Finally, pearl weight was shown to be positively correlated with recipient oyster growth.

1. Introduction

The black-lip pearl oyster *Pinctada margaritifera* (Linnaeus 1758) is farmed to produce black cultured pearls – unique gems generated by a living organism – in several countries in tropical and subtropical regions. In French Polynesia, pearl production is a major industry, with the exportation of pearl products reaching 63 million Euros in 2014 (Talvard, 2016). Production sites are located in the Society, Gambier, and Tuamotu archipelagos, whose pearl production accounts for > 95% of the world's black cultured pearls in terms of value (Cartier et al., 2012). As reported by Southgate et al. (2008a), pearl production involves four phases: (1) preoperative oyster conditioning, (2) the surgical grafting operation, (3) postoperative care, and (4) oyster culture and pearl harvest. Preoperative conditioning consists of reducing the metabolism and gametogenic activity of pearl oysters for 28–40 days prior to grafting (Aji, 2011; Gervis and Sims, 1992; Southgate et al., 2008a). Some pearl producers use preoperative conditioning, including lower water temperature, deliberate over stocking, reduction of food and oxygen levels, and placing of the pearl oysters deeper in the water column prior to the graft operation, as these actions are considered to decrease pearl rejection and improve pearl quality (Aji, 2011; Gervis and Sims, 1992; Southgate et al., 2008a). These rearing practices have not, however, been standardized nor tested under controlled conditions.

Surprisingly, the impact of the environment on cultured pearl biomineralization has been little documented, and previous studies have mainly focused on postoperative maintenance. For instance, the proportion of high-quality pearls harvested 4 months postgrafting was found to be significantly higher in recipient oysters that had undergone a low salinity treatment during the 14 days following the graft operation than in those reared conventionally (Atsumi et al., 2014). Temperature is considered an important factor for obtaining high-quality pearls, and winter is usually considered the best season to harvest pearls. Low temperatures are believed to reduce pearl oyster

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metabolism and lead to thinner mineral lamellae in the final layers of nacre laid down on the pearls, thereby enhancing their luster (Alagarswami, 1987; Menzel, 1991). However, to the best of our knowledge, no study has yet examined the effect of environmental factors experienced during the preoperative conditioning period on the subsequent pearl biomineralization process. The surgical procedure known as "grafting" is carried out by skilled technicians following preoperative conditioning. A small piece of the mantle, the tissue responsible for shell mineralization, is cut from a donor oyster and inserted along with a spherical nucleus (consisting of mollusk shell or synthetic material) into the gonad of another pearl oyster, the "recipient" (Kishore and Southgate, 2016; Southgate et al., 2008a). The external epithelial cells of the graft proliferate and cover the nucleus to form a pearl sac, a process that takes approximately 30 days following the grafting operation (Cochennec-Laureau et al., 2010). The first pearl layers are not homogeneous, as they show high variability in thickness and composition, as well as a remarkable association of organic and mineral materials (Cuif et al., 2008). The basal layer of the pearl, produced by the very first secretion of the pearl sac starting 21 days postgrafting, is usually composed of thin organic layers mostly consisting of proteins, with the mineral material present as dispersed microgranules of aragonite and calcite (Cuif et al., 2011). Two months after grafting, radial microstructures perpendicular to the surface of the nucleus appear due to the formation of organic envelopes. These microstructures form prisms composed of calcite or aragonite. This prismatic aragonite is specific to pearl microstructure and has never been observed in mollusk shells. Finally, a regular and parallel nacreous layer composed of aragonite tablets is established during pearl formation. Its production may occur directly onto the organic layer or may be delayed for a few months (Cuif et al., 2011). Therefore, the mineralization capabilities of the graft could be critical for the development of nacreous layers during the early stages of pearl formation and for obtaining high-quality pearls.

Pearl biomineralization results from complex molecular processes. The pearl sac epithelium synthesizes shell matrix proteins (SMPs), which play a major role in pearl biomineralization. Numerous SMPs have been characterized and some genes encoding these proteins have been identified in pearl oysters (Joubert et al., 2010; Marie et al., 2012; Montagnani et al., 2011; Suzuki et al., 2009). SMPs are thought to partly regulate the formation of the prismatic and nacreous shell layers (Marie et al., 2012). Notable examples of nacreous layer-related proteins include Pif177, known to specifically bind to aragonite crystals (Suzuki et al., 2009); MSI60, which is involved in the formation of aragonite crystal (Sudo et al., 1997); and Pearlin, which exhibits calcium- and chitin-binding properties (Montagnani et al., 2011). In the prismatic layer, Aspein is involved in the calcite precipitation process (Isowa et al., 2012), while Prismalin14 plays an important role in regulating calcification of the prismatic layer (Suzuki et al., 2004). Some proteins such as Nacrein are important for shell formation and are implicated in the mineralization processes of both the aragonitic nacreous and the calcitic prismatic layers (Miyamoto et al., 2005).

Pearl production is also a complex process that involves genetic contributions from two oysters (donor and recipient), which may be affected by the environment. Although the donor oyster is primarily responsible for the expression pattern of biomineralization genes in the pearl sac at both genomic (Arnaud-Haond et al., 2007) and transcriptomic levels (McGinty et al., 2012), the recipient oyster is strongly suspected to regulate pearl sac metabolism (Le Pabic et al., 2016) The grafter skills also influence pearl biomineralization and quality (Ky et al., 2014, 2015b). Recently, significant correlations have been demonstrated between pearl quality traits and some donor and recipient characteristics, such as a positive correlation between pearl nacre deposition and recipient shell growth or significant donor effects on pearl nacre deposition, luster, shape and defects (McDougall et al., 2016). To date, very little attention has been paid to the effects of environmental factors on pearl biomineralization. The purpose of our study was therefore to investigate under controlled conditions the effects of food level (microalgal concentration) and temperature during the preoperative phase to test their influence on: 1) the pearl retention rate, 2) the molecular mechanism involved in biomineralization in both the mantle and pearl sac and 3) pearl quality traits.

2. Material and methods

2.1. Biological material

Wild *P. margaritifera* pearl oysters aged 18 months were obtained by spat collection and transferred by air from Arutua lagoon to Vairao lagoon. These animals were then left in the lagoon for an acclimatization period of at least one month before the trophic and temperature conditioning experiments were conducted.

2.2. Shell labeling and deposition rate

The pearl oysters were immersed for 12 h in a 150 $mg{\cdot}L^{-1}$ calcein (Sigma-Aldrich) solution prepared using 0.1-mm filtered seawater 5–6 days before the conditioning experiments. Both the donor and the recipient shells were sawn along the dorsoventral axis at different sampling time using a SwapTop Trim Saw (Inland, Middlesex, UK). The ventral sides of the shell cross-sections were observed by epifluorescence microscopy using a Leica DM400B microscope (I3 filter block and LAS V.8.0 software for size measurements). The shell deposit rate (SDR, $\mu m{\cdot}day^{-1}$) was calculated by dividing the thickness of the new nacre deposits formed during the experimental time by the number of days that had elapsed since the marking (Linard et al., 2011). A mean of two measurements was calculated for each cross section.

2.3. Experimental design

2.3.1. Experiment 1: microalgal concentration conditioning experiment

A total of 392 pearl oysters with a mean height of 10.5 ± 0.4 cm and a mean weight of 157.1 ± 27.7 g were divided among eight 500-L tanks in which microalgal concentrations were gradually increased over a period of 5 days. The pearl oysters were then reared for 30 days in April 2014. During the 1-month conditioning experiment, the pearl oysters were divided into two groups fed a mixed diet composed of two microalgae: 2/3 *Tisochrysis lutea* (T-iso) and 1/3 *Chaetoceros gracilis*, at an overall concentration of 10,000 or 40,000 $cells{\cdot}mL^{-1}$ supplied continuously using Blackstone dosing pumps (Hanna). Tanks were sampled automatically every 3 min for fluorescence and temperature measurements. The intermediate concentration is considered as an optimal food concentration for *P. margaritifera* (Yukihira et al., 1998) and the high concentration is close to ingestion saturation (Le Moullac et al., 2013). During this experiment, the mean temperature was 28.1 ± 0.5 °C. Twelve pearl oysters (3.1%) died during the conditioning period, 10 (2.6%) were not grafted at the end of conditioning because of their apparently poor health status (weak resistance of the adductor muscle prior to shell opening), 10 were used as donor oysters, and 360 were grafted (Fig. 1, see Section 2.4 for a detailed description of this procedure).

2.3.2. Experiment 2: temperature-conditioning experiment

A total of 378 pearl oysters with a mean height of 10.8 ± 0.5 cm and a mean weight of 175.4 ± 35.5 g were divided among eight 500-L tanks in which temperatures were gradually increased or decreased over a period of 5 days. The pearl oysters were then reared for 30 days in June 2014. Then the pearl oysters were split into two groups, which were exposed to water temperatures of 22 and 30 °C, respectively. In French Polynesia, the monitoring of temperature data over 10 years (Ifremer sources) showed that water temperature is rarely lower than 22 °C and higher than 30 °C. The lower temperature is recorded in the Gambier and Australes archipelagoes whereas the higher is recorded in

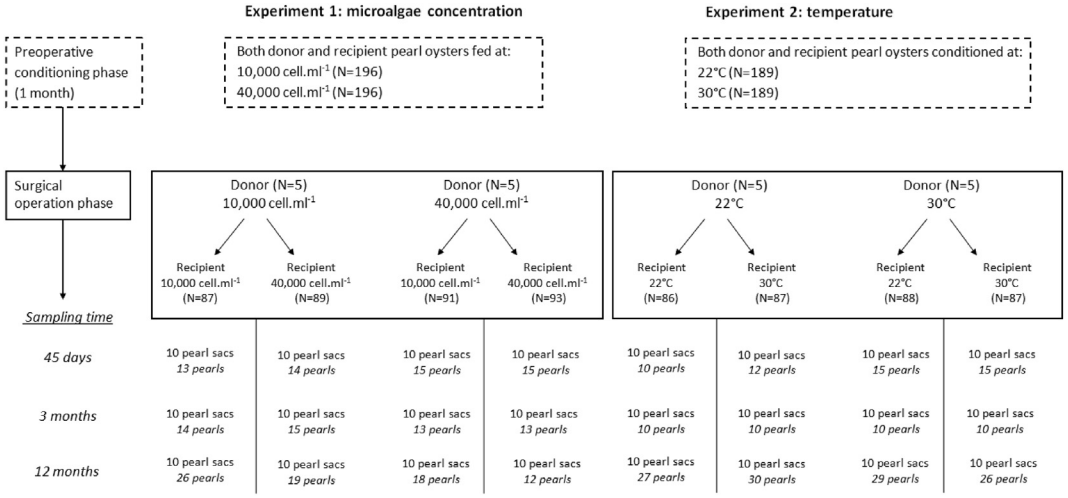

Fig. 1. Experimental design: number of pearl oysters preoperatively conditioned and grafted in experiments 1 and 2 and number of corresponding harvested pearl sacs and pearls (italics) at each sampling time.

Table 1
Forward and reverse primers used for the gene expression analysis.

Gene	GenBank accession numbers	Forward primer (5′-3′)	Reverse primer (5′-3′)
Pmarg-MSI60	SRX022139[a]	TCAAGAGCAATGGTGCTAGG	GCAGAGCCCTTCAATAGACC
Pmarg-PIF 177	HE610401	AGATTGAGGGCATAGCATGG	TGAGGCCGACTTTCTTGG
Pmarg-Pearlin	DQ665305	TACCGGCTGTGTTGCTACTG	CACAGGGTGTAATATCTGGAACC
Pmarg-Aspein	SRX022139[a]	TGAAGGGGATAGCCATTCTTC	ACTCGGTTCGGAAACAACTG
Pmarg-Prismalin 14	HE610393	CCGATACTTCCCTATCTACAATCG	CCTCCATAACCGAAAATTGG
Pmarg-Nacrein A1	HQ654770	CTCCATGCACAGACATGACC	GCCAGTAATACGGACCTTGG

[a] SRA accession number; EST library published by Joubert et al. (2010).

the Tuamotu archipelago. During the experiment, the pearl oysters were fed a mixed diet composed of two microalgae, 2/3 *Tisochrysis lutea* (T-iso) and 1/3 *Chaetoceros gracilis* at a concentration of 25,000 cell·mL^{-1} supplied continuously using Blackstone dosing pumps (Hanna). Tanks were sampled automatically every 3 min for fluorescence and temperature measurements seventeen pearl oysters (4.5%) died during the temperature conditioning experiment, three (0.8%) were not grafted because of their apparently poor health status (weak resistance of the adductor muscle prior to shell opening), 10 were used as donor oysters, and 348 were grafted (Fig. 1; see Section 2.4 for a detailed description of the grafting process).

2.4. Grafting operation and sampling

For each graft experiment, donor oyster selection was based on shell appearance and muscle resistance prior to shell opening (Tayale et al., 2012). Each recipient oyster was grafted using a 2.4 BU "Bio-coat" nucleus (7.27 mm diameter, Hyakusyo Co., Japan). For experiments 1 and 2, donor oysters were used to produce around 30 grafts. For all donors in both experiments, a single experienced grafting technician implanted almost equal numbers of grafts in two batches of randomly selected recipient oysters previously conditioned under differing experimental conditions (Fig. 1).

Grafted oysters were individually placed in net retention bags during the postgrafting phase, which were put in Vairao lagoon. On day 45 postgrafting, grafted oysters were evaluated for pearl retention rates (absence of rejected pearl in the retention bag) and mortalities. Those that had retained their pearls were hung on labeled chaplets (ropes). Pearl oysters were sampled at 45 days (N = 60 from each experiment), 3 months (N = 60 from each experiment), and 12 months postgrafting (this last sample consisted of all the remaining pearl oysters, N = 113 and N = 166 for the microalgal and temperature conditioning experiments, respectively). At each sampling time, 40 pearl sacs, corresponding to four grafted oysters for each combination of tested environmental condition and donor used, were randomly sampled and kept in RNAlater® (Fig. 1).

2.5. Gene expression profiles in mantle and pearl sac tissues

Total cellular RNA was extracted from the mantle of the donor oysters and from the pearl sacs of *P. margaritifera* at each sampling time 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 was treated with DNase (Ambion) to degrade any potential DNA contaminants. The expression levels of six biomineralization-related genes were analyzed using quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis using a set of forward and reverse primers (Table 1). Three other genes out of the following were used as housekeeping genes: 18S rRNA (Larsen et al., 2005), glyceraldehyde 3-phosphate dehydrogenase or GAPDH (Lemer et al., 2015), and an export factor binding protein or REF1 (Joubert et al., 2014). First-strand cDNA was synthesized from 400 ng of total RNA using a Transcriptor First Strand cDNA synthesis kit (Roche) and a combination of random hexamer and oligo (dT) primers in a final reaction volume of 25 μL. Quantitative PCR (qPCR) amplifications were carried out on a Stratagene MX3000P using Brilliant II SYBR Green QPCR master mix (Stratagene) with 400 nM of each primer and 10 μL of 1:100 diluted cDNA template.

The qPCR reactions consisted of an initial step of 10 min at 95 °C followed by 40 cycles (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min). At the end of these steps, an additional cycle was performed from 55 to 95 °C, increasing by 0.1 °C every second, to generate the dissociation curves and verify the specificity of the PCR products. All measurements were performed on duplicate samples. Expression levels were estimated by evaluating the fluorescence signal emitted by SYBR-

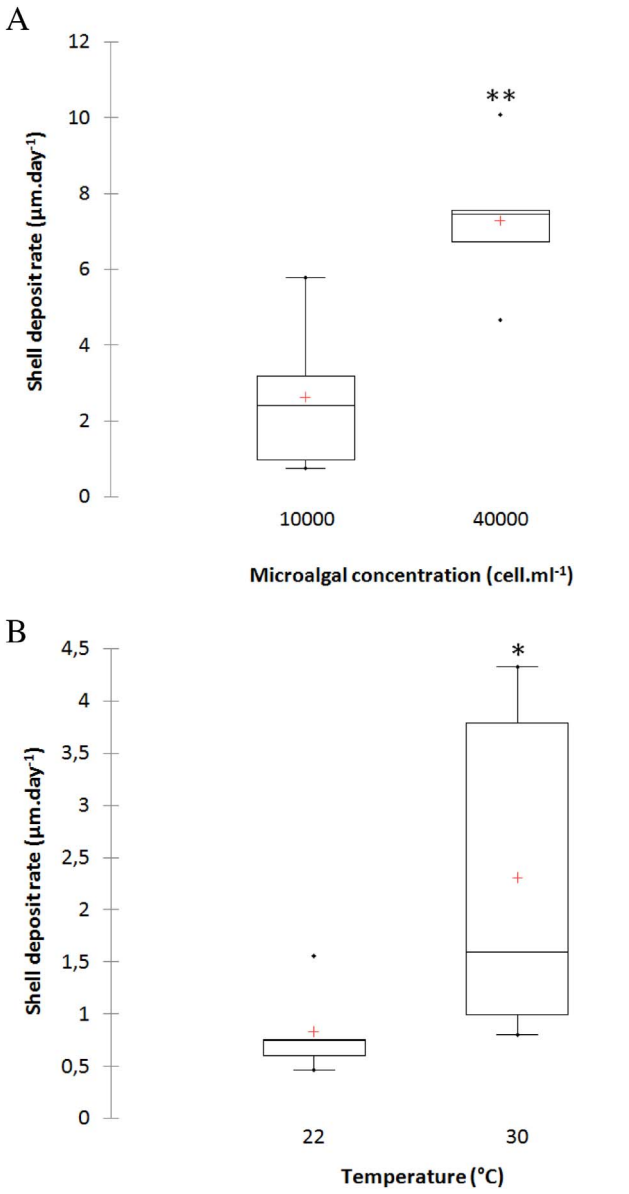

Fig. 2. Mean Shell Deposit Rate (SDR, µm·day^{-1}) inpearl oyster *Pinctada margaritifera* after 1 month at different (A) microalgal concentrations and (B) temperatures. Each plot includes: mean ("+" cross in the box-plot), median (solid bar in the box-plot), 25th to 75th percentile represented in the rectangular box, 1.5 × interquartile range (non-outlier range of the box whiskers), minimum and maximum values (extreme dots), and outlier values (outside box whiskers). Statistical analysis was done using (A) Student *t* and (B) Mann-Whitney tests; *p < 0.05 and **p < 0.01 (N = 5 per condition for each experiment).

Green®. This fluorescent marker binds to double-stranded DNA (dsDNA) and the fluorescence emitted is proportional to the dsDNA present in the reaction mix. Calculations were based on cycle threshold (Ct) values. The relative gene expression ratio of each biomineralization-related gene was calculated following the delta-delta method normalized to three reference genes (Livak and Schmittgen, 2001). This is defined as: ratio = $2^{-[\Delta Ct\ sample\ -\ \Delta Ct\ calibrator]} = 2^{-\Delta\Delta Ct}$, where the ΔCt sample is the ΔCt obtained for a target gene in one sample after normalization to the reference genes and the ΔCt calibrator is the mean of the ΔCt values obtained for all the six genes for each environmental factor (food level and temperature) at each sampling time.

2.6. Measurement of nacre deposition in the early stages of pearl formation, pearl quality traits and deposit weight

Nacre deposition in immature pearls, i.e., those pearls harvested at 45 days and 3 months post grafting, was classed into three categories: absence of nacre, nacre covering < 50% of the pearl surface area, and > 50% of the pearl surface area. Mature pearls, harvested at 12 months post grafting, were cleaned by ultrasonication in soapy water using an LEO 801 laboratory cleaner (2-L capacity, 80 W, 46 kHz) and were then rinsed in water. Four cultured pearl quality traits were determined in mature pearls: shape, surface defects, darkness, and luster. Both the nacre deposition on immature pearls and the quality traits of mature pearls were evaluated visually by two operators who examined the pearls together. The pearl deposit weight was measured using a digital balance, and the final value was calculated by subtracting the nucleus weight from the pearl weight.

2.7. Statistical analysis

The normality of data distribution and homogeneity of variance were tested using the Shapiro-Wilk and Bartlett tests, respectively. For experiment 1, the SDR data followed the conditions for application of parametric tests so the effect of microalgal concentration on SDR was tested using a Student *t*-test. For experiment 2, the SDR did not meet the conditions of application for parametric tests and could not be normalized by mathematical transformation; therefore, the effect of temperature on SDR was tested using a Mann-Whitney test. Differences in pearl retention and mortality rates of the different preoperative conditions were evaluated using Chi-square tests. The effects of preoperative conditions on nacre deposition in the early stages of pearl formation and on pearl quality traits were tested using F-tests. Due to the non-normality of some pearl weight deposit distributions, these values were Box-Cox transformed to satisfy the conditions for parametric tests. Differences in pearl weight deposition were tested using Student *t*-tests. Sperman's correlation coefficient was calculated to measure the strength of the relationship of pearl weight and both donor and recipient oyster growth at the 5% alpha level.

Expression values of genes that met the conditions for parametric tests were analyzed using Student *t*-tests while those that did not were tested using Mann-Whitney tests. In all cases, p values ≤ 0.05 were considered statistically significant, and all data analyses were performed using XLSTAT (version 1.01, 2014).

3. Results

Effects of microalgal concentration and temperature conditioning of future donor and recipient oysters on pearl oyster culture traits.

3.1. Shell biomineralization

Mean shell deposit rate (SDR) was measured following shell calcein marking to analyze the effect of microalgal concentration and temperature on shell growth during the preoperative conditioning phase. Donor oysters fed at 10,000 and 40,000 cells·mL^{-1} had SDR values of 2.6 ± 2.0 and 7.3 ± 1.9 µm·day^{-1} (Fig. 2A), respectively. A Student *t*-test confirmed the significant effect of microalgal concentration on SDR ($p = 0.006$). In the temperature experiment, the mean SDR was 0.8 ± 0.4 and 2.3 ± 1.6 µm·day^{-1} for donor oysters conditioned at 22 and 30 °C, respectively (Fig. 2B). Despite considerable variation in the SDR of pearl oysters conditioned at 30 °C, the Mann-Whitney test showed that SDR was significantly affected by temperature ($p = 0.037$).

3.2. Expression of shell matrix protein genes in mantle

For pearl oysters conditioned at different microalgal concentrations,

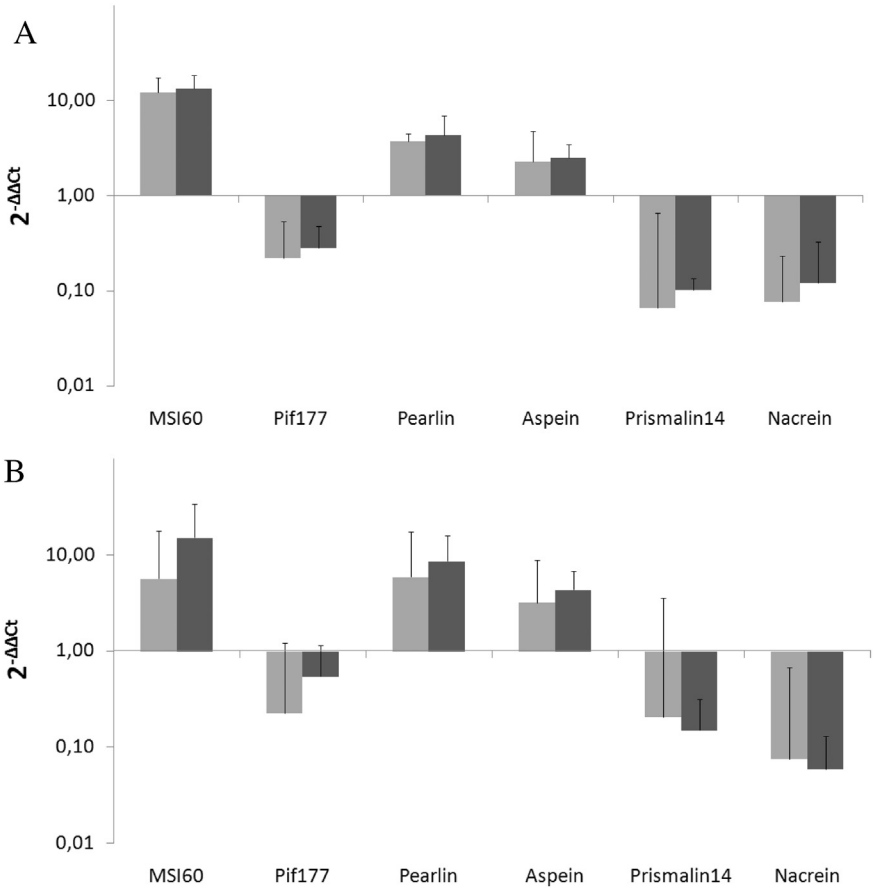

Fig. 3. Mean relative expression of genes coding proteins involved in the formation of the nacreous layer (Msi60, Pif177, and Pearlin), prismatic layer (Aspein and Prismalin14), and both (Nacrein), in mantle following a 1-monthconditioning period at different microalgal or temperature concentrations. (A) Different microalgal concentrations: 10,000 cells·mL^{-1} (light grey) and 40,000 cells·mL^{-1} (dark grey); and (B) different temperatures: 22 °C (light grey) and 30 °C (dark grey). Fold change means were calculated from five individual oysters/treatment. Y-axes are in the logarithmic scale. Error bars indicate standard deviations (SD); statistical analyses used Student *t*-tests (MSI60, Pif177, Pearlin, Aspein, and Nacrein) or Mann-Whitney tests (Prismalin14).

the relative expressions of the six biomineralization-related target genes were not significantly different (Fig. 3A). The genes MSI60, Pearlin, and Aspein were more highly expressed than the others were. Similar results were obtained for the pearl oysters conditioned at different temperatures (Fig. 3B). There were no significant differences in the relative expression levels of any of the studied genes.

3.3. Pearl retention rate

At 45 days postgrafting, for the food level experiment, pearl retention was between 74.2 and 85.4% and was not significantly dependent on the preoperative microalgal concentration supplied to donor or recipient pearl oysters (Table 2A). Mortality values were also independent of the preoperative microalgal concentration of donor oysters (χ^2, p = 0.737). However, mortality values were dependent on the preoperative microalgal concentration of grafted recipient oysters: mortality was lower following intermediate microalgal conditioning (χ^2, p = 0.038) than high microalgal conditioning. For the temperature experiment, pearl retention was between 67.0 and 73.6% and was not significantly dependent on the preoperative temperature at which donor and recipient pearl oysters had been conditioned (Table 2B). Mortality values were also independent of the preoperative conditioning temperature of donor (χ^2, p = 0.083) and recipient pearl oysters (χ^2, p = 0.66).

3.4. Gene expression in the pearl sac during pearl biogenesis

In a preliminary analysis (data not presented), we studied the expression levels of each gene for the four subgroups (combinations of the preoperative conditions of the donor and recipient oysters) for each experiment. We found no significant differences in expression levels when grafts from a given donor preoperative treatment were put in recipient oysters issued from different preoperative conditions, regardless of the genes considered and the food or temperature levels at which the donor oysters had been conditioned. Consequently, we decided to analyze the gene expression levels only as a function of the preoperative donor condition.

The three genes (Pmarg-MSI60, p = 0.029; Pmarg-Pif177, p = 0.009; and Pmarg-Pearlin, p = 0.05) involved in nacre formation were significantly upregulated 45 days postgrafting in pearl oysters implanted with grafts collected from the donors preoperatively fed at 40,000 cells·mL^{-1} (Fig. 4A.1). In addition, one gene involved in prism formation was significantly downregulated (Pmarg-Prismalin14, p = 0.0002). Furthermore, at 3 months postgrafting, two genes involved in prism formation were downregulated (Pmarg-Aspein, p = 0.021 and Pmarg-Prismalin14, Fig. 4A.2, p = 0.009) while, at 12 months postgrafting, only one gene (Pmarg-Prismalin14) was significantly downregulated (Fig. 4A.3, p = 0.017). There were no significantly different effects with donor preoperative conditioning temperature at 45 days or 3 months postgrafting (Fig. 4B.1 and B.2). At 12 months postgrafting (Fig. 4B.3), two genes were significantly upregulated in pearl oysters implanted with grafts collected from donors preconditioned at 30 °C (Pmarg-Pif177, p = 0.014 and Pmarg-Nacrein A1, p = 0.013).

3.5. Nacre deposition quality

At 45 days postgrafting, nacre deposition differed significantly depending on donor preoperative microalgal concentration (F-test, p = 0.038, Fig. 5A.1). Furthermore, donor oysters fed 10,000 cells·mL^{-1} gave rise to a significantly lower proportion of pearls in the nacre > 50% category, whereas those fed 40,000 cells·mL^{-1} produced a significantly lower proportion of pearls without nacre than would be expected by chance. At 3 months postgrafting, nacre

Table 2
Grafted pearl oysters *Pinctada margaritifera* at 45 days postgrafting.

A

Preoperative conditioning: microalgae concentration (cell·ml^{-1})		Number of grafted pearl oysters	45 days postgrafting: Nucleus retention	Nucleus rejection	Mortality
Donor 10,000	Recipient 10,000	87	70 (80.4)	14 (16.1)	3 (3.4)
	Recipient 40,000	89	76 (85.4)	6 (6.7)	7 (7.9)
Donor 40,000	Recipient 10,000	91	73 (80.2)	16 (17.6)	2 (2.2)
	Recipient 40,000	93	69 (74.2)	17 (18.3)	7 (7.5)

B

Preoperative conditioning: temperature (°C)		Number of grafted pearl oysters	45 days postgrafting: Nucleus retention	Nucleus rejection	Mortality
Donor 22	Recipient 22	86	63 (73.3)	20 (23.3)	3 (3.5)
	Recipient 30	87	64 (73.6)	19 (21.8)	4 (4.6)
Donor 30	Recipient 22	88	59 (67.0)	22 (25)	7 (8.0)
	Recipient 30	87	59 (67.8)	20 (23.0)	8 (9.2)

deposition was significantly dependent on the preoperative microalgal concentration fed to the donor oysters (F-test, $p = 0.05$, Fig. 5A.2). Donor oysters preconditioned at 10,000 cells·mL^{-1} produced a significantly higher proportion of pearls without nacre than expected by chance. In contrast, donor oysters preconditioned at 40,000 cells·mL^{-1} produced a significantly lower proportion of pearls without nacre and significantly higher proportion of the nacre > 50% category than would be expected by chance. The preoperative microalgal concentration supplied to recipient oysters showed no effects on any group at 45 days or 3 months postgrafting (results not shown).

In the temperature experiment, no significant effects of donor preoperative conditions were observed on nacre deposition at 45 days or 3 months postgrafting (F-test, $p = 0.264$ and $p = 0.306$, Fig. 6B.1 and B.2, respectively). Furthermore, the preoperative conditioning temperature of recipient oysters showed no significant effects on nacre deposition at 45 days or 3 months postgrafting (results not shown).

The quality of pearls harvested at 12 months post grafting was evaluated using four criteria: shape, number of defects, darkness, and luster (Fig. 6). The microalgal concentration used for donor oysters did not significantly affect the shape ($p = 0.707$), number of defects ($p = 0.188$), darkness ($p = 0.119$), or luster ($p = 0.810$). Similarly, the temperature at which donor oysters were conditioned did not significantly affect pearl shape ($p = 0.108$), number of defects ($p = 0.830$), darkness ($p = 0.051$), or luster ($p = 0.409$). For recipient oyster conditioning, neither microalgal concentration nor temperature treatments had any significant effects on pearl quality criteria (results not shown).

3.6. Pearl deposit weight

For pearl oysters with grafts collected from donor oysters fed 10,000 cells·mL^{-1}, the mean pearl deposit weight was 0.08 ± 0.03, 0.19 ± 0.1, and 0.77 ± 0.28 g at 45 days, 3 months, and 12 months postgrafting, respectively (Fig. 7A). For pearl oysters with grafts collected from donor oysters fed 40,000 cells·mL^{-1}, the mean pearl deposit weight was 0.07 ± 0.03, 0.19 ± 0.08, and 0.66 ± 0.27 g at 45 days, 3 months, and 12 months postgrafting, respectively (Fig. 7A). Mean pearl deposit weight showed no significant difference according to donor microalgal conditioning level at any of the sampling times (45 days, $p = 0.392$; 3 months, $p = 0.775$; and 12 months, $p = 0.052$). Pearl weight was positively correlated with recipient SDR at 45 days and 3 months postgrafting whereas it was not significantly correlated with donor SDR (Table 3A).

Donor oysters maintained at 22 °C during the preoperative phase produced pearls whose mean deposit weights were 0.07 ± 0.03, 0.20 ± 0.09, and 0.65 ± 0.25 g at 45 days, 3 months, and 12 months postgrafting, respectively (Fig. 7B). Donor preconditioning at 30 °C led to pearls whose mean deposit weights were 0.05 ± 0.03, 0.17 ± 0.08, and 0.57 ± 0.21 g at 45 days, 3 months, and 12 months postgrafting, respectively (Fig. 7B). Mean pearl deposit weight showed no significant difference according to donor temperature conditioning at any of the sampling times (45 days, $p = 0.128$; 3 months, $p = 0.290$; and 12 months, $p = 0.098$). Pearl weight was positively correlated with recipient SDR at 45 days, 3 months and 12 months postgrafting whereas it was not significantly correlated with donor SDR (Table 3B).

For recipient preoperative conditions, neither microalgal

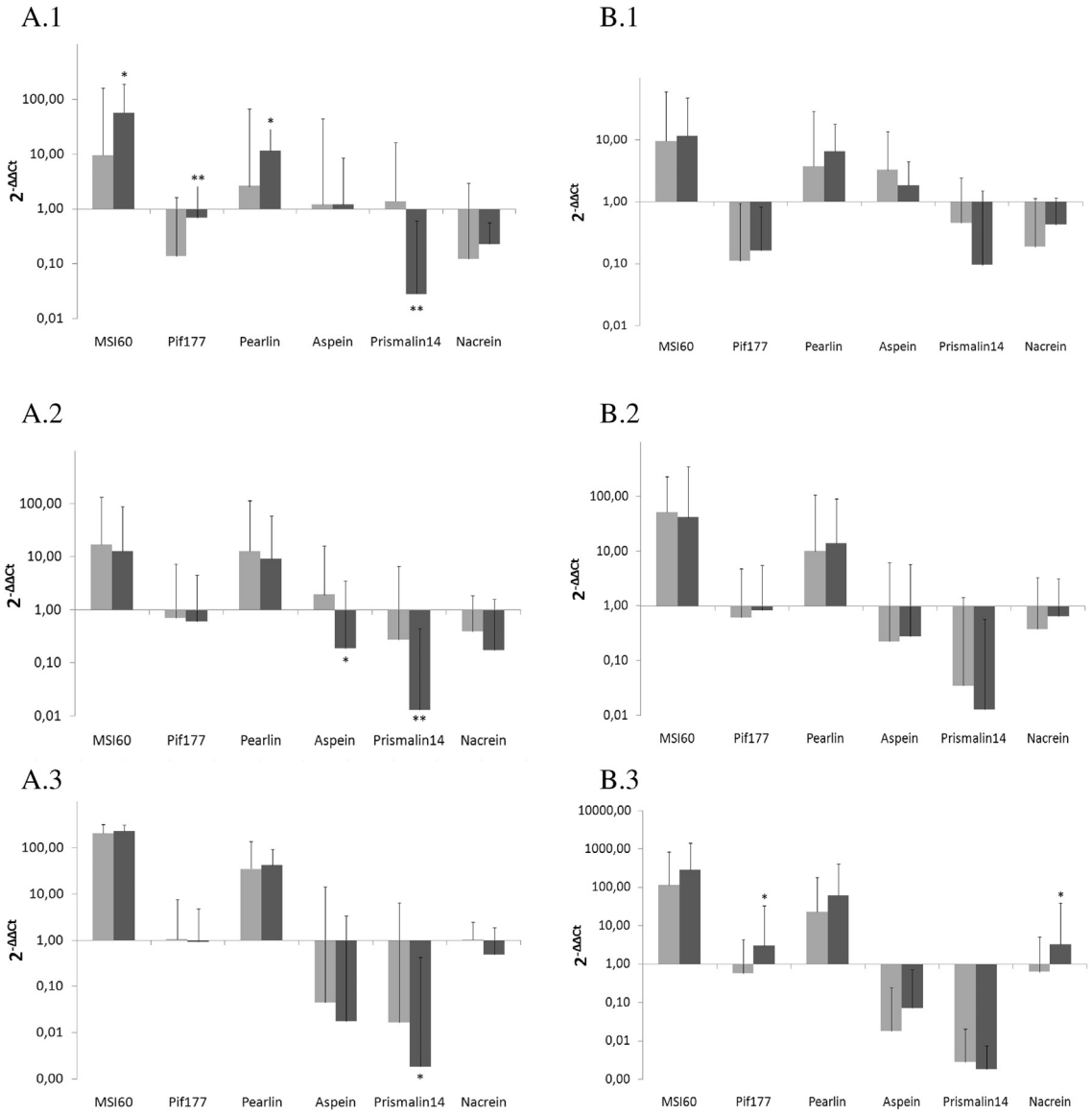

Fig. 4. Mean relative expression of genes coding proteins involved in formation of nacreous layers (MSI60, Pif177, and Pearlin), prismatic layers (Aspein and Prismalin14), and both (Nacrein) in pearl sacs formed from grafts from donor oysters preoperatively conditioned at different microalgal concentrations. (A) Different microalgal concentrations: 10,000 cells·mL^{-1} (light grey) and 40,000 cells·mL^{-1} (dark grey), at (A.1) 45 days, (A.2) 3 months, and (A.3) 12 months postgrafting. (B) Different temperatures: 22 °C (light grey) and 30 °C (dark grey), at (B.1) 45 days, (B.2)3 months, and (B.3) 12 months postgrafting. Fold change means were calculated from 20 individuals per treatment at each sampling time. Y-axes are in the logarithmic scale. Error bars indicate standard deviations (SD); *$p < 0.05$ and **$p < 0.01$.

concentration nor temperature had a significant effect on pearl deposit weight at any of the sampling times (results not shown).

4. Discussion

Our objective was to determine, under controlled conditions, the influence of food level (microalgal concentration) and temperature during the preoperative phase of pearl biogenesis. We analyzed pearl retention rate, expression level of biomineralization-related genes in the pearl sac and pearl quality traits. Our results suggest that, among the preoperative conditioning factors tested, food level has the greatest impact on pearl biogenesis. Indeed, donor oysters fed at high microalgal levels led to pearls sacs with higher biomineralization capabilities and faster nacre establishment during early stages of pearl formation. Surprisingly, we found no significant effect of recipient conditioning in any of the analyses. However, recipient oyster growth at different sampling time was positively correlated to pearl weight for both experiments. As recipient oysters are strongly suspected to regulate pearl sac metabolism, it would be of interest to test the influence of food level and temperature on pearl biogenesis during the postgrafting phase.

4.1. Effect of food level and temperature on shell biomineralization

The physiological process and metabolism of bivalves are mostly controlled by two environmental factors, temperature and food (Kanazawa and Sato, 2008; Laing, 2000; Schöne et al., 2003, 2005; Thébault et al., 2008). In pearl oysters, these two parameters affect growth, reproduction, and biomineralization (Joubert et al., 2014; Southgate et al., 2008b; Teaniniuraitemoana et al., 2015). After only 1 month of conditioning, we found that both microalgal concentration and temperature affected pearl oyster shell growth. In our experiments, SDR was 2.8 times higher at 40,000 cells·mL^{-1} than it was at 10,000 cells·mL^{-1}, and was 2.9 times higher at 30 °C than it was at 22 °C. Our results corroborate those of previous studies showing the effect of microalgal concentration (Linard et al., 2011) and microalgal concentration and temperature combined (Joubert et al., 2014) on *P.*

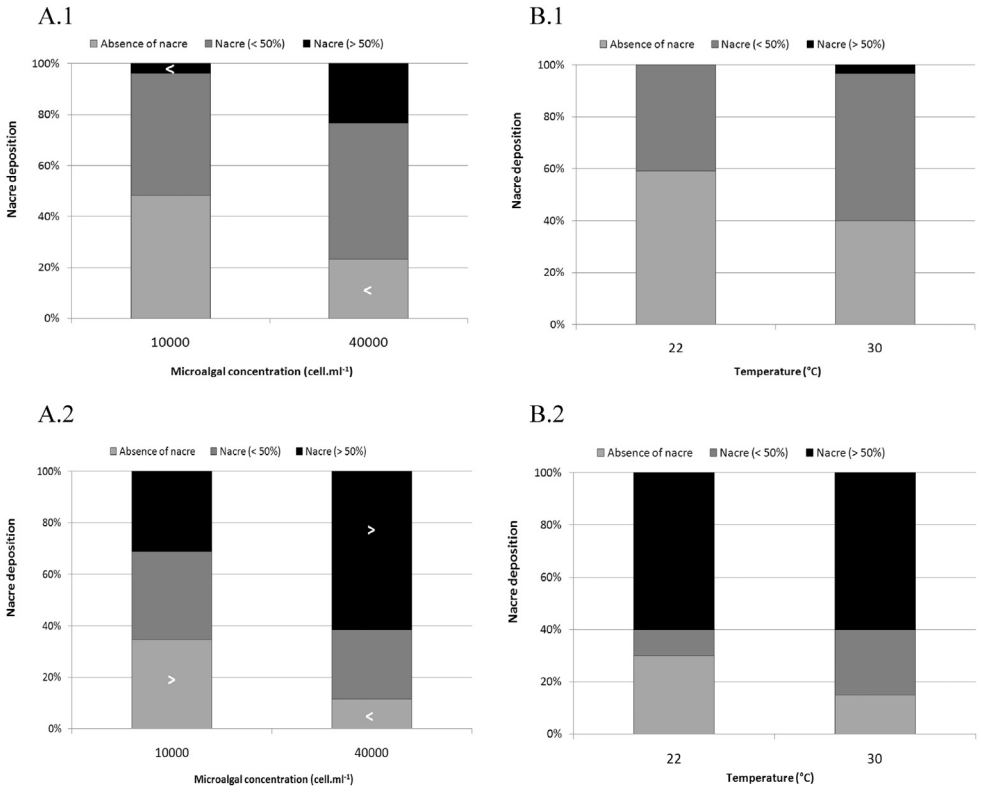

Fig. 5. Proportion of harvested pearls in different categories of nacre deposition with implanted grafts collected from donor oysters preoperatively conditioned at different microalgal concentrations and temperatures. Donors conditioned with (A) different microalgal concentrations: 10,000 and 40,000 cells·mL^{-1} at (A.1) 45 days and (A.2) 3 months postgrafting; and (B) different temperatures: 22 and 30 °C at (B.1) 45 days and (B.2) 3 months postgrafting. Number of harvested pearls is given in Fig. 1. > indicates a significantly higher number and < a significantly lower number than expected between conditions ($p < 0.05$, F-test).

margaritifera SDR. Moreover, Le Moullac et al. (2016) reported that temperature affected the metabolic rate of the pearl oyster *P. margaritifera* and demonstrated an energy gain from 22 to 30 °C, at which metabolic rates were maximized. The energy gain might explain why we observed higher SDR at 30 °C than at 22 °C.

Biomineralization is an energetically costly process, and the synthesis of organic matrix proteins requires higher energy than does the precipitation of mineralized components (Palmer, 1983). However, our study revealed no significant effect of either food level or temperature on biomineralization related genes encoding proteins involved in the formation of nacreous and prismatic layers in the mantle of donor oysters. These results do not correspond to those of Joubert et al. (2014), who reported an upregulation of Pmarg-Pif177 and a downregulation of Pmarg-MSI60 in the mantle of pearl oysters by comparing the effects of two food levels. However, in this previous study, microalgal concentrations were lower than the levels used in the present one (800 vs. 15,000 cells·mL^{-1}) and the experiment was longer (2 months). Regarding temperature, our results are consistent with those of Le Moullac et al. (2016), who showed that the expression level of some of our target genes, such as Pmarg-Aspein and Pmarg-Nacrein A1, were not significantly different between 22 and 30 °C. Nevertheless, we cannot exclude an effect of intermediate temperatures between 22 and 30 °C. Indeed, a significant downregulation of these latter two genes was shown at 30 °C compared to levels recorded at 26 °C (Le Moullac et al., 2016). Finally, the panel of biomineralization-related genes selected in the present study may not be sufficiently sensitive to detect a significant response to the tested temperatures. With the advent of high-throughput sequencing technologies, an overall analysis without a priori knowledge, such as RNA-Seq, would enable the identification of differentially expressed genes related to biomineralization.

4.2. Effect of food level and temperature on pearl retention and mortality

Achieving high retention rates and low mortality following the grafting operation is essential for the pearl production industry. The microalgal concentrations in the preconditioning treatments of this study did not significantly affect pearl retention rate, and the temperature treatments did not significantly affect either pearl retention rate or mortality. The retention and mortality rates we obtained for our two graft experiments were in the same range as the values reported by Ky et al. (2013, 2015a,b). However, we found significantly lower mortality at 45 days postgrafting for recipient oysters that had been fed at the intermediate microalgal concentration than for those that had been fed at the higher microalgal concentration. The reason for pearl oyster mortality following the graft operation is not clear. However, Cochennec-Laureau et al. (2010) examined histological sections of gonads of grafted pearl oysters (*P. margaritifera*) that died following graft operations and found evidence of strong inflammatory reaction. They also pointed out that when mature gonads are incised for nucleus implantation, many gametes are released around the implanted graft. In our study, we hypothesize that grafted recipient oysters fed at intermediate microalgal concentration may have less cellular debris such as gametes around the graft, which could reduce the risk of postoperative infections. However, histological studies are needed to confirm this hypothesis.

4.3. Pearl sac biomineralization capabilities

In order to avoid donor oyster's effect on pearl biomineralization (Arnaud-Haond et al., 2007; McGinty et al., 2012; Tayale et al., 2012), care was taken to split grafts from each donor oyster across the two treatments for each experiment. The profile of gene expression in the pearl sac of recipient oysters was similar to that of donor oysters in the corresponding microalgal preoperative conditions, regardless of the sampling time. The pearl sacs from donor oysters fed at high concentration of microalgae showed significant overexpression of the three genes related to nacre formation (Pmarg-MSI60, Pmarg-Pif177 and Pmarg-Pearlin) and a downregulation of one gene related to prismatic layer formation (Pmarg-Prismalin14) at 45 days postgrafting, compared to the pearl sacs from donor oysters fed at intermediate microalgal concentration. We also observed significant downregulation of Pmarg-Prismalin14 and Pmarg-Aspein at 3 months postgrafting, and

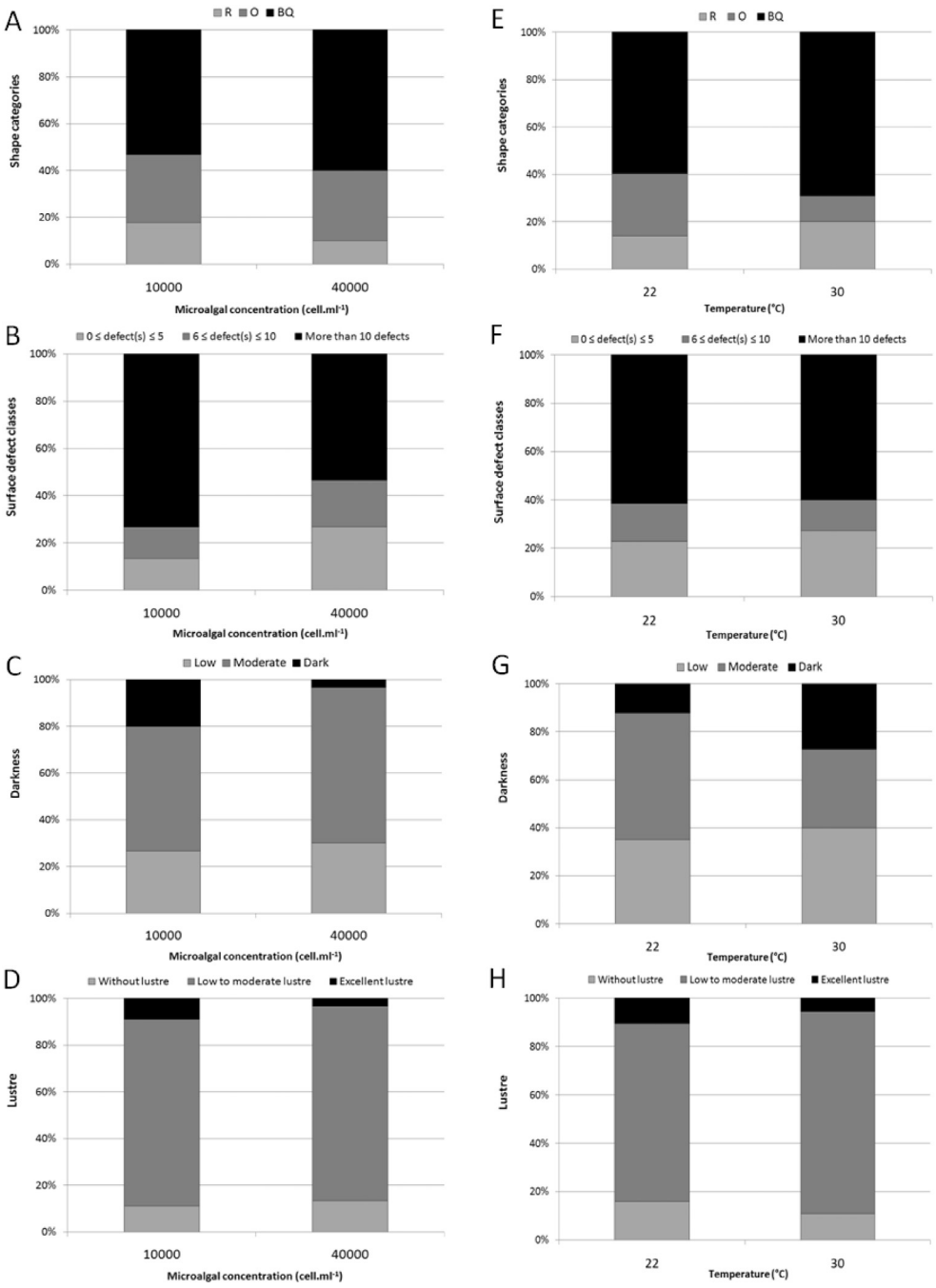

Fig. 6. Proportion of harvested pearls with implanted grafts collected from donor oysters in different pearl quality categories 12 months postgrafting according to preoperative conditioning. Effect of donor preconditioning with 10,000 or 40,000 cells·mL^{-1} on (A) shape (R: round, semi round; O: oval, button and drop; and BQ: semi baroque and baroque), (B) number of defects, (C) darkness, and (D) luster. Effect of donor preconditioning at 22or 30 °C on (E) shape, (F) number of defects, (G) darkness, and (H) luster. Number of harvested pearls is given in Fig. 1.

significant downregulation of Pmarg-Prismalin14 at 12 months postgrafting. Prismalin14 plays an important role in the regulation of calcification of the prismatic layer (Suzuki et al., 2004), and Aspein is involved in the calcite precipitation process (Isowa et al., 2012). The pearl sacs that originated from the mantle tissue of donor oysters fed at high microalgal concentration may produce fewer prisms than the pearl sacs obtained from donor oysters fed at intermediate microalgal concentration. Conversely, the grafts originating from donors fed at high microalgal concentration showed higher biomineralization capabilities than those from donors fed at intermediate microalgal concentration. Interestingly, Sato et al. (2013) showed that MSI60 was strongly expressed at 38 days postgrafting in the pearl sacs of oysters producing pearls with nacreous layers, whereas it was not expressed in pearls exhibiting prismatic layers. In the latter study, the authors hypothesized that, after a transition phase between prismatic and nacreous layers during pearl formation, the nacreous layer formation observed in typical nacreous pearl is associated with strong MSI60 expression during the early stages of pearl formation. Moreover, Pmarg-Pif177 was shown to be positively correlated with pearl nacre weight and thickness (Blay et al., 2016). Inoue et al. (2011a) reported gene expression patterns of shell matrix proteins (including four genes in common with our study: MSI60, Nacrein, Prismalin14, and Aspein) in the mantle graft and pearl sacs harvested 4, 10, 15, and 48 days after implantation. They showed that gene expression pattern changes before and after pearl sac formation. They hypothesized that gene expression patterns are closely related to the type of layer formed on the nucleus (nacreous or prismatic layer) and may be regulated by the donor oyster (Inoue et al., 2011a). Moreover, McGinty et al. (2012) used a xenograft between two different pearl oyster species and showed that the donor mantle tissue (rather than recipient tissue) is the main contributor to the expression of biomineralization genes believed to be involved in pearl formation.

In the temperature experiment, no significant differences in the

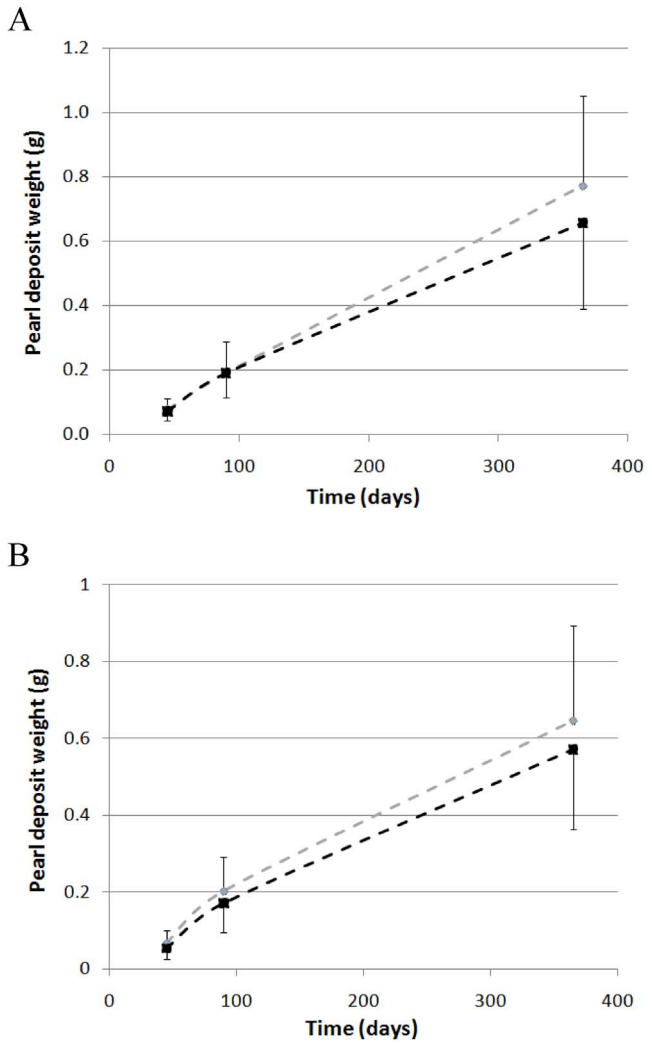

Fig. 7. Pearl deposit weight at different sampling times with grafts collected from donor oysters preoperatively conditioned at different microalgal concentrations and temperatures. (A) Different microalgal concentrations: 10,000 cells·mL^{-1} (light grey) and 40,000 cells·mL^{-1} (dark grey); and (B) different temperatures: 22 °C (light grey) and 30 °C (dark grey). Number of harvested pearls is given in Fig. 1.

Table 3
Correlation between pearl weight and both donor and recipient oyster growth at each sampling time. A: microalgal concentration conditioning experiment. B: temperature conditioning experiment. Spearman's coefficient correlation associated with significant p-value (< 0.05) are in bold. Number of harvested pearls is given in Fig. 1.

Sampling time	Variable associated with pearl weight	Spearman's correlation coefficient	p-Value
A			
45 days	Donor oyster growth	− 0.146	0.281
	Recipient oyster growth	**0.265**	**0.049**
3 months	Donor oyster growth	0.007	0.961
	Recipient oyster growth	0.151	0.265
12 months	Donor oyster growth	− 0.194	0.096
	Recipient oyster growth	**0.549**	**< 0.0001**
B			
45 days	Donor oyster growth	− 0.158	0.33
	Recipient oyster growth	**0.574**	**0.0001**
3 months	Donor oyster growth	− 0.083	0.61
	Recipient oyster growth	**0.345**	**0.03**
12 months	Donor oyster growth	− 0.06	0.528
	Recipient oyster growth	**0.431**	**< 0.0001**

gene expression of the pearl sac were detected at 45 days or 3 months postgrafting. Surprisingly, at 12 months postgrafting, two genes (Pmarg-Pif177 and Pmarg-Nacrein A1) were upregulated for pearl oysters whose grafts originated from donor oysters conditioned at 30 °C temperature compared to those conditioned at 22 °C during the month preceding the graft. The reason why differentially expressed genes were only detected 12 months postgrafting has not been elucidated, but Pmarg-Pif177 and Pmarg-Nacrein A1 showed little expression 45 days and 3 months postgrafting and may be preferentially involved during the latter stages of pearl formation.

Irrespective of the sampling time and experiment, Pmarg-MSI60 was one of the most highly expressed genes, in agreement with the work of Inoue et al. (Inoue et al., 2011b) who showed that MSI60 expression was higher than that of Nacrein and a "prismatic-layer-forming" gene on pearl sac from *P. fucata* harvested 2 months postgrafting.

4.4. Effect of microalgal concentration and temperature on nacre deposition in early stages of pearl formation and on pearl quality

Nacre deposition on the pearl during the early stages of its formation was significantly dependent at both 45 days and 3 months postgrafting on the microalgal concentration at which donor oysters had been conditioned. In general, the donor oysters fed at the high food level produced pearls with a significantly higher proportion of nacre at 45 days and 3 months postgrafting. These results are consistent with the gene expression measurements in the corresponding pearl sacs (see Section 4.3). The pearl oysters whose grafts originated from donor oysters fed at a high microalgal concentration may have higher biomineralization capabilities, resulting in the faster appearance of nacre on the nucleus during pearl formation. The establishment of the nacreous layer may occur directly on the organic layer and can appear some months later (Cuif et al., 2011). Our results indicate that donor oyster food level during conditioning may be critical for pearl sac biomineralization capabilities and nacreous layer establishment. In their study, McGinty et al. (2010) showed that mantle grafts from different pearl oyster species influenced the rate of pearl nacre deposition and pearl nacre weight differently following a xenograft between *P. margaritifera* and *P. maxima*. In our study, we detected both a molecular signature and the dependence of nacre establishment on donor oyster preoperative conditioning.

In contrast, the temperature at which donor oysters were conditioned had no effect on pearl nacre deposition. This environmental parameter may have more influence on the molecular processes involved in pearl biomineralization and consequently pearl quality traits during the latter stages of pearl formation than before nucleus implantation (Alagarswami, 1987; Menzel, 1991). In addition, we showed no significant effect of the preoperative growing conditions of recipient oysters on any of the parameters measured in either of the experiments. As recipient oysters are strongly suspected to regulate pearl sac metabolism, it would be interesting to study the influence of food level and temperature during the postgrafting phase on pearl biogenesis.

Ultimately, we did not detect any differences in pearl quality 12 months postgrafting according to donor or recipient preoperative conditions. Pearl shape has been shown to be dependent on grafter skill (Jerry et al., 2012) and suspected to be influenced by the morphology of the gonad (Southgate et al., 2008a). In our study, a single professional grafter performed all the grafts to minimize the former effect. Pearl luster and darkness may also be linked to the ultrastructure of the pearl's later nacreous layers. Indeed, some pearl producers prefer to harvest pearls during late autumn or winter when temperatures are lower, as this is thought to improve the quality of pearl luster by reducing growth and making the layers of nacre thinner (Alagarswami, 1987; Menzel, 1991). In our study, we did not find any effect of donor or recipient preoperative environmental conditions on pearl weight at any of the sampling times in either experiment. However, recipient oyster growth at different sampling time was positively correlated with

pearl weight for both experiments. This is in agreement with previous studies showing positive correlation between pearl nacre thickness and recipient shell thickness, height and width (Le Pabic et al., 2016) and between cultured pearl size parameters and some characteristics of the recipient oyster (shell height, total weight, and shell weight) in *P. fucata martensii* (Wang et al., 2013). Some pearl parameters such as weight and nacre deposition rate might be under the control of the recipient oysters, with pearl sac metabolism regulated through the food supply.

5. Conclusions

In this study, we confirmed that microalgal concentration and temperature affect *P. margaritifera* shell growth. High temperatures and food level (30 °C and 40,000 cells·mL^{-1} of a mixed diet of 2/3 *Tisochrysis lutea* (T-iso) and 1/3 *Chaetoceros gracilis* respectively) enhanced *P. margaritifera* growth rate. Stimulating this growth rate and thus reaching a size suitable for graft operation more rapidly would be beneficial to pearl industry. Nevertheless, a particular care should be taken for the preoperative conditioning of future recipient oysters since we evidenced lower mortality following graft operation for recipient oysters that had been fed during one month at the intermediate microalgal concentration of 10,000 cells·mL^{-1} (2/3 *Tisochrysis lutea* (T-iso) and 1/3 *Chaetoceros gracilis*). In contrast, the preoperative conditioning of future donor oyster at high food level increased both significantly the nacre-related gene expression in pearl sacs and nacre establishment during the early stages of pearl biogenesis. Furthermore, we did not detect any effect of recipient conditioning on gene expression or pearl characteristics in either experiment. All these combined results taken together, key recommandations for pearl industry should be to use high and intermediate trophic levels for the preoperative oyster conditioning phase culture of future donor and recipient oysters respectively. Further study is needed to test whether the environment influences recipient oysters after the graft by modulating their metabolism and whether this could impact pearl biomineralization.

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RESEARCH ARTICLE

Influence of water temperature and food on the last stages of cultured pearl mineralization from the black-lip pearl oyster *Pinctada margaritifera*

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Abstract

Environmental parameters, such as food level and water temperature, have been shown to be major factors influencing pearl oyster shell growth and molecular mechanisms involved in this biomineralization process. The present study investigates the effect of food level (i.e., microalgal concentration) and water temperature, in laboratory controlled conditions, on the last stages of pearl mineralization in order to assess their impact on pearl quality. To this end, grafted pearl oysters were fed at different levels of food and subjected to different water temperatures one month prior to harvest to evaluate the effect of these factors on 1) pearl and shell deposition rate, 2) expression of genes involved in biomineralization in pearl sacs, 3) nacre ultrastructure (tablet thickness and number of tablets deposited per day) and 4) pearl quality traits. Our results revealed that high water temperature stimulates both shell and pearl deposition rates. However, low water temperature led to thinner nacre tablets, a lower number of tablets deposited per day and impacted pearl quality with better luster and fewer defects. Conversely, the two tested food level had no significant effects on shell and pearl growth, pearl nacre ultrastructure or pearl quality. However, one gene, Aspein, was significantly downregulated in high food levels. These results will be helpful for the pearl industry. A wise strategy to increase pearl quality would be to rear pearl oysters at a high water temperature to increase pearl growth and consequently pearl size; and to harvest pearls after a period of low water temperature to enhance luster and to reduce the number of defects.

Introduction

As in other mollusks, pearl oysters synthesize biomineralized structures, such as their shell, to maintain their soft tissues, and to prevent predation and desiccation [1]. Shell biomineralization results from the activity of an organic matrix, mostly composed of polysaccharides, lipids

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and proteins, secreted by the mantle tissue. The shell is composed of different layers: the periostracum, a thin layer mainly consisting of organic material; the outer prismatic layer made of calcite; and the inner nacreous layer made of aragonite. The nacreous layer has an iridescent and shiny appearance and is of great interest for cultured pearl production. To produce a cultured pearl, a small piece of mantle is cut from a donor oyster and is then implanted together with a nucleus (consisting of mollusk shell or synthetic material) into the gonad of a recipient oyster [2]. The epithelial cells of the graft multiply, ending in the formation of a complete pearl sac covering the nucleus. Histological examinations in *P. margaritifera* revealed that the pearl sac is complete after approximately 14 days following the graft operation [2]. At 18 days post grafting, the pearl-sac cannot be distinguished from the host tissues [2]. As early as 21 days post grafting, the nucleus is partially or totally covered by the first secretions, made of both organic and mineral materials, due to the mineralizing activity of the pearl sac [3]. In this study, huge diversity in the microstructural patterns and mineralogical properties was observed in the first pearl layers of one-month or older pearls, until a homogeneous nacreous layer occurred in most pearls. Another study focusing on the chronological description of pearl-sac development showed that first nacre deposition was recorded at 32 days post grafting [2]. The nacreous layers are composed of aragonite tiles held together by a series of organic matrices [4]. The laminar structure and the thickness of nacre piled on the implanted nucleus are considered as determinant factors for pearl quality [5]. Interestingly, the gene expression patterns of shell matrix proteins (SMP) in pearl sacs are very similar to that of the donor mantle tissue [6]. Shell matrix proteins are known to control shell biomineralization by determining the type of calcium carbonate (calcite or aragonite) that will be deposited and by regulating crystal growth [7]. A large research effort has been conducted to identify and characterize mineralization-related proteins and genes [7–11]. For example, Pif177, MSI60 and Pearlin have been identified as being involved in the formation of a nacreous layer, respectively by specifically binding to aragonite crystals [12], by including a calcium-binding domain [13] and by presenting calcium- and chitin-binding properties that would be involved in nacre crystal structures development [14]. Furthermore, Aspein is related to the prismatic layer and contains an aspartic-rich domain which might be involved in controlling selective precipitation of calcite [15–17]. Other proteins are involved in both the formation of nacreous and prismatic layers, such as Nacrein, which is thought to act as a calcium concentrator [18].

Pearl production in French Polynesia is an important industry, with production sites located in the Society, Gambier and Tuamotu archipelagos. Tahitian cultured pearls produced from the pearl oyster *Pinctada margaritifera* are harvested after approximately 18 to 24 months. Their quality is evaluated using different criteria: size, surface quality, luster, shape, color and darkness level [19]. Sizes range from 9 to 20 mm; with average size around 8–12 mm [20]. Surface quality is an assessment of the different imperfections on the surface of the pearl, such as spots, bumps and wrinkles. Luster is considered as the brilliance of the pearl, where high luster corresponds to mirror-like appearance and low luster corresponds to dull appearance. The most valuable pearls are the lustrous, large, round pearls with no defects on the surface. The darker a Tahitian pearl is, the more valuable it will usually be [21]. However, only 5–10% of cultured pearls are considered to be of high quality [22]. The potential for pearl quality improvement is thus considerable.

Environmental parameters, such as food level and water temperature, have been shown to be major factors influencing pearl oyster shell growth and molecular mechanisms involved in this biomineralization process [23]. It has also been reported that food level influences the thickness of nacre tablets in the shell [24] and that pearl oyster metabolism is dependent on water temperature [25]. As a recipient oyster is strongly suspected to regulate pearl sac metabolism [26], food level and water temperature may influence pearl biomineralization. However,

to our knowledge, few publications have addressed the issue of environmental influence on pearl biomineralization. Water temperature is suspected to be determinant for obtaining high-quality pearls; therefore, pearls are preferentially harvested in winter [27,28]. Indeed, lower water temperatures are assumed to reduce pearl oyster metabolism and to influence nacreous structure in the last layers of the pearl, with thinner nacre tablets that would result in higher luster [28,29]. Recently, pearl nacre growth and tablet thickness have been shown to be influenced by water temperature [30]. Finally, the iridescence and color of the shell depend on the last few layers of nacre and on the structure of nacre [31]. Therefore, the last stages of pearl mineralization may be crucial to obtaining high-quality pearls, regarding surface quality, luster and iridescence.

The present study investigates the effect of food level and water temperature, in laboratory controlled conditions, on the last stages of pearl mineralization and on pearl quality. To this end, grafted pearl oysters *P. margaritifera* were fed at different food levels and subjected to different water temperatures during the preharvest phase. We studied the influence of these environmental parameters on biomineralization-related gene expression in the pearl sac, and on pearl and shell growth, pearl nacre tablet thickness and pearl quality traits.

Material and methods

Biological material

Cultivated pearl oysters *P. margaritifera* of approximately two years old were reared in Rangiroa atoll (Tuamotu Archipelago, French Polynesia). A first graft was conducted in March 2014 and a second graft in May 2014. For each graft experiment, donor oyster selection was based on shell appearance and muscle resistance prior to shell opening [32]. Each recipient oyster was grafted using a 2.4 BU "Bio-coat" nucleus (7.27 mm diameter, Hyakusyo, Japan). A single professional grafter performed the two experimental grafts.

Grafted pearl oysters were then placed individually in net retention bags, and at 45 days post-grafting, nucleus retention rates (absence of rejected pearl in the retention bag) were evaluated. The pearl oysters that had retained their nucleus were hung on labeled chaplets (ropes) [22,33]. For each of the two experimental grafts, pearl oysters were transferred by plane to Ifremer Center in Vairao lagoon (Society Archipelago, French Polynesia) 9.5 months postgrafting. Grafted pearl oysters stayed in Vairao lagoon for six weeks before the experiments were conducted 11 months postgrafting.

Experimental design

Experiment 1: Microalgae concentration experiment. A total of 210 pearl oysters of a mean height of 12.1 ± 1.1 cm (dorso-ventral axis) and mean weight of 257.0 ± 57.3 g were cultured in eight 500 L tanks (3 tanks with 26 pearl oysters and 1 tank with 27 pearl oysters for each condition). Pearls oysters were divided into two groups for which microalgal concentrations were gradually increased over a period of 5 days before the beginning of the experiment. Pearl oysters were then fed a mixed diet composed of two microalgae: 2/3 *Tisochrysis lutea* (T-iso) and 1/3 *Chaetoceros gracilis* at a concentration of 7,000 cell mL^{-1} (n = 105 individuals) or 28,000 cell mL^{-1} (n = 105 individuals) supplied continuously using Blackstone dosing pumps (Hanna) during one month. These two food levels are contrasted in terms of ingestion, with the higher food level being close to the maximal assimilation efficiency for *P. margaritifera* [34]. Homogenization of the water was achieved in the tanks by "air-lifts," and photoperiod was maintained at 12:12. Seawater was renewed at a rate of 96L. h^{-1}. Tanks were cleaned two times per week and they were sampled automatically every 3

min for fluorescence and water temperature measurements. During this experiment, the mean water temperature was 28.5 ± 0.5˚C.

Experiment 2: Water temperature experiment. A total of 116 pearl oysters of a mean height of 12.0 ± 1.2 cm (dorso-ventral axis) and mean weight of 256.0 ± 64.3 g were cultured in eight 500 L tanks. Pearl oysters were divided into two groups for which water temperatures were gradually increased or decreased over a period of 5 days before the beginning of the experiment. Pearl oysters were then exposed to a water temperature of 22˚C (n = 56 pearl oysters, divided into 14 pearl oysters per tank) or 30˚C (n = 60 pearl oysters, divided into 15 pearl oysters per tank) for one month. The pearl oysters were fed a mixed diet composed of two microalgae: 2/3 *Tisochrysis lutea* (T-iso) and 1/3 *Chaetoceros gracilis* at a concentration of 15,000 cell mL^{-1} supplied continuously using Blackstone dosing pumps (Hanna). Homogenization of the water was achieved in the tanks by "air-lifts," and photoperiod was maintained at 12:12. Seawater was renewed at a rate of $96L.h^{-1}$. Tanks were cleaned two times per week and they were sampled automatically every 3 min for fluorescence and water temperature measurements. In French Polynesia, the water temperature rarely drops below 22˚C or exceeds 30˚C. The lower water temperature is recorded in the Gambier and Austral archipelagoes, whereas the higher water temperature is recorded in the Tuamotu archipelago.

Sampling

In order to avoid a donor oyster effect on pearl biomineralization [6,32,35], care was taken to split grafts from each donor oyster across the two treatments for each experiment (food and water temperature). From 20 grafted pearl oysters, with five donors per condition, a total of 40 pearl sacs were kept in RNAlater® for each experiment (food and water temperature). A random selection of nine recipient oysters per condition and per experiment, that were implanted with grafts collected from three of the previously mentioned five donor oysters, were analyzed for pearl growth, pearl nacre tablet thickness and number of tablets deposited per day.

Pearl sac gene expression

Total cellular RNA was extracted from the pearl sacs of *P. margaritifera* 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 was treated with DNase (Ambion) to degrade any potential DNA contaminants. The expression levels of five biomineralization-related genes were analyzed using quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis using a set of forward and reverse primers (Table 1). Three other genes were used as housekeeping genes: 18S rRNA [36], glyceraldehyde 3-phosphate dehydrogenase or GAPDH [37], and an export factor binding protein or REF1 [23]. First-strand cDNA was synthesized from 500 ng of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) and a combination of random hexamer and oligo(dT)primers in a final reaction volume of 25 μL. Quantitative PCR (qPCR) amplifications were carried out on a Stratagene MX3000P using Brilliant II SYBR Green QPCR Master Mix (Stratagene) with 400 nM of each primer and 10 μL of 1:100 diluted cDNA template.

The qPCR reactions consisted of an initial step of 10 min at 95˚C followed by 40 cycles (95˚C for 30 s, 60˚C for 30 s, and 72˚C for 1 min). At the end of these steps, an additional cycle was performed from 55 to 95˚C, increasing by 0.1˚C every second, to generate dissociation curves and verify the specificity of the PCR products. All measurements were performed on duplicate samples. Expression levels were estimated by evaluating the fluorescence signal emitted by SYBR-Green®. This fluorescent marker binds to double-stranded DNA (dsDNA) and the fluorescence

Table 1. Set of forward and reverse primers used for gene expression analysis.

Gene	GenBank Accession Numbers	Forward primer (5′-3′)	Reverse primer (5′-3′)
Pmarg-MSI60	SRX022139*	TCAAGAGCAATGGTGCTAGG	GCAGAGCCCTTCAATAGACC
Pmarg-PIF 177	HE610401	AGATTGAGGGCATAGCATGG	TGAGGCCGACTTTCTTGG
Pmarg-Pearlin	DQ665305	TACCGGCTGTGTTGCTACTG	CACAGGGTGTAATATCTGGAACC
Pmarg-Nacrein A1	HQ654770	CTCCATGCACAGACATGACC	GCCAGTAATACGGACCTTGG
Pmarg-Aspein	SRX022139*	TGAAGGGGATAGCCATTCTTC	ACTCGGTTCGGAAACAACTG

*SRA accession number; EST library published in Joubert et al., 2011.

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emitted is proportional to the dsDNA present in the reaction mix. Calculations were based on cycle threshold (Ct) values. The relative gene expression ratio of each biomineralization-related gene was calculated following the delta-delta method normalized to three reference genes [38]. This is defined in the following equation: ratio = $2^{-[\Delta Ct\ sample-\Delta Ct\ calibrator]} = 2^{-\Delta\Delta Ct}$, where ΔCt sample is the ΔCt obtained for a target gene in one sample after normalization to the reference genes, and ΔCt calibrator is the mean of the ΔCt values obtained for all five genes as in [23,39,40].

Shell and pearl labeling and deposition rate

The pearl oysters were immersed for 12 h in a 150 $mg.L^{-1}$ calcein (Sigma Aldrich) solution prepared with 0.1 mm filtered seawater. Due to technical constraint, all the pearl oysters could not be labeled at one time. Pearl oysters were divided into 4 groups that were labeled successively during 2 days. The pearl oysters then stayed in the lagoon for at least five days. Then 300 μL of calcein solution (200 $mg.L^{-1}$, 0.1 mm filtered and sterilized seawater) was injected in the gonad of the pearl oysters using a sterile syringe. The pearl oysters stayed in the lagoon for five additional days before being transferred to the tanks. Microalgal concentrations and water temperatures were gradually increased or decreased over a period of 5 days before the beginning of the experiments. Once the microalgal concentrations and water temperatures were set, pearl oysters were fed at different microalgal concentrations (experiment 1) or subjected to different water temperatures (experiment 2) during one month. After the one-month experimental period, all shells and a selection of pearls were sawn along the dorsoventral axis and in half, respectively, using a "SwapTop Trim Saw" machine (Inland, Middlesex, United Kingdom). The ventral sides of shell cross sections and pearl sections were observed by epifluorescence microscopy using a Leica DM400B UV microscope (I3 filter block and LAS V.8.0 software for size measurements) (Fig 1). The shell and pearl deposition rates (SDR and PDR), expressed in μm day^{-1}, were calculated by dividing the thickness of the new nacre deposits formed by the number of days elapsed since the marking [24].

Pearl nacre tablet thickness and number of tablets deposited per day

Fractures of pearl were observed at 15 kV (in charge-up reduction mode) using a Hitachi TM 3030 scanning electron microscope (SEM) at the Université de la Polynésie française. High magnification images (10,000x) of fractured pearl were taken to measure the thickness of nacre tablets. For each pearl, the mean of nacre tablets thickness was calculated based on 30 measurements among the last deposited nacre layers on three different pictures (10 measurements/picture). The number of tablets deposited per day was calculated by individually dividing the pearl daily deposition rate by the mean tablet thickness.

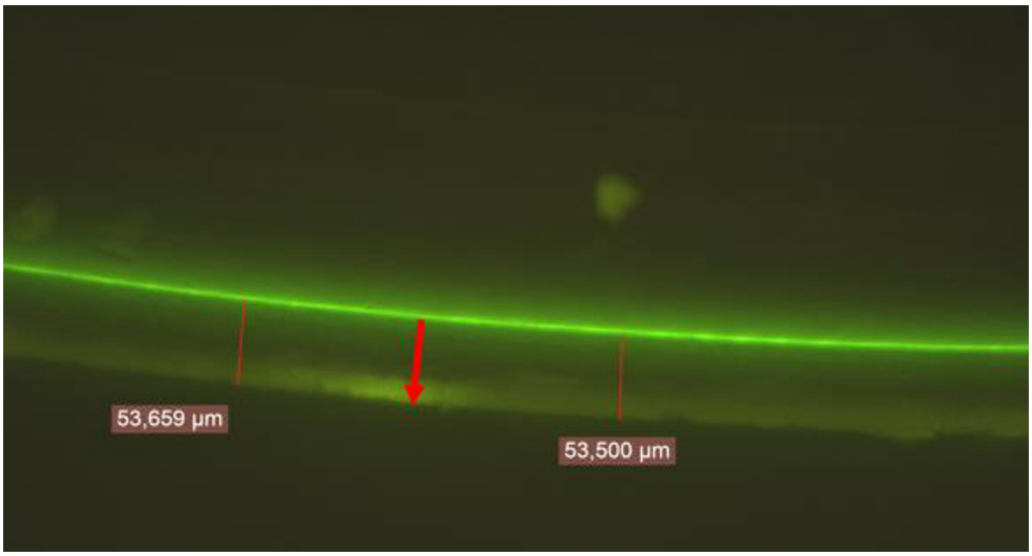

Fig 1. Picture of calcein marking (green fluorescent line) on a pearl cut in half and observed under an epifluorescence microscope (×400). The red arrow indicates the growth direction of nacre.

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Evaluation of pearl quality

Cultured pearls were cleaned by ultrasonication in soapy water using an LEO 801 laboratory cleaner (2 L capacity, 80 W, 46 kHz) and were then rinsed in water. Four cultured pearl quality traits were determined: surface defects, color, darkness and luster. Surface defects were classified into one of three categories: 0 to 5 defects, 6 to 10 defects and up to 10 defects. Some pearls develop pearl surface anomaly corresponding to the presence of circle. Such circle was classified as a defect. The visually-perceived color was classified into seven color categories: grey, white, yellow, green, aubergine (red/purple), blue, and peacock (a mix of aubergine and green); and into three darkness categories: low, moderate and high. Luster was classified into three categories: low (matte appearance), moderate and high (mirror-like reflectivity). These quality traits were evaluated visually by two operators who examined the pearls together.

Statistical analysis

The normality of data distribution and homogeneity of variance were tested using the Shapiro-Wilk and Bartlett tests, respectively. The SDR, PDR and number of tablets deposited per day followed the conditions for application of parametric tests for both experiments, so the effect of microalgal concentration and water temperature on these parameters was tested using a Student's *t*-test. The nacre tablet thickness in pearls did not meet the conditions of application for parametric tests and could not be normalized by mathematical transformation; therefore, the effect of microalgal concentration and water temperature on nacre tablet thickness was tested using a Mann-Whitney test. The effects of the two environmental parameters on pearl surface defect classes, color and luster was tested using F-tests, whereas their effect on darkness level was evaluated using a Chi-square test. Expression values of genes that met the conditions for parametric tests were analyzed using Student's *t*-tests, while those that did not were tested using Mann-Whitney tests. Spearman's correlation coefficients were calculated to measure the strength of the relationships between gene expression in pearl sacs and both PDR and nacre tablet thickness, as well as between the PDR and nacre tablet thickness, at the 5% alpha level. A Pearson's correlation coefficient was calculated to measure the linear correlation

between the SDR and the PDR at the 5% alpha level with critical value r = 0.468. In all cases, p values $\leq$ 0.05 were considered statistically significant, and all data analyses were performed using XLSTAT (version 1.01, 2014).

Results

Gene expression in the pearl sac

Among the studied genes, only one, Aspein, was significantly affected by food level with a down-regulation for pearl oysters fed at 28,000 cell mL^{-1} compared to 7000 cell mL^{-1} (Fig 2A). Concerning the water temperature experiment, the relative expressions of the five genes were not significantly different between the two tested water temperatures (Fig 2B).

Pearl and shell deposition rate

Mean shell deposition rate (SDR) and pearl deposition rate (PDR) were measured following calcein marking to analyze the effect of microalgal concentration and water temperature on shell and pearl growth during the last stages of pearl formation. Recipient oysters fed at 7000 cell mL^{-1} and 28,000 cell mL^{-1} had a mean SDR of 2.63 ± 1.6 μm day^{-1} and 3.22 ± 2.1 μm day^{-1}, respectively (Fig 3A). Concerning the pearl, pearls oysters fed at 7000 cell mL^{-1} and 28,000 cell mL^{-1} produced a PDR of 0.78 ± 0.58 μm day^{-1} and 0.98 ± 0.51 μm day^{-1}, respectively (Fig 3B). Student's *t*-tests did not reveal significant differences in the SDR (p = 0.069) and in the PDR (p = 0.449) between the two microalgal concentrations.

The mean SDR was significantly lower for pearl oysters maintained at 22˚C compared with those maintained at 30˚C, with values of 2.13 ± 1.29 μm day^{-1} and 3.76 ± 1.88 μm day^{-1}, respectively (Student's *t*-test, p < 0.0001) (Fig 3C). Recipient oysters subjected to 22 and 30˚C had PDR values of 0.51 ± 0.4 μm day^{-1} and 1.38 ± 1.1 μm day^{-1} respectively (Fig 3D). A Student's *t*-test confirmed the significant effect of water temperature on PDR (p = 0.01).

For both experiments, SDR and PDR were strongly correlated (r = 0.668, p = 0.003 for the microalgae concentration experiment, and r = 0.659, p = 0.003 for the water temperature experiment. The critical value of Pearson's r for both experiments was 0.468).

Pearl nacre tablet thickness and number of tablets deposited per day

Recipient oysters fed at 7000 and 28,000 cell mL^{-1} produced pearls with mean nacre tablet thickness of 307.6 ± 11.8 nm and 312.9 ± 11.2 nm, respectively (Fig 4A). The microalgal concentrations supplied to recipient oysters showed no significant effects on nacre tablet thickness in pearls (Mann-Whitney test, p = 0.121). The pearl oysters fed at 7000 and 28,000 cell mL^{-1} exhibited a mean number of tablets deposited per day of 2.53 ± 1.87 and 3.16 ± 1.68, respectively. The microalgal concentration showed no significant effects on the mean number of tablets deposited per day in the pearls (Student's *t*-test, p = 0.461).

The mean nacre tablet thickness was significantly lower for pearl oysters maintained at 22˚C than the pearl oysters maintained at 30˚C, with values of 262 ± 36.7 nm and 331.1 ± 99.4 nm, respectively (Mann-Whitney test, p = 0.042) (Fig 4C). Recipient oysters subjected to 22˚C and 30˚C exhibited a mean number of tablets deposited per day of 1.98 ± 1.84 and 4.05 ± 2.46 (Fig 4D). A Student's *t*-test confirmed the significant effect of water temperature on the number of tablets deposited per day in the pearls (p = 0.023).

Among the tested genes, Pmarg-MSI60 was found to be strongly positively correlated with the PDR (ρ = 0.507, p = 0.033) and Pmarg-Aspein was negatively correlated with the PDR (ρ = -0.668, p = 0.003) for the microalgal concentration experiment (Table 2). The PDR and the tablet thickness were not significantly correlated (ρ = 0.055, p = 0.827). Concerning the

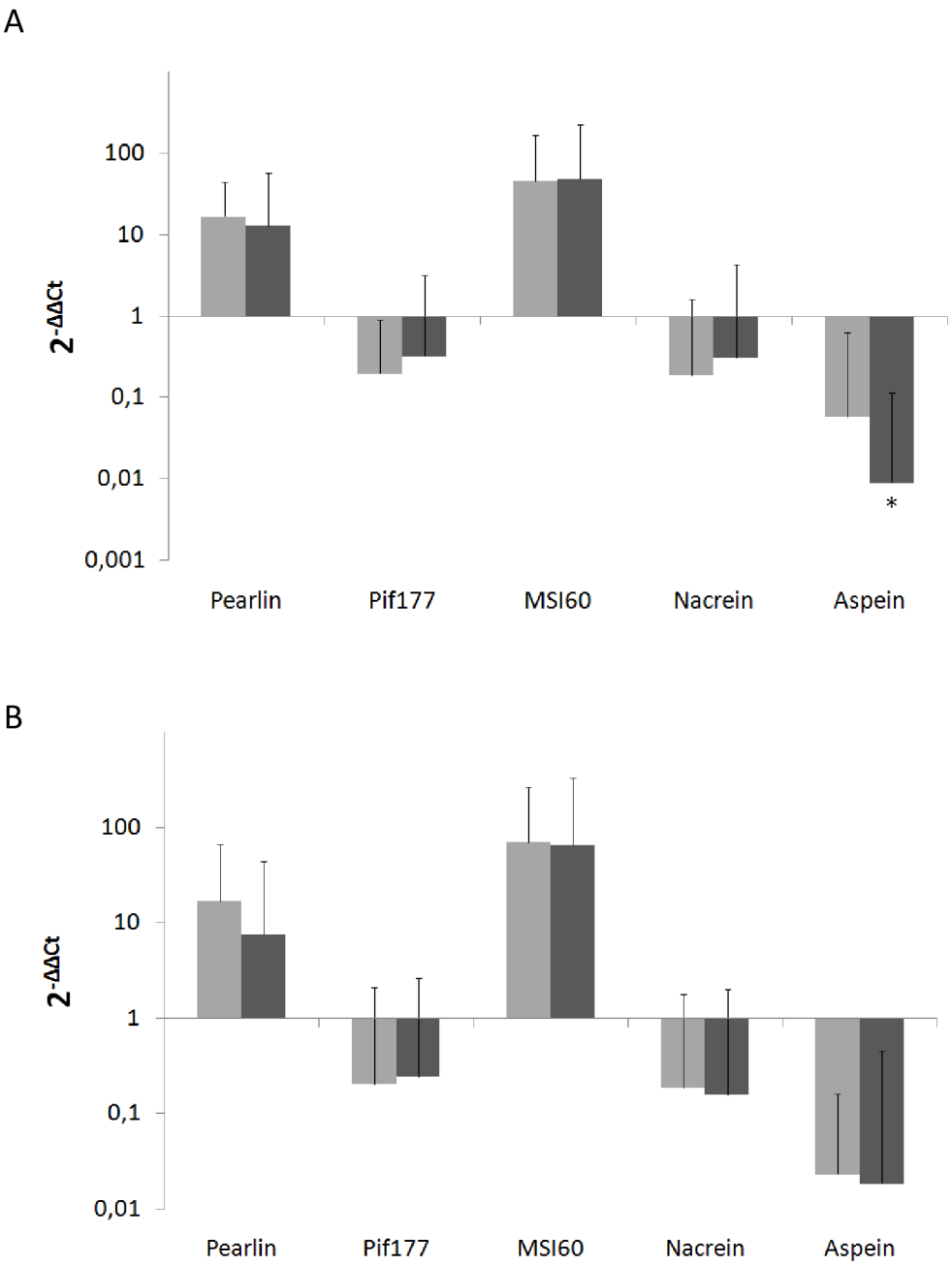

Fig 2. Mean relative expression of genes encoding proteins involved in the formation of the nacreous layer (Pearlin, Pif177 and MSI60), prismatic layer (Aspein) and both (Nacrein) in pearl sacs following a one-month experiment at different microalgal or water temperature conditions. (A) Different microalgal concentrations: 7,000 cell mL^{-1} (light grey) and 28,000 cell mL^{-1} (dark grey); and (B) different water temperatures: 22˚C (light grey) and 30˚C (dark grey). Fold changes were calculated from 20 individuals per condition. Y-axes are in the logarithmic scale. Error bars indicate standard deviations (SD); statistical analyses used Student's *t*-tests (A: Pearlin, Pif177, MSI60 and Aspein; B: Pearlin, Pif177 and Nacrein) or Mann-Whitney tests (A: Nacrein; B: MSI60 and Aspein). Statistical significance is indicated by asterisks as follows: * $p < 0.05$.

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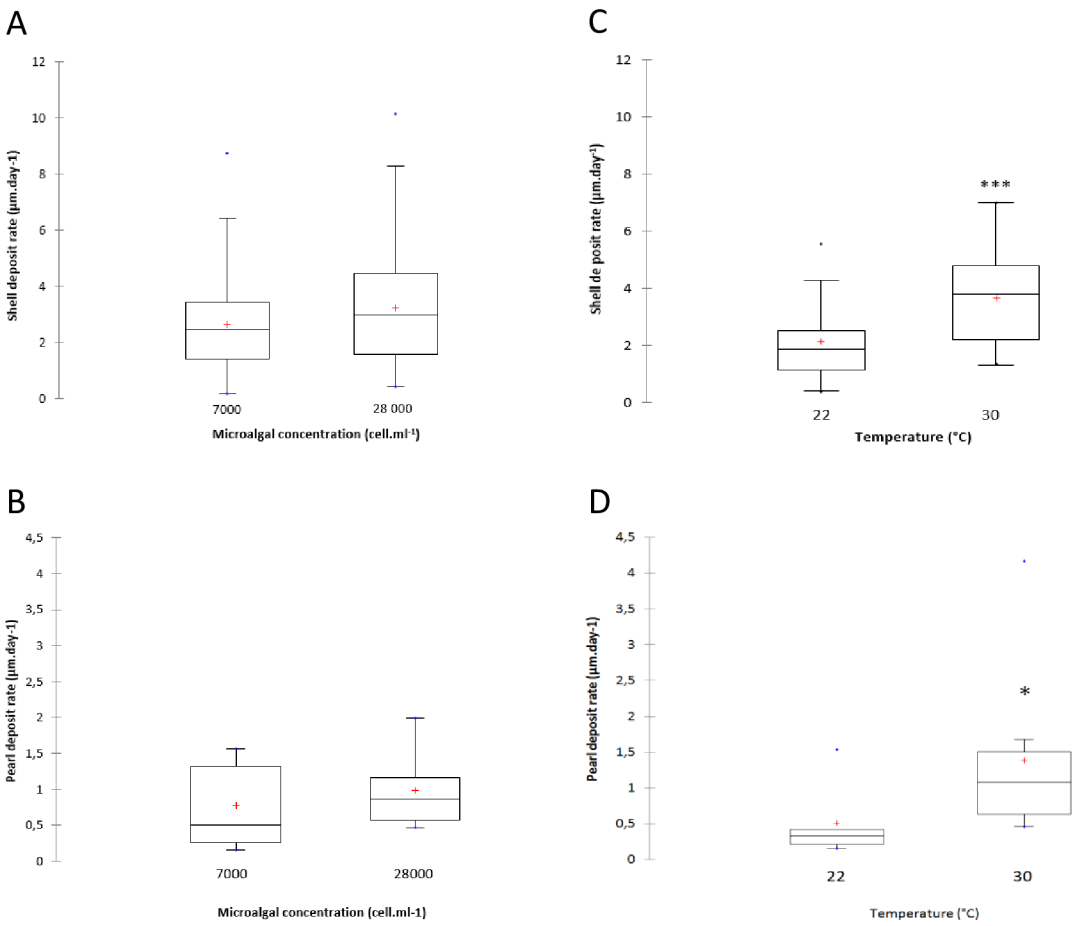

Fig 3. Mean shell and pearl deposition rate (SDR and PDR, µm day^{-1}) in the pearl oyster *Pinctada margaritifera* after one month at different (A and B) microalgal concentrations and (C and D) water temperatures. Each plot includes mean ("+" cross in the box-plot), median (solid bar in the box-plot), 25th to 75th percentile represented in the rectangular box, 1.5×interquartile range (non-outlier range of the box whiskers), minimum and maximum values (extreme dots) and outlier values (outside box whiskers). Statistical analysis is based on the Student's *t*-test. Statistical significance is indicated by asterisks as follows: $^{*}p < 0.05$, $^{**}p < 0.01$. (A: n = 88 and n = 87 for pearl oysters fed at 7000 cell mL^{-1} and 28,000 cell mL^{-1}, respectively; C: n = 46 and n = 50 for pearl oysters subjected to 22°C and 30°C, respectively; and D: n = 9 per condition).

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water temperature experiment, we found no significant correlations between the gene expression levels and the PDR or nacre tablet thickness (Table 3). The PDR and nacre tablet thickness were not significantly correlated ($\rho = 0.346$, $p = 0.159$).

Pearl quality traits

The quality of pearls harvested at 12 months postgrafting was evaluated using four criteria: surface quality as the number of defects, visual color and darkness level, and luster (S1 Data and Fig 5).

In the microalgal concentration experiment, no significant effects of pearl oyster preharvest conditions were observed on either the number of defects (F-test, $p = 0.877$), color (F-test, $p = 0.623$), darkness level ($\chi^2 = 0.957$) and luster (F-test, $p = 0.297$). (Fig 5A, 5B, 5C and 5D, respectively).

In the water temperature experiment, no significant effects of pearl oyster preharvest conditions were observed on either the color (F-test, $p = 0.836$) or darkness level ($\chi^2 = 0.509$)

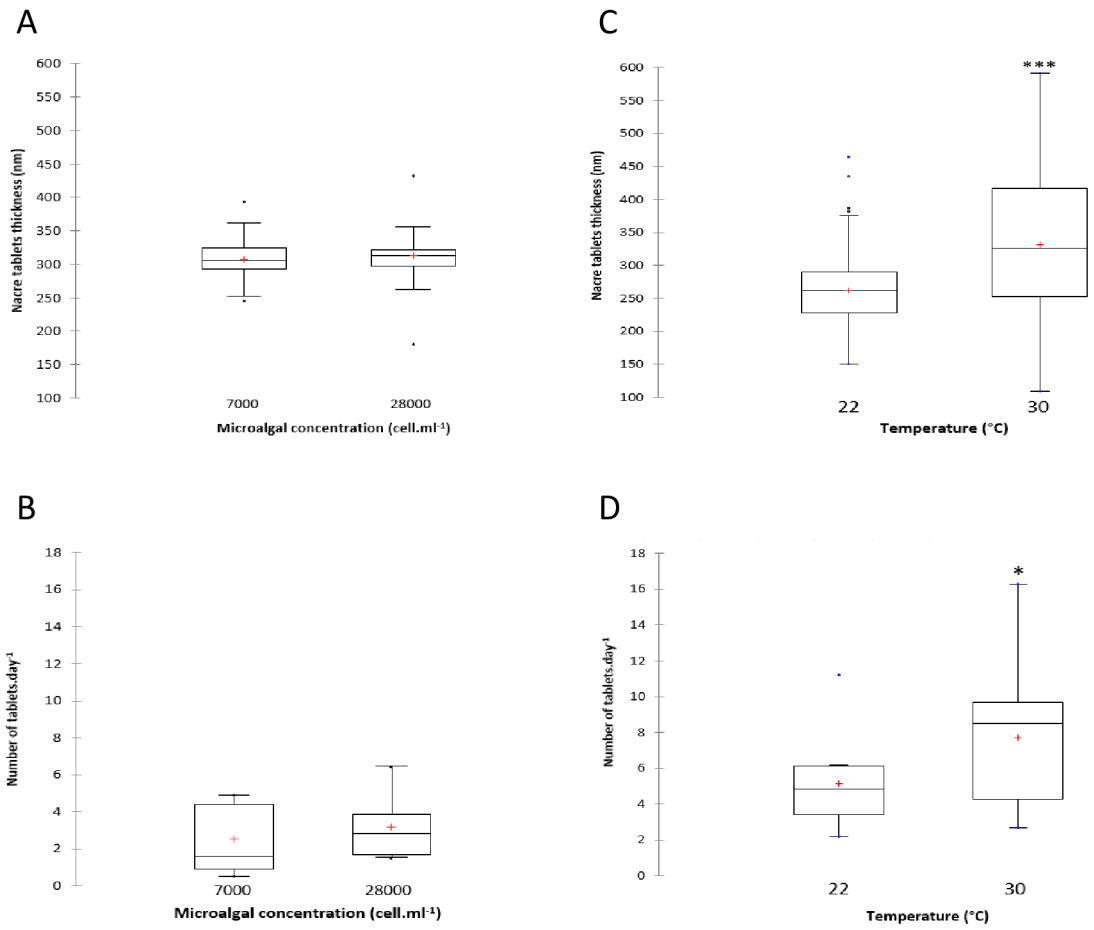

Fig 4. Pearl nacre tablet thickness and number of tablets deposited per day in pearl oyster *Pinctada margaritifera* after one month at different (A and B) microalgal concentrations and (C and D) water temperatures. Each plot includes: mean ("+" cross in the box-plot), median (solid bar in the box-plot), 25th to 75th percentiles represented in the rectangular box, 1.5×interquartile range (non-outlier range of the box whiskers), minimum and maximum values (extreme dots) and outlier values (outside box whiskers). Statistical analysis is based on the Mann-Whitney test for pearl nacre tablet thickness and on the Student's *t*-test for number of tablets deposited per day. Statistical significance is indicated by asterisks as follows: $^{*}p < 0.05$, $^{***}p < 0.001$. (A and C: n = 270 per condition; B and D: n = 9 per condition).

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(Fig 5F and 5G, respectively). However, the number of defects and luster were significantly dependent on water temperature (F-test, p = 0.047 and p = 0.006, respectively) (Fig 5E and 5H, respectively). Furthermore, pearl oysters maintained at 22˚C gave rise to a significantly lower proportion of pearls in the category with >10 defects and a significantly higher proportion in

Table 2. Correlation between relative gene expression and pearl deposition rate and nacre tablet thickness for microalgal concentration experiment.

Gene	PDR		Nacre tablet thickness	
	ρ	p-value	ρ	p-value
Pmarg-Pearlin	0.282	0.255	-0.245	0.327
Pmarg-Pif177	0.212	0.395	-0.212	0.398
Pmarg-MSI60	**0.507**	**0.033**	-0.352	0.152
Pmarg-Nacrein A1	0.251	0.312	-0.418	0.827
Pmarg-Aspein	**-0.668**	**0.003**	-0.063	0.446

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Table 3. Correlation between relative gene expression and pearl deposition rate and nacre tablet thickness for water temperature experiment.

Gene	PDR		Nacre tablet thickness	
	ρ	p-value	ρ	p-value
Pmarg-Pearlin	-0.195	0.436	-0.104	0.680
Pmarg-Pif177	0.067	0.789	0.059	0.815
Pmarg-MSI60	0.071	0.776	0.232	0.350
Pmarg-Nacrein A1	-0.212	0.398	0.247	0.320
Pmarg-Aspein	0.079	0.751	-0.117	0.664

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the category 6–10 defects, whereas pearl oysters maintained at 30˚C produced a significantly lower proportion in the category 6–10 defects than expected. Finally, pearl oysters maintained at 22˚C produced a significantly lower proportion of pearls without luster and a significantly higher proportion of pearls with high luster than expected by chance. Pearl oysters subjected to 30˚C produced a significantly higher proportion of pearls without luster and a significantly lower proportion of pearls with high luster than expected by chance.

Discussion

This study is the first analysis of the influence of food level (microalgal concentration) and water temperature, under controlled conditions, during the preharvest phase of pearl formation. We measured the expression levels of biomineralization-related genes in the pearl sacs, shell and pearl deposition rates, pearl nacre ultrastructure and quality traits. Our results show that, among the preharvest experimental factors investigated, water temperature had the greatest impact on pearl ultrastructure and quality. Indeed, pearl oysters maintained at 22˚C during the last stages of pearl formation produced pearls with thinner nacre tablets, higher quality of luster and fewer defects than expected. However, pearl growth was higher for pearl oysters maintained at 30˚C. Food level was shown to modulate the expression levels of the Aspein gene, with a significantly lower transcript abundance for pearl oysters fed at 28,000 cell mL^{-1} than those fed at 7000 cell mL^{-1}. Nevertheless, we did not observe differences in pearl ultrastructure and quality between the two microalgal concentrations.

Pearl sac biomineralization-related gene expression levels

As the donor oyster is the main genetic contributor to the expression of biomineralization-related genes involved in pearl formation [6], pearl oysters with grafts collected from each donor oyster were split across the two treatments for each experiment. Among the tested genes, only *Pmarg-Aspein* expression in the pearl sacs decreased when pearl oysters were fed abundantly (i.e., 28,000 cell mL^{-1} *T. lutea/C. gracilis*). This gene is involved in the calcite precipitation process [15] and is responsible for the formation of calcite in the prismatic shell layer [17]. This downregulation may indicate less calcite production and this could be advantageous for pearl production, as calcite is not desirable on the pearl surface and leads to a drop in quality. It would therefore be useful to measure other gene expression levels related to the formation of calcite. Food level was shown to significantly change the expression levels of several biomineralization-related genes in the mantle of *P. margaritifera* after a two-month exposure; for example, *Pmarg-MSI60* and *Pmarg-Pif177* showed a significant increase and decrease, respectively, for pearl oysters fed at 15,000 cell mL^{-1} in comparison to pearl oysters fed at 800 cell mL^{-1} [23]. In contrast, we did not provide evidence for such a food level effect on these

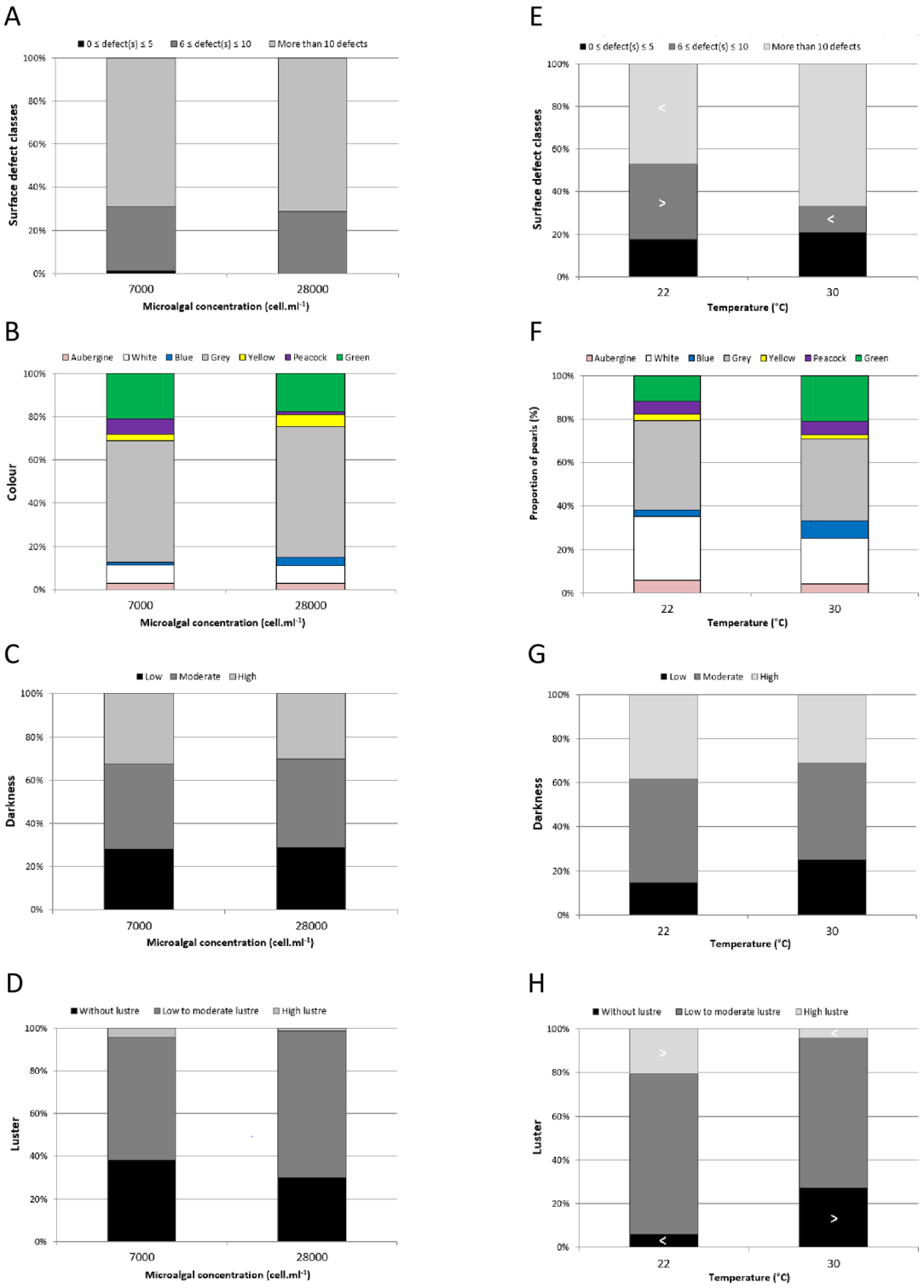

Fig 5. Proportion of harvested pearls in different pearl quality categories 12 months postgrafting following one month at different microalgal concentrations and water temperatures. Effect of pearl oyster fed at 7,000 or 28,000 cells mL^{-1} (n = 71 and n = 73, respectively) on (A) the number of defects, (B) color, (C) darkness and (D) luster. Effect of pearl oyster subjected to 22 or 30˚C (n = 34 and n = 48, respectively) on (E) the number of defects, (F) color, (G) darkness and (H) luster. > indicates a significantly higher number and < a significantly lower number than expected by chance (F-test, $p < 0.05$).

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same genes in the pearl sacs. Nevertheless, the microalgal concentrations we tested were not the same (7000 and 28,000 cell mL^{-1}) and our experiment was shorter (one month).

In this study, the water temperature did not impact biomineralization gene expression in the pearl sac. This is in agreement with previous studies on the same five genes in the mantles of *P. margaritifera* subjected to 22 and 30˚C [39,41]. However, in a previous study [41], a downregulation of *Pmarg-Aspein* and *Pmarg-Nacrein A1* was reported at 30˚C in comparison to 26˚C. Thus, it would be of interest to expand the range of tested water temperatures on the pearl sac biomineralization gene expression levels. Finally, with the advent of high-throughput sequencing technologies, an overall analysis without *a priori* knowledge, such as RNA-Seq, would be useful to identify differentially expressed genes related to biomineralization in response to food level and water temperature conditions.

Shell and pearl deposition rate

Food level has been reported to strongly influence shell growth rate of several mollusk species [42–44]. In pearl oysters *P.margaritifera* and *P.maxima* fed with T-Iso from 10 to 100 × 10^3 cell.mL^{-1}, optimal food concentrations for maximum scope for growth were 10 to 20 × 10^3 cell.mL^{-1} and 20 to 30 × 10^3 cell.mL^{-1} respectively [45]. Moreover, it was shown that different microalgal concentrations between 800 and 15,000 cell.mL^{-1} (composed of a mix of T-Iso and *Chaetoceros gracilis*) influences shell growth, molecular mechanisms underlying shell biomineralization and nacre tablet thickness in the shell of *P. margaritifera* [23,24].

In this study, we did not find differences of shell and pearl growth rates between our two microalgal concentrations. The shell deposition rate was lower than in two other studies, despite higher microalgae concentration [23,24]. This could be explained by a difference in growth potential related to the difference in sizes of individuals in the three experiments (mean shell height: 85 ± 6 mm in [23]; 85 ± 5.7 mm in [24]; 121 ± 11 mm for experiment 1 and 120 ± 12 mm for experiment 2 in this study). Indeed, the daily rate of nacre deposition declined with the size of pearl oyster *P. margaritifera* [46]. Moreover, in young pearl oysters *P. margaritifera* (< two years old), the reproductive effort is low and almost all the energy is invested in somatic and shell tissue [47]. Shell deposition rate was higher for 23-month-old pearl oysters in comparison to 40 month old pearl oysters, with values of 12.2 μm day^{-1} and 2.15 μm day^{-1}, respectively [48]. In this study, however, pearl oysters were approximately three years old and may have invested energy in both reproduction and shell growth. We did not report differences in pearl deposition rate between the two microalgal concentrations. We measured a pearl deposition rate of 0.78 ± 0.58 μm day^{-1} for pearls oysters fed at 7000 cell mL^{-1} and of 0.98 ± 0.51 μm day^{-1} for pearl oysters fed at 28,000 cell mL^{-1}. These deposition rates for 12 month old pearls were in the same range as a previous study that found pearl deposition rates of 9.21 × 10^{-2} ± 0.01 and 1.44 ± 0.04 μm day^{-1} for 24-month-old and 4-month-old pearls, respectively, after a two-month exposure at 10,000 cell mL^{-1} [24].

The microalgal concentrations of this study did not affect pearl nacre tablet thickness or the number of nacre layers deposited per day. Conversely, trophic level was shown to influence nacre tablet thickness in the shell with thicker nacre tablets for pearl oysters fed at 15,000 cell mL^{-1} than the pearl oysters fed at 800 cell mL^{-1} after a two-month exposure [24]. The authors of the study concluded that higher shell deposition measured at 15,000 cell mL^{-1} seemed to be due to the combination of thicker aragonite tablets and an increasing shell deposition rate [24]. Testing a wider range of microalgal concentrations that was used in the present study would help to better understand the effect of food level on pearl biomineralization.

Water temperature is a key parameter for bivalve shell growth [49–52]. In this study, we reported a higher SDR for pearl oysters subjected to 30˚C than those subjected to 22˚C, with a

gain of around 75%. Such an increase in shell growth with increased water temperature was also shown in the pearl oyster *P. margaritifera* with an increase by a factor of three between 21˚C and 28˚C [23] and by a factor of 2.9 between 22˚C and 30˚C [39]. Bivalve metabolic activity increases with an increase of water temperature until it reaches an optimum, beyond which it decreases [53,54]. The thermal optimum range was studied for several pearl oyster species [45,55]. For *P. margaritifera*, an energy gain from 22 to 30˚C was reported, with metabolic rates maximized at 30˚C [25]. This energy gain would explain the shell growth differences. Water temperature also influenced pearl growth rates, which was significantly higher for pearl oysters subjected to 30˚C than to 22˚C, with values of 1.38 ± 1.1 and 0.51 ± 0.4 μm day^{-1}, respectively. These results confirm the influence of water temperature that was suspected to partially explain size differences in pearl nacre thickness and weight produced in locations with contrasted water temperatures [26].

In our study, water temperature significantly affected both pearl deposition rate and the number of tablets deposited per day, which were higher for pearl oysters subjected to 30˚C than pearl oysters subjected to 22˚C. These results are consistent with previous studies, which have shown that high water temperatures promote pearl nacre growth [30,56]. Moreover, a recent study has provided support for the assertion that water temperature influences pearl nacre tablet thickness [30]. In their study, the authors reported that the thickness of the nacre tablet layer was influenced by cold water, which induced thinner tablets [30]. This is in accordance with another study, which stated that low water temperature results in thinner laminar nacreous layers and that high water temperature led to faster pearl oyster growth and a higher rate of nacre deposition [57]. Finally, a strong correlation was found between nacre crystal misorientations and water temperature in a study including several mollusk species from different environments [58]. The strong correlation was only found with maximum water temperature, which suggests that higher water temperatures increase nacre deposition and that aragonite tablets grow faster [58].

For both experiments, pearl oyster growth and subsequent pearl deposition rates were positively correlated, which confirmed previous results from studies demonstrating positive correlations between pearl parameters (size, thickness or weight) and recipient oyster biometric parameters [26,39,59,60]. The energy allocated to pearl deposition rate is likely to depend on recipient oyster metabolism.

A strong positive correlation was also found between *Pmarg-MSI60* expression levels and PDR, and a negative correlation was found between *Pmarg-Aspein* expression levels and PDR. MSI60 is involved in the formation of the nacreous layer and includes a calcium-binding domain [13], whereas Aspein is related to the formation of the prismatic layer and contains an aspartic-rich domain, which might be involved in controlling the selective precipitation of calcite [15–17]. MSI60 could therefore be a potential molecular marker of the pearl nacre deposition rate.

Pearl quality traits

The surface defects were not dependent on food level but on water temperature. Pearl oysters maintained at 22˚C gave rise to a low proportion of pearls in the category of >10 defects, whereas pearl oysters maintained at 30˚C produced a low proportion in the category of 6–10 defects. Rapid growth may result in pearls with a beaten surface appearance, referred to as "hammering" [19]. The slower growth rate of pearls at low water temperature may thus have resulted in fewer defects on the surface of the pearl. However, care should be taken when considering surface defects since some of them find their origin in the first stages of pearl formation [3].

The visual color and the darkness were not dependent on either food or water temperature. Pearl color has been reported to depend on donor oyster [32], on family effect [61] and is reported to be influenced by the environmental conditions where the recipient oysters grow [4,28]. The strength of the iridescent color has been shown to depend on the last layers of nacre and on its nacre structure in the shell [31]. The nacre structure was influenced by water temperature, with thinner nacre layers for pearl oysters reared at 22˚C than at 30˚C. However, there was no dependence of pearl color and water temperature. The darkness level has been found to be correlated with pearl nacre thickness and weight, with the palest pearls being the smallest [21,32]. These two pearl quality traits may be more dependent on the whole process of pearl mineralization rather than on the last stages of pearl formation.

Luster was dependent on water temperature but not food level. At 22˚C, pearl oysters produced more pearls with high luster. Thinner nacre layers on the top of the pearl was suspected to enhance the luster [28], and several studies have reported that pearls are preferentially harvested when water temperature is low because of their better luster [19,27]. Moreover, the quality of half-pearls (mabe) produced from *Pteria penguin* was the best during a period of relatively low water temperature [62]. We verified this hypothesis experimentally and confirmed that water temperature influences both pearl nacre ultrastructure and luster, with thinner nacre tablets and better luster at low water temperatures. In French Polynesia, this could partially explain that pearls produced in the Gambier archipelago, where water temperature is lower than in the Tuamotu archipelago, are renowned for their high quality of luster.

Conclusion

The last stage of pearl mineralization is crucial to obtain high-quality pearls and may be influenced by environmental parameters. Our results show that water temperature influences both pearl and shell deposition rates, with higher growth rates at higher water temperatures. Moreover, we confirmed that lower water temperature induces thinner nacre tablets, and we show that low water temperature gives rise to better luster and fewer defects. Nevertheless, we found no significant differences between the two tested microalgal concentrations on shell growth, pearl growth, nacre ultrastructure and quality traits. These results could have major implications for the pearl industry. A wise strategy to increase pearl quality would be to rear pearl oysters at high water temperatures to increase pearl growth and consequently pearl size; and to harvest pearls after a period of low water temperature to enhance luster and to reduce the number of defects.

Supporting information

S1 Data. Supporting_information.xlsx.
(XLSX)

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Writing – review & editing: Oïhana Latchere, Nabila Gaertner-Mazouni, Gilles Le Moullac, Denis Saulnier.

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Crossing Phenotype Heritability and Candidate Gene Expression in Grafted Black-Lipped Pearl Oyster *Pinctada margaritifera*, an Animal Chimera

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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 during *Pinctada margaritifera* pearl production. Genetic variance was evaluated at 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 biparental crosses and the F1 generation. Donor-related genetic parameter estimates clearly demonstrate heritability for nacre weight and thickness, darkness and color, and 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; whereas in pearl sac, heritability estimates were low ($h^2 < 0.10$; except for *CALC1* and *Aspein*). 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.

Subject areas: Quantitative genetics and Mendelian inheritance, Molecular adaptation and selection
Keywords: gene expression, heritability, pearl oyster, phenotype, *Pinctada margaritifera*

Quantitative genetics is a powerful framework to explore the complex genetic architecture of phenotypic traits (Kruuk and Hadfield 2007). The fraction of the phenotypic variability that is of transmittable genetic origin is called heritability (Falconer and Mackay 1996; Roff 1997; Lynch and 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 (Falconer 1989; Lynch and Walsh 1998; 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 graft-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 and 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 (Paultre 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 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 *Hyriopsis cumingi* (Jin 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 *Pinctada 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 with 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 2 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 understand 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 multicross design that made it possible to consider parental and segregating progeny contributions at 3 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 biparental crosses, making it possible to evaluate character transfer in a 2-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*, *Shematrin*, and *Prismalin*); and 3) for proteins implicated in both layers, *Nacrein* (Joubert et al. 2010; Marie et al. 2012; Xiang et al. 2013).

Materials and Methods

Experimental Design

Nine biparental *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. 2015a). Nine families were produced in 2 distinct periods (i.e., 2 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 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).

Individuals of the 9 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 9 progenies were taken from the rearing station and stored ready for use in the grafting procedure.

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 (color of the visceral mass and gills),

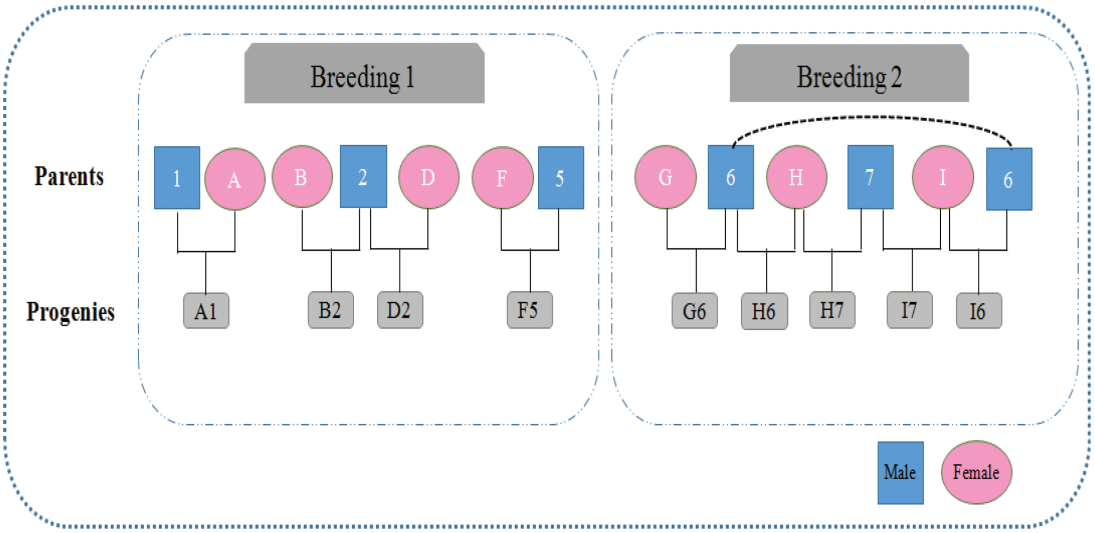

Figure 1. *Pinctada margaritifera* crossbreeding design for the production of the 9 half-sib families used as graft donors. Females and males were named with letters and numbers, respectively.

shell size appearance, and muscle resistance when prising the shells slightly open.

A total of 4 different experimental grafts were performed: 2 using the parents of the 2 breeding designs (1 per breeding) and 2 others using the progenies (1 per breeding). For breeding #1, all the 5 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 labeled according to the corresponding donor oyster for traceability.

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 5 variables were measured to characterize their quality (Figure 2):

- The size of the cultured pearls was assessed by measuring nacre thickness and weight.
- Cultured pearl shape was characterized in 2 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 color of the pearls was evaluated on the basis of the darkness of their color and their visually perceived color category, which is conferred by pigments (body color: gray, white, and yellow) and secondary colors (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 luster (components of cultured pearl grade) were determined separately so that they could be analyzed 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 2 operators working together and cross-checking.

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 (Miyamoto et al. 2005; Suzuki et al. 2009; Joubert et al. 2010; Montagnani et al. 2011; Marie et al. 2012; Huang et al. 2013; Miyamoto et al. 2013; Shi et al. 2013; Suzuki and Nagasawa 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). 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 a thin (<0.5 mm) layer tissue surrounding the pearls remained and immediately transferred and preserved into 2.0-ml tubes with RNAlater®. We then evaluated relative gene expression by screening aragonite-related genes (*Pif-177*, *MSI60*, and *Perline*), calcite-related genes (*Aspein*, *Shematrin5*, *Shematrin9*, and *Prismalin*), and one gene implicated in both layers (*Nacrein*) (Blay et al. 2017) (Table 1). 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. For each sample, 3 µg of total RNA were treated with DNase using a DNA-free Kit (Ambion). 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

Figure 2. *P. margaritifera* cultured pearls from different colors, showing the main quality traits variables. Shapes and A grade pearls were illustrated in (**a**), (**b**), (**c**), and (**d**), with respective circle, baroque, drop, and round samples. Pictures (**e**), (**f**), and (**g**) represented respectively pearls with numerous surface defects, pearls without luster, and rebut pearls.

"housekeeping genes": *REF1* (Joubert et al. 2014) and *GAPDH* (Lemer et al. 2015). The amplification reaction details are provided in Blay et al. 2017. 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 2 reference genes *GAPDH* and *REF1*, by the 2–ΔCt method (Livak and Schmittgen 2001), as follows: Relative expression $_{(\text{target gene, sample x})} = 2^{\wedge-(\Delta Ct \text{ sample, sample x}-\Delta Ct \text{ calibrator, sample x})} = 2^{-\Delta\Delta Ct}$. 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; Andersen et al. 2004).

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).

Table 1. Set of forward and reverse primers used for the biomineralization gene expression analysis in *Pinctada margaritifera*

Gene name	Function	NCBI accession numbers	Forward primer (5′–3′)	Reverse primer (5′–3′)
Pif-177	Aragonite formation	HE610401	AGATTGAGGGCATAGCATGG	TGAGGCCGACTTTCTTGG
MSI60	Aragonite formation	No accession number but described by Marie et al. 2012	TCAAGAGCAATGGTGCTAGG	GCAGAGCCCTTCAATAGACC
Perline	Aragonite formation	DQ665305	TACCGGCTGTGTTGCTACTG	CACAGGGTGTAATATCTGGAACC
Aspein	Calcite formation	No accession number but described by Marie et al. 2012	TGGAGGTGGAGGTATCGTTC	ACACCTGATACCCTGCTTGG
Prismalin	Calcite formation	HE610393	CCGATACTTCCCTATCTACAATCG	CCTCCATAACCGAAAATTGG
Shematrin5	Calcite formation	HE610376	GTCCGAAACCAAATCGTCTG	CTGTGGTGATGGTGACTTCG
Nacrein	Aragonite and calcite formation	HQ896199	CTCCATGCACAGACATGACC	GCCAGTAATACGGACCTTGG
Shematrin9	Calcite formation	No accession number but described by Marie et al. 2012	TGGTGGCGTAAGTACAGGTG	GGAAACTAAGGCACGTCCAC

Table 2. Significance of the fixed family effect on pearl quality traits and gene expression in *P. margaritifera* progenies

Pearl traits (n = 1918)	Nacre weight	Nacre thickness	Circle	Shape	Surface defect(s)	Luster	Grade	Darkness	Color
	***	***	**	*	***	ns	***	*	**
Graft (n = 164)	*Pif-177*	*MSI60*	*Perline*	*Nacrein*	*Aspein*	*Shematrin9*	*Prismalin*	*Shematrin5*	
	**	***	***	***	***	***	***	***	
Pearl sac (n = 73)	*Pif-177*	*MSI60*	*Perline*	*Nacrein*	*Aspein*	*Shematrin9*	*Prismalin*	*Shematrin5*	
	ns	*	ns	*	*	*	ns	ns	

1918 harvested pearls were examined for 9 pearl quality traits, and 8 genes coding for proteins potentially involved in the construction of the nacreous layer (*Pif-177, MSI60,* and *Perline*), the prismatic layer (*Aspein, Shematrin9*, *Prismalin,* and *Shematrin5*), and both the prismatic and the nacreous layers (*Nacrein*) were studied in 164 graft tissue pieces and 73 pearl sacs

***$P < 0.0001$; **$P < 0.01$; *$P < 0.05$; ns = not significant.

We first evaluated the "family effect" on culture pearl trait and gene expression among the progenies of the controlled breeding (Table 2). 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, luster, 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 color 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 as follows:

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

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 3 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).

Results

Cultured pearl quality traits were recorded and analyzed 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 Supplementary Tables S1–S4, supporting information. Results are presented in 3 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.

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 Supplementary Table S1, supporting information. Very highly significant "family effects" were demonstrated for the nacre thickness and weight ($P < 0.0001$) (Table 2). The cultured pearl quality traits are described in Supplementary Table S2, supporting information. Overall, analyses comparing differences in pearl quality indicators among the 9 families showed a significant "family effect" for all traits except luster (Table 2).

Analyses comparing differences in relative gene expression of the graft from the 9 families showed a highly significant "family effect," with the main differences between the 9 families (Table 2). 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 8 genes are given in Supplementary Table S3, supporting information. All differences between the 9 families for each gene are shown in Supplementary Figure S1, supporting information.

The comparison of the relative expression of the 8 genes in the pearl sac showed a significant "family effect" for 4 genes *Aspein* ($P = 0.01$), *MSI60* ($P = 0.01$), *Shematrin9* ($P = 0.01$), and *Nacrein* ($P = 0.04$) (Table 2). Pearl sac relative gene expressions are given in Supplementary Table S4, supporting information. All differences between the families for each gene in pearl sac are shown in Supplementary Figure S2, supporting information.

The high variability across samples for the gene expression data could be explained by the correlation between the gene expressions with the pearl's surface quality (Blay et al. 2017). Moreover, in *P. margaritifera*, we have a lot of variability in pearl culture quality at the time of harvest, with surface defects, surface deposits, and grade classification, which could justify the range of standard deviation observed.

Heritability of Cultured Pearl Traits

Heritability estimates for donor-derived pearl quality traits are given in Table 3. 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]), color ($h^2 = 0.16$; 95% CI [0.11, 0.23]), and luster ($h^2 = 0.12$; 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.

Table 3. Heritability (and CI 95%) for 1) pearl quality traits in *P. margaritifera* ($N = 2241$); 2) mantle graft tissue relative gene expression levels ($N = 176$); and 3) pearl sac tissue relative gene expression level ($N = 80$)

1) Pearl quality traits	Nacre weight	Nacre thickness	Circle	Shape	Surface defect(s)	Luster	Grade	Color	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)
2) Graft tissue relative gene expression	*Pif-177*	*MSI60*	*Perline*	*Nacrein*	*Aspein*	*Shematrin9*	*Prismalin*	*Shematrin5*	
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)	
3) Pearl sac relative gene expression	*Pif-177*	*MSI60*	*Perline*	*Nacrein*	*Aspein*	*Shematrin9*	*Prismalin*	*Shematrin5*	
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)	

Eight genes were studied coding for proteins potentially involved in the construction of the nacreous layer (*Pif-177*, *MSI60*, and *Perline*), prismatic layer (*Aspein*, *Shematrin9*, *Prismalin*, and *Shematrin5*), or both (*Nacrein*).

Heritability of Relative Gene Expression Levels

Heritability estimates for donor-derived relative gene expression in the graft are given in Table 3. 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 = 0.33; 95% CI [0.14, 0.51]), *Prismalin* (h^2 = 0.44; 95% CI [0.27, 0.6]), *Shematrin5* (h^2 = 0.35; 95% CI [0.21, 0.52]), and *Shematrin9* (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 3. 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).

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).

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 1998). 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* (Blay et al. 2017; Ky 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 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 2001; Li et al. 2013; Cao et al. 2016; Wang et al. 2017). 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. 2017). 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. 2017). 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 nonadditive 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 nongenetic sources of heritable phenotypes play a role in phenotypic variations (Jablonka and 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 and 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 nongenetic 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.

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; Cheung et al. 2003; Schadt et al. 2003). In such studies, mRNA levels are considered as a phenotypic value, which is subjected to variation (as every phenotype) due to experimental, environmental, and/or

genetic sources. These variations and associated heritability could thus 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 (Cheung et al. 2003; Schadt 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 biomineralization 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 2 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 (Liu et al. 2012; Miyamoto et al. 2013; Suzuki and 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, luster, and grade) and with color. Furthermore, a significant correlation was found between *Nacrein* relative gene expression with color of the pearl (and no significant correlation in graft tissue; Blay et al. 2017). These 2 candidates seem to be involved in pearl quality and color 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.

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 color, surface defects and grade, signifying an important donor oyster effects with a genetic basis, whereas 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. 2015b). 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 nonadditive 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, color, darkness, surface defects, and grade should be achievable through targeted donor oyster selection, whereas further work is required to understand the role of the recipient oysters on pearl phenotypic variance and gene expression variance.

Conclusion

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 interactions 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.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data Availability

In accordance with the *Journal of Heredity* data archiving policy, we have deposited the primary data underlying these analyses as follows:

- Cultured pearl quality traits for each individual: Dryad (10.5061/dryad.4gr1303)
- Gene expression levels for each individual: Dryad (10.5061/dryad.4gr1303)

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ORIGINAL ARTICLE

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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Abstract

Nucleated pearls are produced by molluscs of the *Pinctada* genus through the biomineralisation 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 3 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 Biomineralisation · Relative gene expression · Pearl sac · Biomarkers · Pearl quality · *Pinctada margaritifera*

Introduction

Biomineralisation 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 organisation 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 biomineralisation. 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 (Funabara et al. 2014). To understand biomineralisation mechanisms, one must possess knowledge on the structures of the organic matrix components together with the entire process.

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Nucleated cultured pearl production from the *Pinctada* genus 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 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 biomineralisation process similar to development of inner shell regulated by the mantle (Zhan et al. 2015). The role of the pearl sac in nacreous layer biomineralisation 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). The quality of Tahitian cultured black pearl 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 (Tayalé 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 biomineralisation of the nacreous layer of great economic interest. The regulation of shell matrix proteins in biomineralisation during the pearl culture and the impact of this process on pearl quality are research topics of high priority.

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) (Table 1). Other genes involved in prismatic calcite include *Aspein* (Tsukamoto et al. 2004; Takeuchi et al. 2008), *Prismalin-14* (Suzuki et al. 2004; Suzuki and Nagasawa 2007) and the *Shematrin* 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; Suzuki and Nagasawa 2013). The role of elaborate organic matrix of protein embedded in calcium carbonate crystals of either aragonite or calcite has been examined (Addadi and Weiner 1985; Belcher et al. 1996; Falini et al. 1996; Levi et al. 1998; Weiner and Hood 1975). Shell matrix is not formed simply by self-assembling silk-like proteins but by diverse proteins through complex assembly and modification processes that may involve haemocytes and exosomes (Zhang et al. 2012). 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 surface characteristics that impact pearl quality. Detailed knowledge of the variation in gene expression of protein constructing the matrix in the pearl sac during pearl formation is an essential step towards 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 eight 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 explores 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.

Materials and Methods

Experimental Animal and Tissue Sampling

Wild donor and recipient *P. margaritifera* were collected as spat in the lagoon of Mangareva (Gambier Archipelago, French Polynesia) 2 years prior grafting process. Their collection and culture are detailed in Ky et al. (2014). At this age, the dorso-ventral and antero-posterior measurements of shells average 7.85 ± 1.2 cm and 7.3 ± 1.1 cm, respectively. 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

Table 1 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′)	Efficiency
PIF	Pif-177	Aragonite formation	HE610401	AGATTGAGGGCATAGCATGG	TGAGGCCGACTTTCTTGG	2.02
MSI60	MSI60	Aragonite formation	No accession number but described by B. Marie et al. 2012	TCAAGAGCAATGGTGCTAGG	GCAGAGCCCTTCAATAGACC	2.08
PERL1	Pearlin	Aragonite formation	DQ665305	TACCGGCTGTGTTGCTACTG	CACAGGGTGTAATATCTGGAACC	2.04
ASP	Aspein	Calcite formation	No accession number but described by B. Marie et al. 2012	TGGAGGTGGAGGTATCGTTC	ACACCTGATACCCTGCTTGG	2.01
PRISM	Prismalin 14	Calcite formation	HE610393	CCGATACTTCCCTATCTACAATCG	CCTCCATAACCGAAAATTGG	1.92
SHEM5	Shematrin	Calcite formation	HE610376	GTCCGAAACCAAATCGTCTG	CTGTGGTGATGGTGACTTCG	2.11
SHEM9	Shematrin	Calcite formation	No accession number but described by B. Marie et al. 2012	TGGTGGCGTAAGTACAGGTG	GGAAACTAAGGCACGTCCAC	1.95
CALC1	Nacrein	Aragonite and calcite formation	HQ896199	CTCCATGCACAGACATGACC	GCCAGTAATACGGACCTTGG	1.96

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 minimise 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.

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 no. 30, 26 July 2001) from the most to the least valuable quality: A, B, C, D and “Rejects” (R). 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 five 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 Fig. 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.

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*, *Shematrin*, *Prismalin*) and one gene implicated in both layers (*Nacrein*). Primers used for amplification are given in (Table 1).

After removing the RNA later by pipetting and absorption, total cellular RNA was extracted from the individual pearl sac

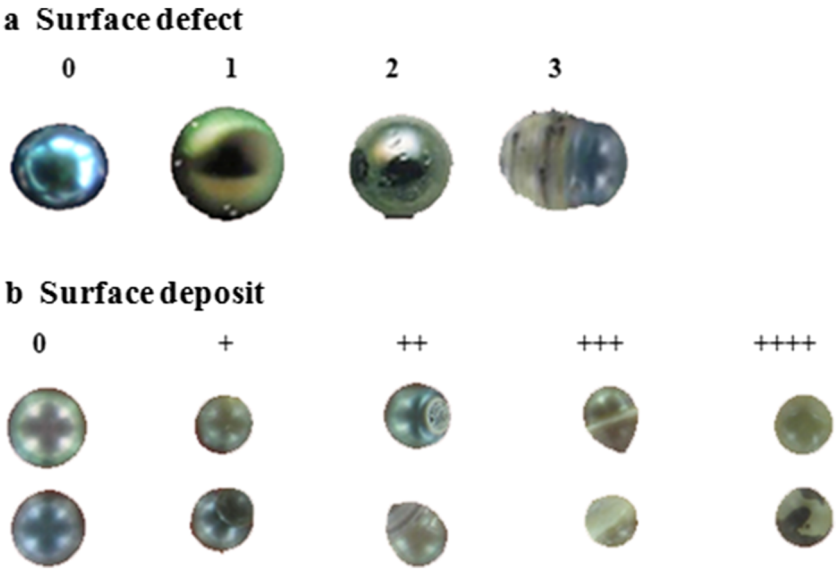

Fig. 1 Cultured pearl description. **a** Surface defect levels "0": no defects, "1": 1 to 5 defect(s), "2": 6 to 10 defects and "3": more than 10 defects. **b.** Surface deposits ("0" no deposits to "++++" in which deposits cover the whole surface)

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, normalised with two reference genes SAGE and GAPDH (Blay et al. 2017), to compare the relative expression results (Livak and Schmittgen 2001) as follows: relative expression $_{(\text{target gene, sample x})} = 2^{\wedge\text{-}(\Delta\text{Ct sample, sample x}-\Delta\text{Ct calibrator, sample x})} = 2^{-\Delta\Delta\text{Ct}}$. Here, the ΔCt calibrator is the mean of the ΔCt values obtained for the tested gene.

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. 2013) 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 (Team RC 2015).

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.

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 Fig. 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 ($p =$

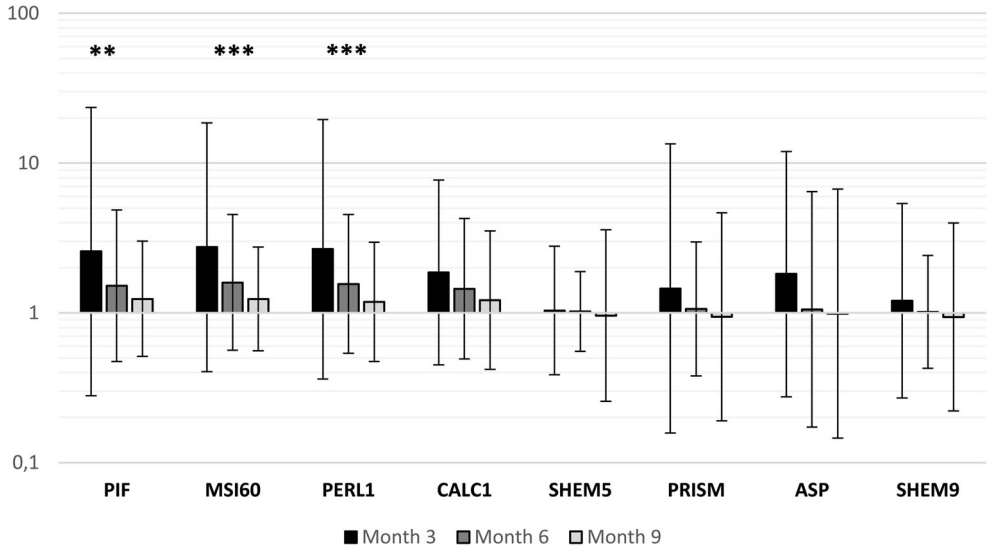

Fig. 2 Relative expression levels of matrix proteins in the pearl sac at different stages of pearl development. Histograms in dark grey represent data for all pearls at 3 months and histograms in light grey represent data at 9 months. *Y* axes are in the logarithmic scale. Error bars indicate standard deviations. Statistical differences between the phenotypes are indicated by asterisks: * for $0.05 < p < 0.005$, ** for $0.005 < p < 0.0005$, *** for $p < 0.0005$

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. The temperature at harvest time was 27.0, 27.0 and 25.2 °C after 3, 6 and 9 month of culture, respectively.

Analysing kinetics data over the 9 months following the graft, we did not find any preferential harvest time when focusing on changes in surface defects (χ^2 test, $p = 0.590$). 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 2).

Data analysis showed a highly significant difference in pearl surface deposits between harvest times (χ^2 test, $p < 0.0001$). 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 2).

There were also significantly more reject 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 1).

Impact of Biomineralisation Gene Expression Level on Pearl Surface Quality

Among the eight candidate genes studied in the pearl sac at month 3, the expressions of four of them were significantly different in pearl sac between the surface defect categories (Online Resource 1). PIF, MSI60 and PERL1 gene expression levels were significantly 5.2, 3.4, 4.2 times higher, respectively for pearls with less defect (0 to 9 defects), in comparison to pearls having more than 10 defects, which had a fold change inferior to 0.6 ($p < 0.0001$). Inversely, SHEM5 showed

Table 2 Cultured pearl quality traits from the experimental graft. Percentages of cultured pearls (and number in brackets) at each harvest time (3, 6 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	31.2	25.0	39.1	11.0	14.1	18.7	18.7	37.5	46.9	15.6	6.2	12.5	18.8
	(3)	(20)	(16)	(25)	(7)	(9)	(12)	(12)	(24)	(30)	(10)	(4)	(8)	(12)
Month 6	2.6	48.0	16.9	32.5	11.7	35.0	20.8	18.2	14.3	76.6	10.4	1.3	6.5	5.2
	(2)	(37)	(13)	(25)	(9)	(27)	(16)	(14)	(11)	(59)	(8)	(1)	(5)	(4)
Month 9	4.3	40.0	22.9	32.8	10.0	20.0	42.9	17.1	10.0	82.9	10.0.	2.9	1.4	2.8
	(3)	(28)	(16)	(23)	(7)	(14)	(30)	(12)	(7)	(58)	(7)	(2)	(1)	(2)

significantly greater expression in pearls having more than 10 defects, with a 3.0-fold change compared with pearls having 0 to 5 defect(s), which had a 0.4-fold change ($p = 0.003$). At month 6, the relative expression of gene encoding matrix protein of Aspein and Shematrin families in pearl sac were significantly different between the categories of surface defect. ASP and SHEM9 showed significantly higher fold change 9.0 and 2.5, respectively for pearls with more than 10 defects compared to other pearls which had fold change inferior at 0.5 for ASP and inferior at 1 for SHEM9 ($p < 0.001$ and $p = 0.024$ respectively). After 9 months of culture, only the relative expressions of gene encoding matrix protein of Aspein family were significantly different between the surface defect categories. ASP gene expression level in pearl sac showed significantly higher relative expression levels for pearls with more than 10 defects (fold change = 4.7) compared to other pearls (fold change < 1) ($p = 0.001$).

After 3 month of culture, only CALC1 encoding a nacrein protein matrix was not significantly different among the different level of surface deposit on the pearls. In fact, the three genes encoding aragonite protein PIF, MSI60 and PERL1 showed higher relative gene expression level in pearl "without deposit" (fold change = 6.0, 4.0, 4.7, respectively), compared to "++++" pearl category (fold change = 0.1, 0.6, 0.4, respectively) ($p < 0.0001$, $p = 0.001$ and $p = 0.0001$, respectively). Inversely, SHEM5, PRISM, ASP and SHEM9 encoding protein playing a role in calcite formation, had higher expression levels for pearls with overall deposit (fold change = 7.9, 40.5, 13.3, 20.1, respectively) than pearls "without deposit" and pearls with "+" low deposit (fold change < 1) (Online Resource 2). At month 6, three genes encoding aragonite protein, PIF, MSI60 and PERL1 were significantly different according to the deposit level on pearls ($p = 0.004$, $p = 0.006$, $p = 0.006$, respectively). Data analysis showed a lower expression of these genes for the pearls in the "++++" category. Inversely, pearls with high surface deposit "++++" showed higher relative expression level of gene encoding protein taking part in calcite formation such as PRISM ($p = 0.001$), ASP ($p < 0.0001$) and SHEM9 ($p = 0.002$) (fold change = 175.1, 698.3, 122.4, respectively) than pearls "without deposit" (fold change < 1). After 9 month of culture rearing, PRISM, ASP and SHEM9 expression levels were significantly different among the levels of deposit on the pearls ($p < 0.0001$, $p = 0.0002$, $p = 0.0001$, respectively). PRISM, ASP and SHEM9 had higher expression levels for pearls with deposit ("+" to "++++") (mean fold change = 68.3, 221.0, 33.5, respectively) than pearls "without deposit" (fold change < 0.6) (Online Resource 2).

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 reject pearls (fold change = 3.0, 7.5, 5.4, 5.2, respectively) than for grade "A" pearls (fold change < 0.4) (Fig. 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 reject 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 reject pearls (fold change = 53.3, 89.9, 13.5, respectively) than for A, B and C pearls (fold change < 1) (Fig. 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 Fig. 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*, *CALC1* 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 *CALC1* 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

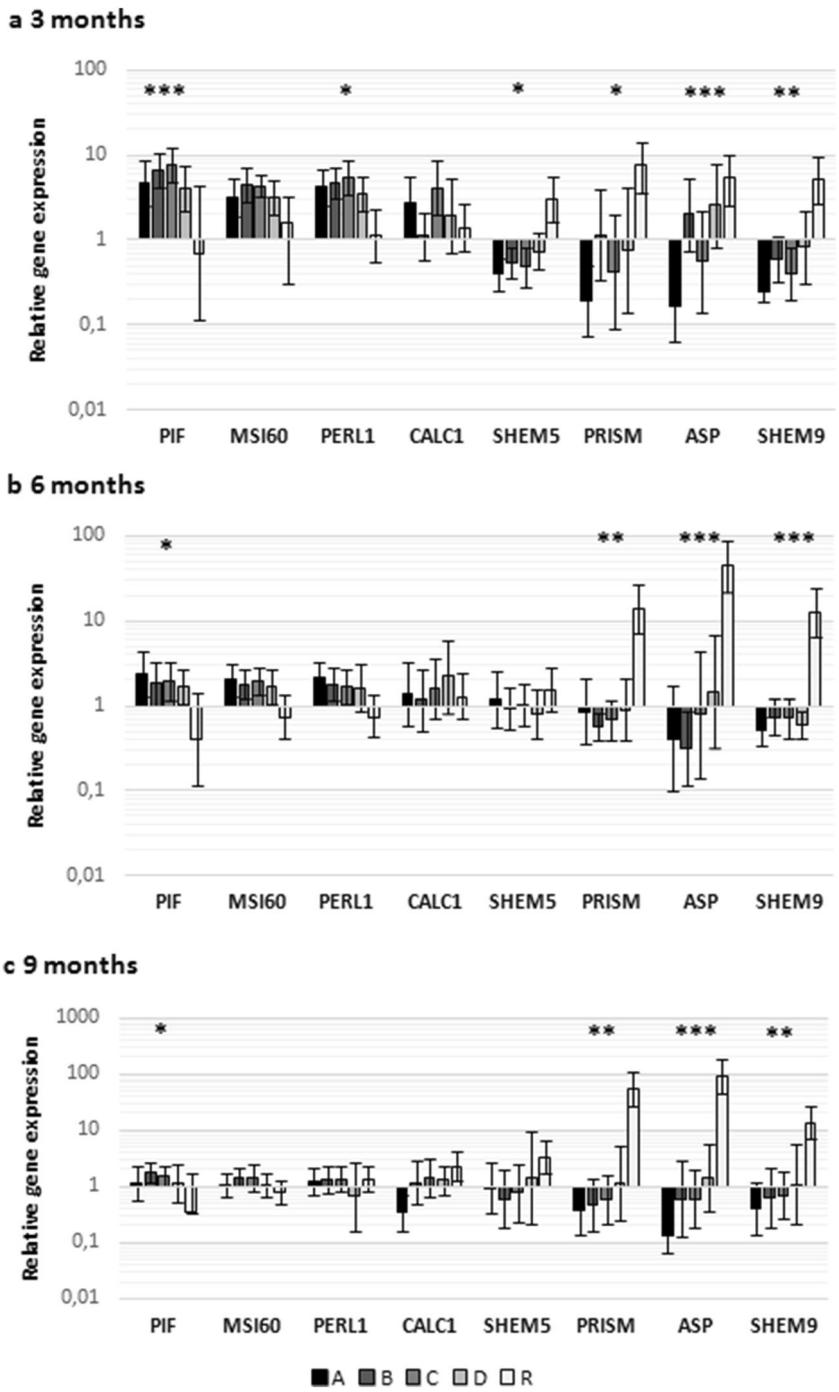

Fig. 3 Relative expression of 8 biomineralisation genes in the pearl sac of *P. margaritifera*: **a** at 3 months, **b** 6 months and **c** 9 months. Histograms in dark grey show data for "A" grade pearls and light grey histograms show data for the "R" grade pearls (rejects). Y axes are in the logarithmic 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 $p < 0.0005$

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*. 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 reject 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

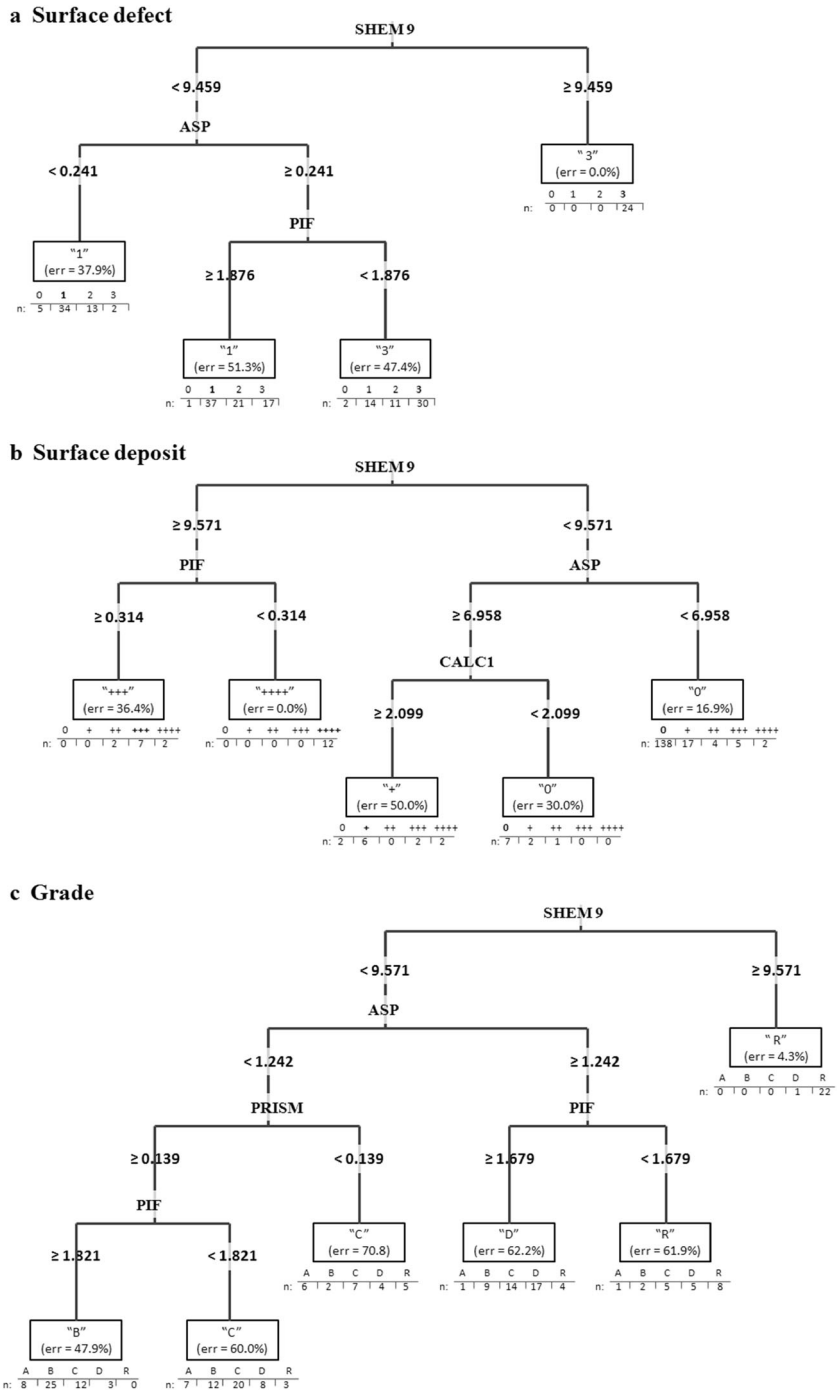

Fig. 4 Multivariate regression trees of pearl quality categories ($n = 211$). Candidate predictor/explanatory variables are the relative expression ratio of the eight potential marker genes, and the response variables are **a** the four sub-clusters of surface defect without to more than 10 defect on 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

of three common genes (i.e. *SHEM9*, *ASP* and *PIF*), which relate to surface defect, surface deposit and grade.

Discussion

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 3 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 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.

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 biomineralisation process (Suzuki et al. 2009; Miyamoto et al. 2005, 2013; Montagnani et al. 2011; Huang et al. 2013; Shi et al. 2013; Suzuki and Nagasawa 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. An RNAi (RNA interference) 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 method would be useful to investigate functions of genes and their impact on cultured 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. 2011a, b). 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. Shematrin is a family of glycine-rich shell matrix proteins known to be present in the prismatic microstructure of several pearl oyster species (Gardner 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, Prismalin-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; Suzuki and Nagasawa 2007). These findings suggest when the pearl had surface deposits or defects, *Shematrin* and *Aspein* and *Prismalin 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.

For the first time, cultured pearl biogenesis was followed over the first 9 months of *P. margaritifera* pearl culture examining both pearl phenotype and pearl sac molecular parameters. Results showed that the first 3 months of pearl biogenesis in *P. margaritifera* is primordial in determining pearl surface quality. In addition, three genes encoding proteins involved in the biomineralisation process (*SHEM9*, *ASP* and *PIF*) were implicated in the surface quality signature of the cultured pearl. The findings provide a basis for future research towards developing improved pearl culture practice and pearl quality.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

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RESEARCH ARTICLE

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

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Abstract

Ageing is defined as the progressive decline in tissue and organ functions 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 grafting experiments 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 family 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; and 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. These results indicate that donors aged between 12 and 18 months have high potential for biomineralisation and nacre deposition, and likely produce larger and higher quality cultured pearls than older donors.

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 [1–10]. 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

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mortality [11–15]. 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 [16,17]. Another study examined biological and environmental factors that control root dynamics and function through the effect of root ageing on grapevines [18].

Since the development of artificial pearl cultured 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 [19]. The marine pearl industry is primarily based on the culture of pearl oysters from the genus *Pinctada* spp or *Pteria* spp (Family: Pteriidae) [20], with an estimated annual global value of over US$397million in 2013 [21]. 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 donor (two distinct genomes) that create a genetic chimera with each genome maintaining its own genetic identity throughout the grafted organism [22]. 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 (~4mm^2) of mantle tissue cut from a donor oyster. This small piece of donor mantle tissue is called saibo. The saibo 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. As only small pieces of saibo are required for grafts, a single donor oyster can provide material for multiple recipients. 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 [19].

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 culture. 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 [23]. Studies equally demonstrated a donor [24] or family [25] 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 [26–29]. A recent study evaluated the effect of donor shell size at a specific age on pearl quality traits [30] 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. The study found that pearls produced from 2 year old donors were significantly better quality than those produced by 5 year old donors [31]. No study has examined the impact of using a younger donor oyster ($<$ 2 years old) and the age effect on cultured pearl size.

This 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. To do this, we conducted grafting experiments using donors from a single biparental family at 12, 18, 24 and 30 months of age, and assessed resulting pearl quality from each cohort. To determine whether our results were reproducible (and to assess even younger donors), we conducted a second series of experiments in a separate location, in which donors

from a second unrelated family were used as grafts at 7, 12, 18 and 24 months of age. Several phenotypic measurements were made: 1) the shell biometric growth parameters of the donor families, 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.

Materials and methods

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. The authority who issued the permission for pearl oysters transfers is: "Direction des Ressources Marines et Minières". No endangered or protected species is involved in the present study, and no specific permission is required for the location of the culture experiment, as it is in public maritime area.

Grow-out site locations: Rangiroa atoll and Mangareva 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. 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 km^2. The site of culture is located at Avaturu village (Gauguin's pearl farm).

Mangareva Island is located in the Gambier Archipelago (23˚09′S, 134˚58′W), about 1590 km southeast of Tahiti. The island surface area is 27 km^2. The island is the central and largest of the archipelago and has a large lagoon 24 km in diameter containing reefs. The culture site was located at Regahiga Pearl Farm.

Animal sources

Two bi-parental *P. margaritifera* families were produced at the Ifremer hatchery facilities (Vairao—Tahiti, French Polynesia), using wild 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 [32]. Artificial breeding, larval rearing and oyster culture procedures were conducted using the protocol developed by Ky et al. [25]. After approximately 45 days of age, the seed oysters were transferred to the nursery and reared in raceways of 90 × 20 × 20 cm (corresponding to a volume of 30 liters). Unfiltered seawater was added with a suspension of algae produced in outdoor tanks at a renewal rate of 100 L h-1. The juveniles were detached once they reached an average size of 3.0 ± 0.8 mm (dorso-ventral measurements) and were pierced and tied together onto a CTN (Cord Technical Nakasai) rearing system, where they were left until their transfer at 6 months old. The CTN involves drilling a small hole through the base of the shell in the dorsal-posterior region. This process does not affect living tissue. The CTN were protected using plastic mesh to prevent predation in the lagoon.

CTN from the two families, used as donors, were randomly selected and transferred (at six months old) by plane to Rangiroa atoll for F_RGI family and Mangareva Island for F_GMR family to allow the oysters to adapt (1 month) and grow in local environmental conditions before they were randomly selected for the grafting procedure. These pearl oysters (attached to CTN) were regularly cleaned by high pressure sea water spray

Experimental design and grafting procedure

Donors from the F_RGI family were used in grafting experiments atthe 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 used in grafting experiments 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 [33]. The recipient oysters were sourced from a local natural spat collection at each culture site. They were selected based on visible health status (colour of the visceral mass and gills), shell appearance (black colour without any damage, and with visible concentric growth lines) and muscle resistance when the shells were pried open. A total of 8 different grafting experiments were performed with all 80 donors for F_RGI and F_GMR families. These donors were used to perform 1400 and 1000 grafts, respectively (Table 1). All recipient oysters were seeded using 2.4BU nucleus size in Rangiroa and 1.8BU nucleus size in Mangareva. A larger number of grafts were performed in Rangiroa because the increased size of the donors enabled more pieces of saibo to be cut from the mantle. At 45 days post-graft, recipient oysters were checked to estimate nucleus retention and oyster mortality rates as described in [34]. 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 by high sea water spray every six months of culture at both culture sites, in order to remove biofouling (epibiota), which can hinder healthy oyster growth and pearl production.

Cultured pearl quality phenotypes

Pearl phenotype categories were recorded to characterize the quality of the pearl [25]:

- 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).

Table 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), total number of grafted oysters. *Harvest*: date of harvest (18 months post grafting), percentage of grafted oysters that produced a pearl and number of pearls and keshi. *Sample*: Number of graft tissues at graft time and number of pearl sac tissues at harvest time.

			Graft			Harvest				Sample	
Location	Experiment name	Age of donor (month)	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

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- 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.

Biomineralisation gene expression phenotype

In order to assess expression levels of known biomineralisation genes in donor tissue of different ages, we sampled graft tissues (3 to 5 pieces per donor) during the graft operation and pearl sac tissues during the harvest. 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 a thin (< 0.5 mm) layer of tissue surrounding the pearls remained, and immediately transferred and preserved into 2.0 ml tubes with RNAlater". 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*, *Shematrin5*, *Prismalin*, *(for graft and pearl sac tissues) and Shematrin9* (only for pearl sac tissues)) and one gene implicated in both layers (*Nacrein*) (S1 Table). 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 [35] and GAPDH (GAPDHS: AGGCTTGATGACCACTGTCC/ GAPDHR: AGCCATTCCCGTC AACTTC) [36]. The relative stability of the GAPDH and SAGE combination was confirmed using NormFinder (Stability value for best combination) (Results in S1 Appendix).

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.) and the quality of the RNA was checked to exclude degradation using an agilent 2100 bioanalyser. The RIN values were between 6.50 and 7.40 corresponding to a sufficient quality for quantitative real-time PCR analysis. 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. A no-RT control was screened by qPCR using a housekeeper gene to ensure there was no DNA contamination. The amplification reaction contained 5 μL LC 480 SYBR Green I Master Mix (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. We allowed a difference of less

than 0.5 ct between our two replicates. If the difference is superior to 0.5 ct, the qPCR reaction was repeated, and the sample was removed if congruent ct values were once again not obtained.

The relative expression ratio (R) of a target gene was calculated based on E and the CP deviation of an unknown sample versus a "control", and expressed in comparison to a reference gene as follows: $R = E_{(target)}^{\Delta Ct\ target\ (control—sample)} / E_{(ref)}^{\Delta Ct\ ref\ (control—sample)}$ [37]. Here, the control represents the mean of the values obtained for the tested gene [38]. 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% (S1 Table). A total of 136 graft tissues and 135 pearl sac samples were used for the analyses (last two columns in Table 1). The graft from GMR7 and pearl sac sample from the GMR12 experiment were not sampled due to technical problems.

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 [28]. 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, the donor oyster 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 [27]. Pearl thickness was measured using a digital micrometer and nacre thickness = [(cultured pearl average diameter)—(nucleus diameter)]/2. The diameter of non-round pearls was taken as the averageof measurements from the thinnest and thickest points.

Statistics

The normality of the data distribution and homogeneity of variance were tested for pearl size, donor oyster biometric parameters and relative gene expression ratio 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 relative gene expression ratio. 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, Kruskall-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)[39].

Results

A total of 1086 pearls 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 family shell growth, pearl size, pearl quality traits and relative gene expression in the graft and pearl sac tissue.

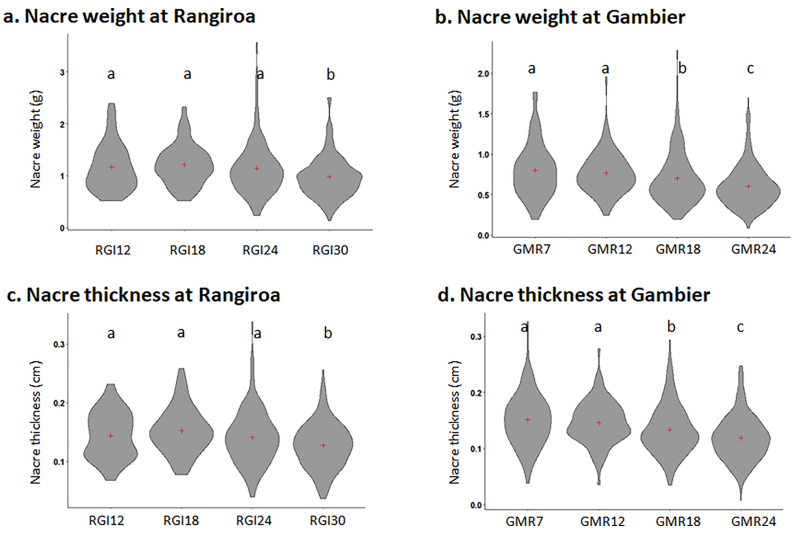

Fig 1. Pearl size harvested for the two sites across four age classes. Pearl nacre weight pearls in Rangiroa (a) and Gambier (b) and mean pearl nacre thickness in Rangiroa (c) and Gambier (d) measured across four age classes. "+" Cross represent the mean in the violin plot. Letter indicates significant difference between the age group ($p < 0.05$). Datas available on S2 Appendix.

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Age effect on pearl size

Results of pearl size in Rangiroa and Gambier for each donor age category are illustrated in Fig 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 pearls 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.

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.

A significant age effect was recorded for "lustre" in Gambier and Rangiroa ($p < 0.0001$ and $p = 0.003$, respectively). Cultured pearls from GMR18 and RGI24 recorded the highest level of lustre pearl, but also the same amount of pearl without any lustre (Fig 2A).

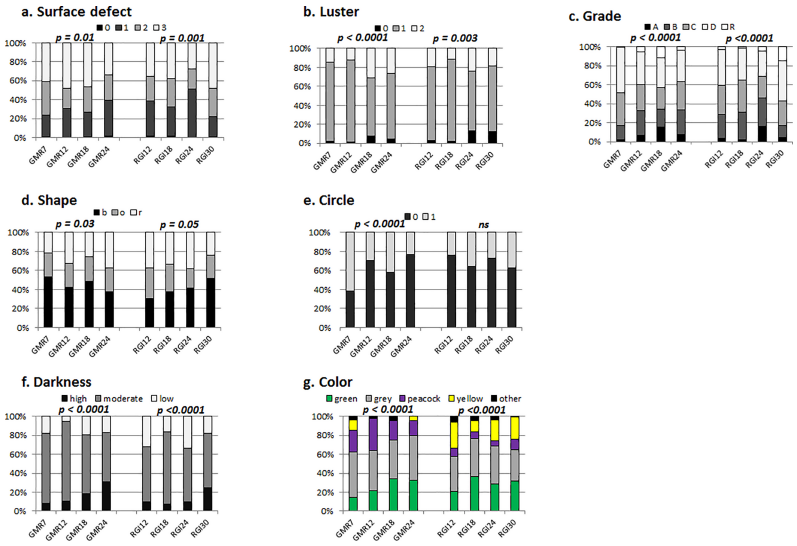

Fig 2. Cultured pearl quality traits from the experimental graft distribution. Percentage of cultured pearls for each experiment in Gambier (GMR) and Rangiroa (RGI) for different donors in different age groups (7, 12, 18, 24 and 30 months old) and the p-value in Gambier and Rangiroa with the following variables: a. surface defect classes ("0" = 0 defects, "1" = 1–5 defects, "2" = 6–10, and "3" = >10 defects), b. luster levels ("0" = absence of luster, "1" = moderate luster, and "2" = high luster), c. classification grade ("A" to "D" and Rejects), d. shape categories ("b" for baroque and semi baroque, "o" for oval and drop," r" for round and semi round pearls), e. pearl circles ("0" = absence and "1" = presence), f. darkness level (low, moderate and high darkness) and g. visual color categories ("green", "grey", "peacock", "yellow" and "other", corresponding to white, blue and aubergine pearls). Datas available on S2 Appendix.

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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 Fig 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 circled than in other experiments with 62% of circled pearls compared to 31% of circled 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 Fig 2.

Age effect on biomineralisation process

Relative gene expression for the panel of protein coding genes implicated in calcite and aragonite layers at Rangiroa and Gambier in the graft and pearl sac tissue are illustrated in Fig 3.

Concerning the graft tissue, the relative gene expression ratio for the seven candidates varied at Rangiroa and were significantly different among the three donor ages (12, 18 and 24

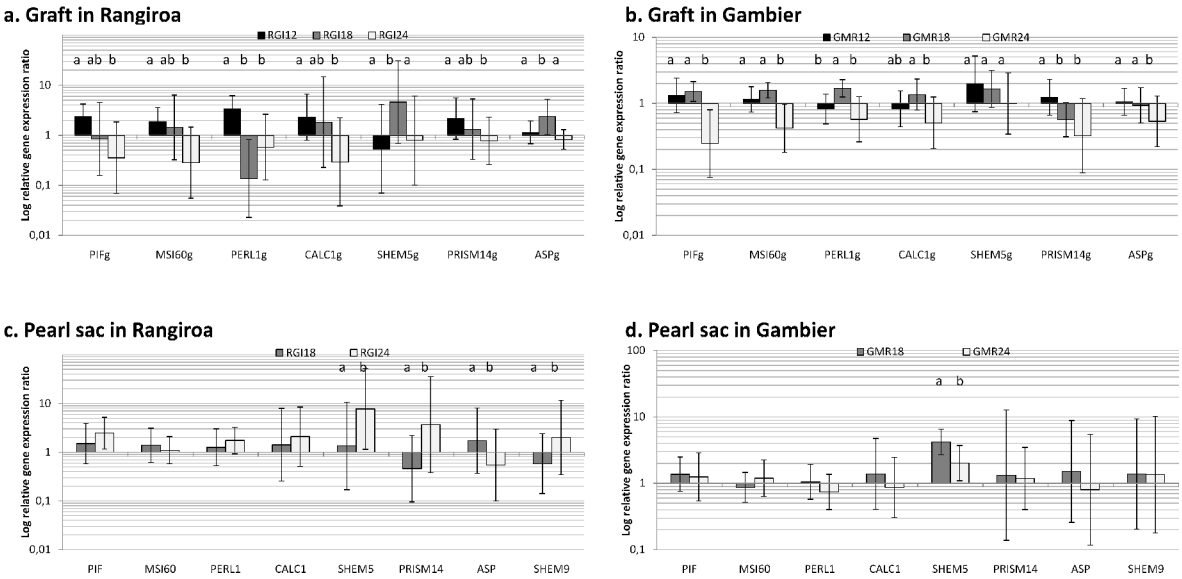

Fig 3. Relative gene expression ratio of biomineralisation genes in *P. margaritifera*. Relative expression of 7 genes in the saibo at Rangiroa (a) and Gambier (b) for donors aged 12, 18 and 24 months (dark to light grey). Relative expression of 8 biomineralization genes in the pearl sac in Rangiroa(c) 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 ($p < 0.05$). Datas available on S2 Appendix.

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months). PIF, MSI60, PERL1 and CALC1 relative gene expression ratio were significantly higher for the 12 month old donors (2.40, 1.94, 3.51 and 2.24, respectively), compared to the 24 month old donors which had a relative gene expression ratio 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 relative gene expression ratio were significantly higher for GMR18 compared with GMR24 which had a relative gene expression ratio inferior to 0.6 for all genes ($p < 0.0001$).

In Rangiroa, four candidate genes PIF, MSI60, PERL1 and CALC1were not significantly different in the pearl sac between the two donor age categories. ASP had higher relative gene expression ratio in pearl sac from younger donor tissue (1.73).

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 ($p = 0.0002$). SHEM5 and ASP had higher relative gene expression ratio in pearl sac from younger donor tissue (4.68 and 1.5, respectively).

Age effect on shell growth

At the time of grafting, the donor oyster width, height and thickness were recorded to provide average growth measurements for the two families. Results for shell growth with respect to donor age and culture site are described in Fig 4. No difference in growth rates between the two sites were observed in oysters younger than 24 months, *i.e.*, only 24 month 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,

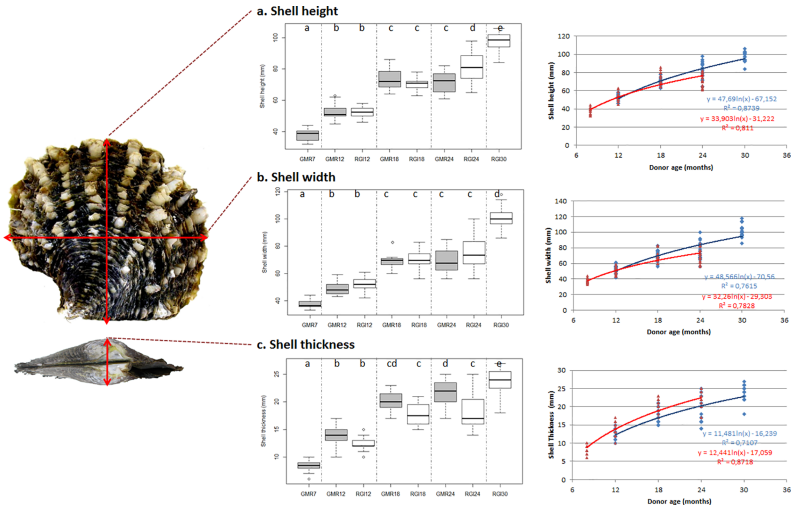

Fig 4. *Pinctada margaritifera* donor oyster shell biometric parameters. a. Shell height, b. width and c. thickness were measured at each graft time (GMR7 corresponds to 7 months old at Gambier location, GMR12 and RGI12 to 12 months old, GMR18 and RGI18 to 18 months old and GMR24 and RGI24 to 24 months 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. Datas available on S2 Appendix.

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respectively). The growth curves show the maximum slope (*i.e.*, maximum growth rate) for donors younger than 18 months old at both Rangiroa and Gambier.

Discussion

This study is the first to evaluate the impact of pearl oyster donor 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 donor 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 relative gene expression ratio of aragonite-related genes (*Pif-177*, *MSI60*, *Perline*), calcite-related genes (*Aspein*, *Shematrin5*, *Prismalin*,) as well as the gene implicated in both layers (*Nacrein*) for graft tissue. For pearl sac tissues only calcite-related genes (*Aspein*, *Shematrin5*, *Prismalin*, *Shematrin9*) were impacted by donor age.

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 [40]. Pouvreau et al. [41] 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 [42]. 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 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 [30]. 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 lead 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 [43]. In Pouvreau et al. [40], increments in nacre deposition equalled 7.2 μm. d^{-1} during the second year of the life cycle and decreased with the age of the pearl oyster, reaching a mean value of 3.1μm.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 nacre deposition. In French Polynesia, donor oysters are currently sourcedfrom wild spat collection and used in operations after 24 months of age. Our results suggest that better results would be obtained by using younger oysters. The results were consistent with the graft biomineralisation analysis where in GMR30, the oldest donor oysters showed the lowest level of expression for the aragonite candidate genes (PIF, MSI60 and PERL). PIF was previously found to be positively correlated with shell deposition rates in *P. margaritifera* [35] and thus with pearl size (nacre weight and thickness) [27]. Among the various phenotypes of pearl quality traits surveyed in this work, those concerning grade and surface defects deserve special consideration. Indeed results concerning lustre are difficult to interpret. Lustre is known to depend on environmental factors such as temperature during cultivation period [25–26]. Our results clearly demonstrate that poorer pearls grade (more D-R pearls) and increased pearl defects (> 5)were observed when originated from donor oysters older than 24 months and younger than 12 months. For pearls produced by donors younger than 12 months, we propose a technical problem verses a physiological one. We hypothesise that a 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 [28,30,44]. 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. [31] where 2-year-old graft cells improved pearl grade, predominantly through a higher proportion of zero 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 (the current age in

commercial production is > 24 months). This 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.

Supporting information

S1 Table. Set of forward and reverse primers used for the biomineralization gene expression analysis in *Pinctada margaritifera*.
(DOCX)

S1 Appendix. Relative stability of the GAPDH and SAGE genes combination confirmed using NormFinder (Stability value for best combination).
(DOCX)

S2 Appendix. All quantitative and qualitative figures datas.
(XLSX)

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