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A histological examination of grafting success in pearl oyster *Pinctada margaritifera* in French Polynesia

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Abstract – Pearl oyster grafting is a complex surgical operation that should lead to pearl formation after approximately eighteen months. Although this technique has been used for many years in French Polynesia, the grafting process is still not standardised. While studies have been carried out in order to improve graft performance and yield, these remain highly variable due to post-grafting mortality, nucleus rejection and unreliable pearl quality, all of which constrain pearl farm profitability. The present study uses histological analysis to monitor oysters that either rejected or retained their nuclei. Both groups of oysters are compared in terms of evolution of the graft, which could influence retention, and the development of a pearl sac in cases where grafting was successful. Data show that rejection phenomena are linked to a number of causes, notably an inflammatory reaction in the "receiving" oyster, the presence of numerous tissue lesions and the quality of the grafted tissue. These results suggest that study is needed on the different concomitant elements of the grafting process: the graft "donor" oysters, the nucleus and the "receiving" oyster and their interactions.

Key words: Grafting process / Pearl formation / Pearl oyster / Pteriidae / *Pinctada margaritifera*

Résumé – La greffe est un acte opératoire complexe qui doit permettre d'aboutir à la formation d'une perle en dix-huit mois environ. Bien que ces opérations soient effectuées depuis de nombreuses années en Polynésie française, la technique de greffe reste aujourd'hui un savoir-faire non standardisé. Différentes études ont été menées en vue d'améliorer les performances de greffe. Toutefois, l'hétérogénéité des rendements de greffe, mortalité post-opératoire, rejets des nucleus et celle de la qualité des perles restent autant de facteurs limitants pour la rentabilité des exploitations. Cette étude montre que les phénomènes de rejet sont associés à des causes multiples : réaction inflammatoire de l'huître receveuse, présence de lésions tissulaires et qualité des greffons. Ces résultats suggèrent la nécessité d'étudier de manière concomitante les différents compartiments intervenant dans la greffe : huître donneuse de greffons/nucleus/huître receveuse et d'analyser leurs interactions.

1 Introduction

Pearl culture makes a major contribution to the Polynesian economy. Polynesian pearl production is based on the culture of the black-lip pearl oyster, *Pinctada margaritifera*, var. *cummingi* (Pteriidae). As the largest export industry in French Polynesia (88 million euros in 2007; www.isfp.pf), the pearl culture industry employs about 5000 people and counters population migration from the atolls to Tahiti. The increased availability of maritime leases from the start of the 1990s and the spread of grafting skills (techniques previously mastered only by Japanese grafters) among local people led to the multiplication of farms. At the same time, other countries like Australia, China, Indonesia and the Cook Islands entered the black pearl market, with the advantage of lower production costs. This strengthening of international market led Polynesia to rapid increase in black pearl production. The result was an intensification of the number of low quality pearls. Since 2002, the Polynesian pearl culture agency (*Service de la Perliculture*) has established a set of regulations in order to stabilize the industry. These measures cover the management of maritime areas, management of the pearl culture industry itself and pearl quality control. The *Service de la Perliculture* established a classification system to define and

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improve the quality of pearls for exportation (*Journal Officiel de la Polynésie, délibération* n° 2005-42, 4th February 2005). Pearls must have a nacreous layer with a minimum thickness of 0.8 mm built up on the nucleus. The gems are then classified based on different criteria: size, shape, surface quality and lustre.

To produce cultured pearls, a spherical bead of shell material, the nucleus, and a piece of mantle tissue, the graft (about 4 mm^2) from a sacrificed "donor" oyster is implanted into the pearl pouch of a "receiving" pearl oyster; this process is known as "grafting" (Acosta-Salmon et al. 2005; Acosta-Salmon and Southgate 2005, 2006). Ideally, the graft tissue proliferates to form a layer of secretory epithelium (pearl sac), responsible for deposition of successive layers of organic matrix that control and determine the mineral deposits around the nucleus (Aoki 1966; Dix 1973; Mamangkey and Southgate 2009). The organic matrix deposited is the starting point of the future pearl. The deposition process results in the formation of a cultured pearl after approximately 18 months (Caseiro 1993, 1995). However, in many cases, good gem quality is not achieved. The receiving oyster may die soon after the grafting process or, even if it survives, the pearls obtained can be of low quality with an irregular shape or organic layer.

The importance of pearl culture for the Polynesian economy led the *Service de la Perliculture* to conduct several research projects. Collaboration agreements were signed with Ifremer (French Research Institute for Exploitation of the Sea) to define new research actions that would optimize the grafting operation and Tahitian pearl quality. Following these objectives, a large-scale research program was designed in which the understanding of the cellular mechanisms of pearl formation is a fundamental step needed before any kind of improvement strategy can be developed. The present paper provides the first description of nucleus rejection and the early post-grafting processes that lead either to pearl formation or its failure. The responses of "receiving" oysters to transplanted grafts and surgery wounds were studied. Subsequent changes in the implanted graft were then examined in cases of normal and abnormal pearl sac evolution.

2 Materials and methods

2.1 Biological material

The grafting experiment was conducted in Takapoto Island (an atoll of the Tuamotu Archipelago) on the experimental farm of the *Service de la Perliculture.* Three hundred 2-year-old *P. margaritifera* oysters were grafted. Each "receiving" oyster were individually maintained in panel (small pocket) nets on a long-line. These pockets have a mesh that allows the retention of the rejected nucleus. The checking for the rejected nucleus can be done daily by diving.

2.2 Methods

2.2.1 Post-grafting monitoring analysis

A total of seven "donor" oysters were used in the experimental grafting operation. For each "donor" oyster, the "grafting delay", time elapsed between oyster opening, graft preparation and surgery were recorded. Each of the "receiving" oysters was individually marked to facilitate sampling.

By diving, mortality and nucleus rejection were then monitored daily for a period of three months post-grafting. Every day, all the oysters that had rejected their nuclei and two randomly-selected oysters that had retained their nuclei were transferred from the long-line to the *Service de la Perliculture* laboratory for histological preparation.

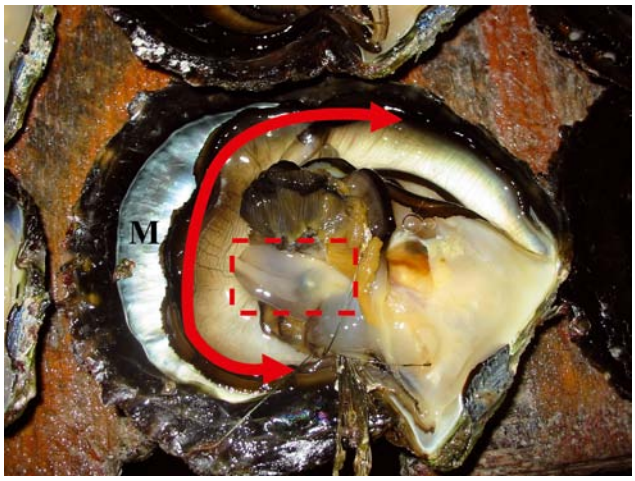


Fig. 1. *Pinctada margaritifera* with one shell valve removed to show the mantle edge (M). The line indicates the section of mantle tissue used for graft preparation in a "donor" oyster. The area within the dotted line shows the part of pearl pouch (gonad) used for histological study in a "receiving" oyster. The pearl can be seen inside the pouch, through a layer of tissue.

2.2.2 Histological analysis

Graft tissue pieces are prepared from the mantle edge of the two valves of a "donor" oyster (Fig. 1). The marginal zone of mantle with the inner, middle and outer folds are eliminated. The strip of mantle obtained is then smoothed over with specific tool to facilitate the cut of small portions which are used as the grafts. For this histological study, two grafts were sampled from each "donor" oyster: one taken before surgery on "receiving" oysters was started (valve 1) and one corresponding to the first graft used from the mantle of the second valve (valve 2). The pearl pouch (gonad) in the "receiving" oysters sampled was sectioned together with the nucleus for standard histological preparation (Fig. 1). A total of 120 oysters that had retained their nuclei were sampled at different times post-grafting (day +1, day +3, day +6, day +12, day +30 and day+45). Another 52 oysters that had rejected their nuclei and 32 recently dead oysters were also collected. Samples were placed in Davidson's fixative (Shaw and Battle 1957) for 24 h in paraffin. Sections (3 μm) were placed in an oven at 60 °C, deparaffinized in xylene for 10 min, and stained with a conventional haematoxylin and eosin stain.

Table 1. Summary information on the grafting process. (a) grafts of these donor oysters (1D and 2G) have not been used in totality and data are not used for statistical analysis of mortality and nucleus rejection rates. The time (hour) between valve 1 and 2 is the time required for opening the "donor" oyster, the preparation of grafts from valve 1 and valve 2 and use of first (valve 1 and valve 2) grafts. Mortality and nucleus rejection rates were compared for time lower and higher than 2 hours. The criterion of statistical significance is indicated by asterisks as follow: ** $p < 0.001$.

Day of transplant	Donor oyster	Grafting description							
		Valve 1				Valve 2			
		First graft use (hour)	Number o of grafts prepared	% mortality	% nucleus rejection	First graft use (hour)	Number Number prepared	% mortality	% nucleus rejection
1	A	+0.10	25	8	20	+2.10	24	25	55**
1	B	+2.10	23	9	48**	+4.00	22	10	58**
1	C	+3.50	25	9	67**	+5.45	22	17	44**
1	D	+0.10	19(a)	17	59	-	-	-	-
2	E	+0.10	31	7	32	+1.20	35	7	35
2	F	+0.10	29	31	28	+2.45	33	0	67**
2	G	+0.10	12(a)	0	67	-	-		

2.3 Statistical analysis

Analysis of variance (ANOVA) or Kruskall-Wallis tests (when data were not normal but variance was equal) were performed to determine the difference between the mortality and nucleus rejection rates according to "donor" oyster and time elapsed before graft use. Prior to the variance analysis, percentages were transformed using an arcsin(✓) function. Table 1 gives the original data.

3 Results

3.1 Mortality and nucleus rejection rates

Before examining the results of mortality and nucleus rejection rates with respect to the grafting conditions, we verified that the death of these oysters was not a direct consequence of nucleus rejection. Twenty non-grafted oysters and twenty oysters that had rejected their nuclei were randomly selected and kept on a long-line to monitor mortality over three months, and respectively zero (0%) and one of these oysters died (5%).

Table 1 gives the information recorded during the two days of grafting operations: the number of grafts taken from "donor" oysters, grafting delay (time elapsed between the opening of the "donor" oyster and the use of the first graft from valve 1 and valve 2). Mortality rates varied between 0 and 31% according to the "donor" oyster and nucleus rejection rates varied between 20 and 67%. No statistically significant differences were observed between "donor" oysters for either mortality or rejection rate ($p = 0.957$ and 0.713, respectively).

The time until use of the first graft varied between +0.10 and +3.50 hours for valve 1 graft tissue pieces and between +1.20 and +5.45 hours for valve 2 graft tissue pieces. These variations occurred because graft preparation was heterogeneous. Ideally, grafts are prepared just before the grafting operation and it takes about 1 or 2 minutes to insert the graft and nucleus into the "receiving" oyster. We hypothesised that all grafts should be inserted less than 2 hours after cutting.

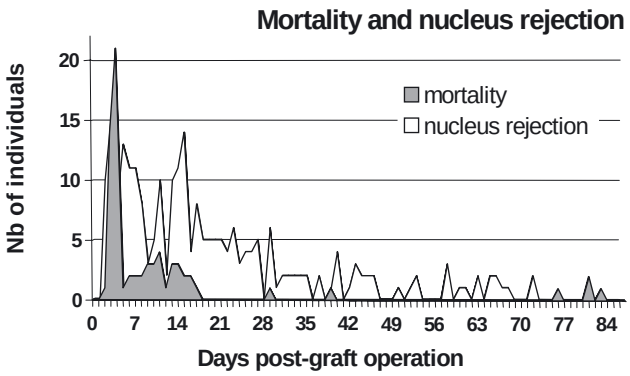


Fig. 2. Monitoring of mortality (dark grey) and nucleus rejection (pale grey) during the three month post-grafting period.

Mortality and nucleus rejection rates were compared between grafting delay times of more or less than 2 hours. A statistical difference was observed for nucleus rejection rates according to the time between the opening of the "donor" oyster and the use of the graft ($p = 0.001$) (Table 1), but no statistically significant effect of this time was observed for "receiving" oyster mortality rates ($p = 0.725$).

3.2 Mortality and nucleus rejection kinetic

Three months post-grafting, the mortality rate was 12.3%. Nucleus rejection rate was much higher and reached 45.4%. Daily monitoring showed that the majority of deaths occurred in the first two weeks post-grafting, with a peak of mortality in the first week (Fig. 2). Nucleus rejection started from the first day after surgery and was still being observed at 73 days post-grafting. However, the majority of nucleus rejections occurred during the first month and daily variations could be observed.

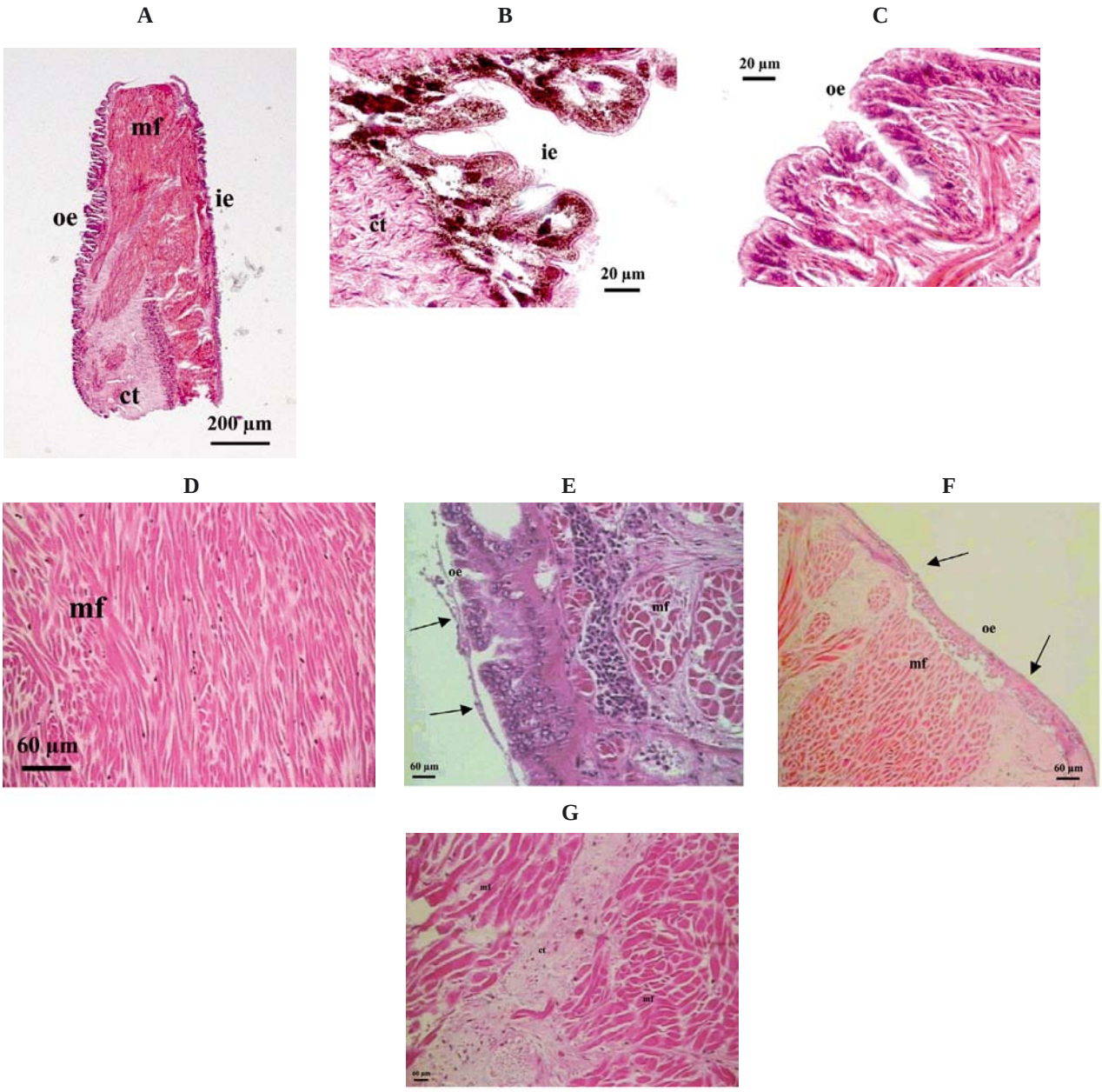


Fig. 3. Photomicrographs of a 3 μm radial paraffin section of the grafts used in the grafting operation. Haematoxylin-eosin stain. (A) A graft composed of inner (ie) and outer epithelia (oe) enclosed muscular fibres (mf) and connective tissue (ct). (B) Single layer of inner ciliated columnar epithelium (ie). This epithelium is typically pigmented with fine granules. (C) Single layer of columnar outer epithelium (oe). (D) Muscle fibres of a first used graft (valve 1) showing normal tissue structure (mf). (E) Portion of "crushed" outer epithelium (oe) showing the presence of cellular debris and a layer of mucus (arrows) partly due to the smoothing technique. (F) "Eroded" portion showing the disappearance of the outer epithelium layer (oe) (arrows) partly due to the smoothing techniques. (G) Distended muscle fibres (mf) of a graft from valve 2 of a "donor" oyster partly due to the time elapsed between oyster opening, graft preparation and surgery. Ct: connective tissue.

3.3 Histological analysis

3.3.1 Prepared grafts

Graft tissue pieces were taken from the marginal zone of mantle. Their cut were facilitate by a smoothing technique. It consisted to stretch the strip of mantle to cut small portions which are used as the grafts. Grafts were composed predominantly by muscular tissue surrounded by inner and outer (shell side) columnar epithelial layers (Fig. 3A to D). The inner epithelium had dense cilia, melanin pigmentation in the cytoplasm and deeply stained basal ovoid nuclei (Fig. 3B). Cilia and cytoplasmic pigmentation were absent from the outer columnar epithelium and the ovoid nuclei were located basally to centrally (Fig. 3C).

Most of the graft histological analyses showed tissue anomalies. Cut edges of grafts may present tears and epithelial alterations. In some grafts the outer epithelium presented

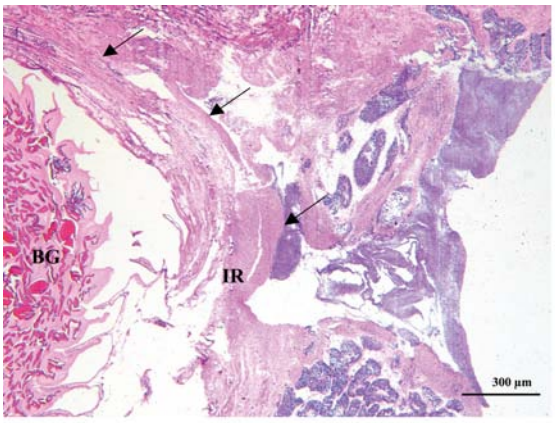


Fig. 4. Photomicrograph of a histological section of a "receiving" oyster pearl pouch observed one day post-grafting showing the area of incision (arrows). IR: inflammation reaction; NC: nucleus cavity; BG: byssal gland. Haematoxylin-eosin stain.

a "flattened" appearance with abundant mucus and/or cellular debris covering the epithelial cells (Fig. 3E). Others presented no external epithelial cells (Fig. 3F). Numerous grafts presented an "eroded" inner epithelia with a loss of pigmentation in the cytoplasmic cells. All grafts that were prepared a long time before the grafting process showed dilation and muscle fibre disorganization associated with connective tissue lyses (Fig. 3G).

3.3.2 Histological analysis of pearl sac formation

Using histological analysis to monitor oysters that either rejected or retained their nuclei allowed us to compare these groups in terms of evolution of the graft, which could influence retention, and the development of a pearl sac in cases where grafting was successful.

- *Analysis of "receiving" oysters that retained their nuclei*

The first day post-grafting, an intense haemocyte infiltration reaction was observed in "receiving" oyster tissues including digestive gland connective tissue, gonads and the pearl pouch (Fig. 4). These areas were also zones through which the grafting tools, graft and nucleus were passed. The presence of numerous haemocytes corresponded to the defensive reaction of the "receiving" oyster to injuries and characterizes the healing process. The speed and extent of this defensive reaction illustrated the large extent of the injuries sustained by the tissues during such surgery. According to the individual "receiving" oyster considered, the gonadal tissue surrounding the digestive gland were at different maturity stages. During the incision in receiving oysters with mature gonads, the gonads were became injured and many gametes were released from acini into the nucleus cavity (Fig. 4).

Pearl pouch histological examinations showed at least three different graft locations. On the side and at the bottom of the pearl pouch, the graft was away from the wounded incision zones. In these conditions, graft epithelium was observed extending itself and occupying surrounding connective tissue of the pearl pouch (Fig. 5A, B).

Histological examination indicated a graft located near the incision zone becomes trapped by haemocytes and/or gametes released during grafting surgery (Fig. 5C).

Six days post-grafting, incised tissues showed healing. Haemocyte infiltration was often resolved (Fig. 6). Graft epithelium progressed along the connective tissue. Graft muscle fibres and connective tissue were still observed. The pearl sac grew to surround nucleus taking on its round form (Fig. 6). Haemocytes and gametes were still observed and were trapped in the nucleus cavity.

After twelve days, the pearl sac was almost complete. It consisted of a single uniform layer of non-ciliated, cubical or flattened epithelial cells, supported by a fine fibrous stroma and closely attached to the receiving pearl pouch connective tissue. The pearl sac showed cellular similarities with the outer epithelium of the graft. Organic matrix was observed within the nucleus cavity showing the early stage of mineralization process (Fig. 7A). However, this organic matrix was sometimes distorted by cellular debris present within the nucleus cavity (Fig. 7B).

Figure 8 illustrates and summarizes the formation of a complete pearl sac, taking into account the major graft location observed in the pearl pouch in this study. Ideally, thirty days post-grafting, a complete pearl sac was already established and can be observed lining the pearl pouch. At this time, the location of the original graft tissue could no longer be detected and the pearl sac presented a homogeneous structure (Fig. 8A). In some cases, large amounts of haemocyte and/or gametes could be found in the cavity with the nucleus (Fig. 8B). The presence of such cellular components was associated with pearl sac structural deformation.

- *Analysis of receiving oysters that rejected their nuclei*

Histological observation of the pearl pouch of oysters that had rejected their nuclei revealed numerous anomalies. The main anomaly was degenerative lesions of the transplanted graft within the pearl pouch (Fig. 9A). Rejection of the nucleus was associated with a lack of fusion between the graft tissue and "receiving" oyster connective tissue. Another observed anomaly was distension of the receiving oyster connective tissue associated with the presence of numerous haemocytes all around the incision zone and the nucleus (Fig. 9B).

- *Analysis of grafted oysters that died*

The majority of dead oysters presented irreversible injuries of digestive tract. Such accidental damage, made during the grafting operation, was accompanied by a strong inflammatory reaction.

4 Discussion

Grafting operations have been performed in Polynesia since the 1960s. However, even though the basis of pearl

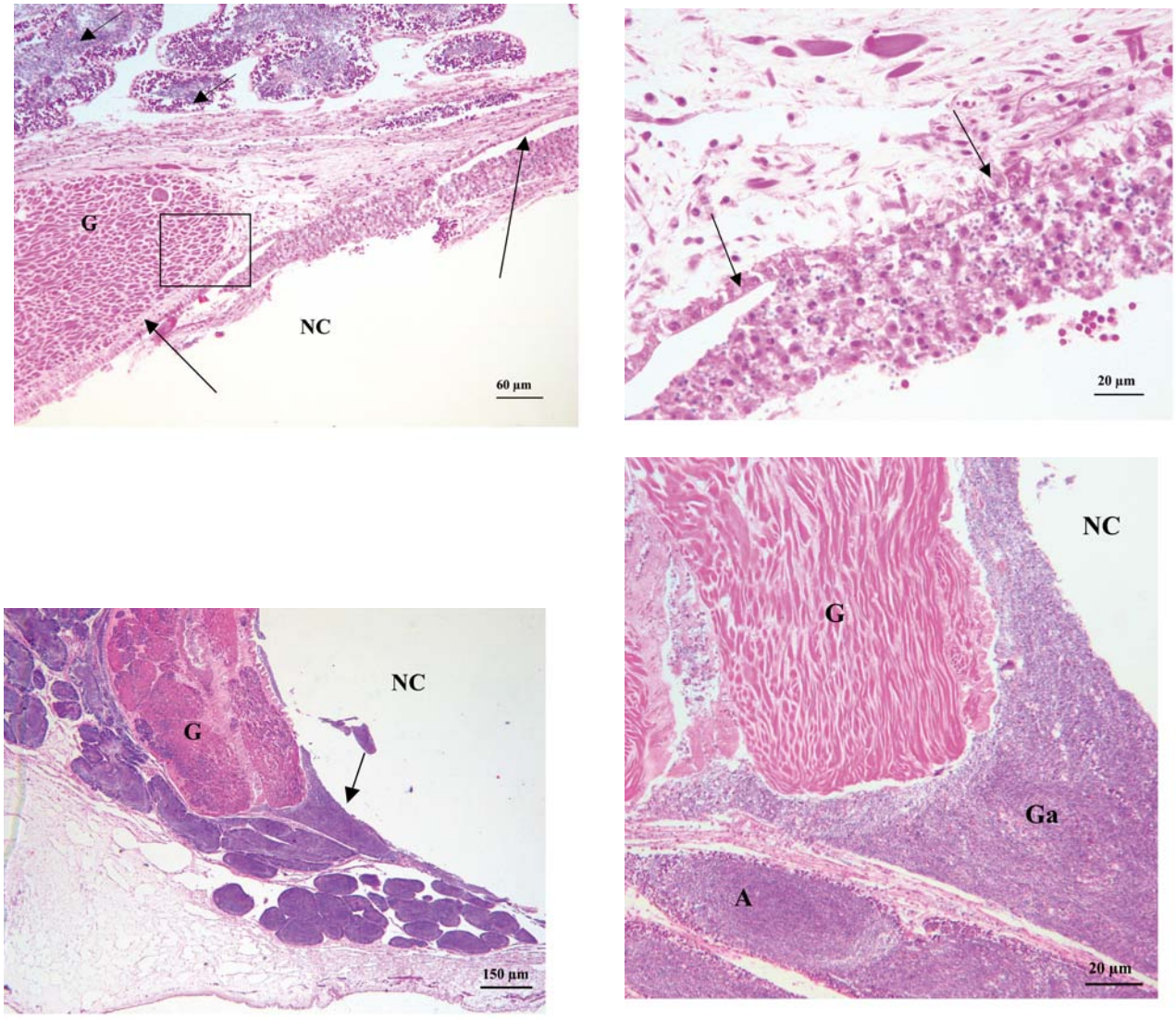


Fig. 5. Photomicrographs of graft histological section. Haematoxylin-eosin stain. Cell extensions from the epithelial edge of the graft (G) surrounding connective tissue of the pearl pouch (arrows). NC: nucleus cavity. Detail of the epithelial layer extension (arrows). Graft (G) trapped in gametes present inside the nucleus cavity (NC) (arrow). A: acini. Detail of the gametes (Ga) released from acini (A) into the nucleus cavity (NC). G muscle fibres of the graft.

production technology is known, transplant practices are not entirely controlled. Indeed, the pearl grafters initially came mainly from Japan, but more Chinese grafters have progressively moved to Polynesia, with highly specialized skills and closely guarded techniques. Without any real sharing of knowledge, different techniques have multiplied and led to highly variable levels of post-transplant mortality, nucleus rejection and probably pearl quality. Until now there were no data available in the literature on rates of post-grafting mortality and nucleus rejection. Nevertheless, after more than half of century of Polynesian grafting, it is surprising that very few studies have been made to analyse the success or failure of the processes leading to pearl formation. To reduce post-transplant mortalities and nucleus rejection in *P. fucata* and *P. margaritifera*, Norton et al. (1996, 2000) proposed the use of anaesthetics. Oyster rearing techniques also seemed to have an influence on mortality and growth during subsequent grafting operations in *P. margaritifera* (Le Duc 1997), and also on the quality of half-pearls in *Pteria sterna* (Ruiz-Rubio et al. 2006). In any case, the techniques from grafting operation to harvest were often different from one oyster farm to another and practices are far from standardized. The present study, which is essentially descriptive, examines the grafting operation in the pearl oyster *P. margaritifera* in greater detail, and the early post-grafting processes that lead either to pearl formation or its failure. It allowed to investigate possible hypotheses that would explain successful grafting processes and pearl formation.

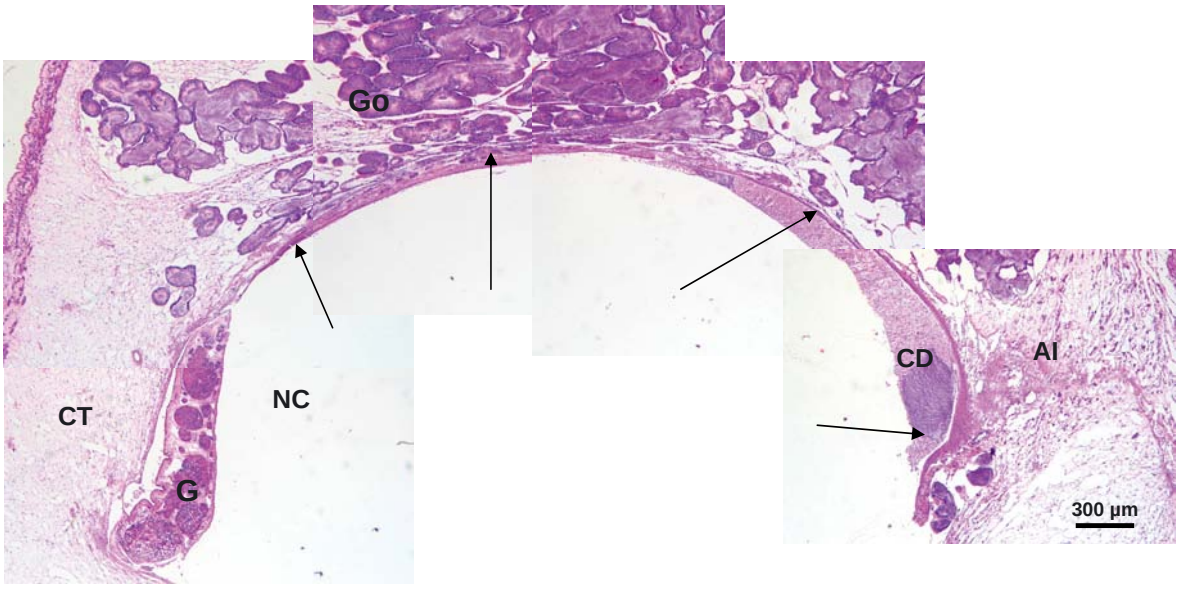


Fig. 6. Photomicrograph of the establishment of pearl sac (arrows). Haemocytes and gametes (names cellular debris CD) are present within the nucleus cavity (NC); CT: connective tissue, Go: gonads, AI: area of incision, G: graft.

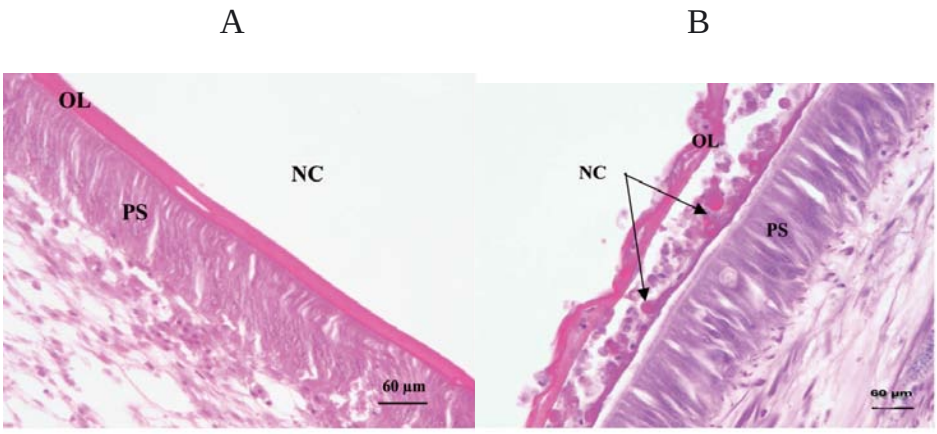


Fig. 7. Photomicrographs of the early mineralization process. (A) An organic layer (OL) secreted by the pearl sac (PS) can be observed in the cavity of the nucleus (NC). (B) However, organic layers (OL) may be distorted by debris (arrows). Haematoxylin-eosin stain.

4.1 Choice of donor oyster and preparation of the grafts

One grafter emphasized the close attention he paid to the selection and preparation of donor oysters. However, although the choice of "donor" oyster, techniques used (as smoothing technique) ant tissue quality of prepared grafts are decisive at the time of the transplant operation, they are neither managed nor technically defined. This study demonstrated that nucleus rejection rates are directly related to the delay before a graft is put to use. Moreover, tissue anomalies were observed in relation to the delay between the opening of the "donor" oyster and the use of the graft. In particular, muscle fibre relaxation and necrotic tissue zones were observed for grafts prepared a long time in advance. Furthermore, the technique of "smoothing" the strip of mantle, commonly practiced to facilitate mantle cutting, may cause tissue disorganization or sometimes a total erosion of the inner and/or outer layers of the graft epithelium. The fate of such grafts after the operation is unlikely to aid successful pearl development: the graft may be rejected, the pearl sac may not be established or be incompletely formed, and/or poor pearl quality may be induced.

4.2 Outcome of the graft and development of the pearl sac

This study was made to monitor oysters that either rejected or retained their nuclei and to compare these groups in terms of evolution of the graft, which could influence retention, and

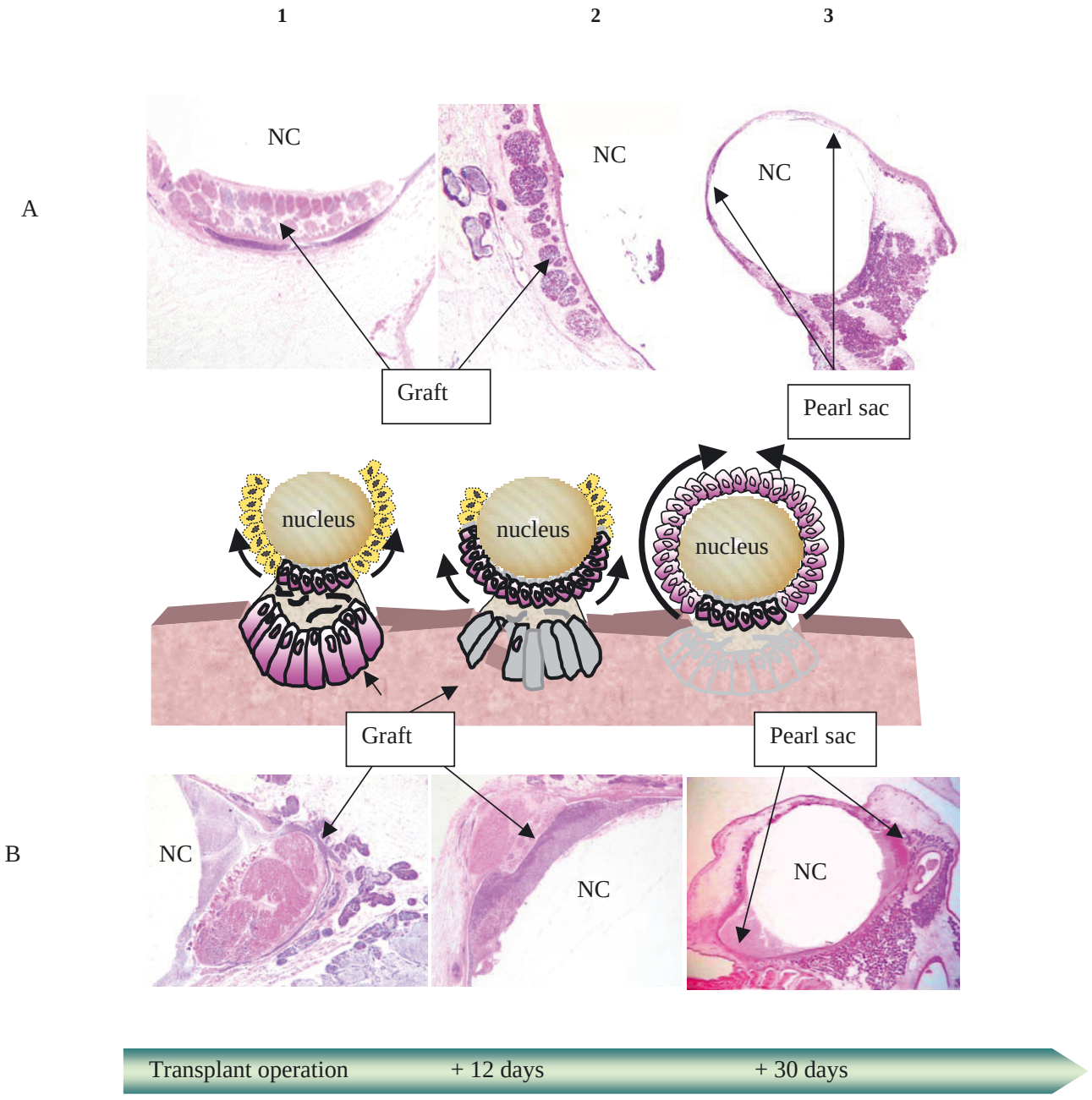
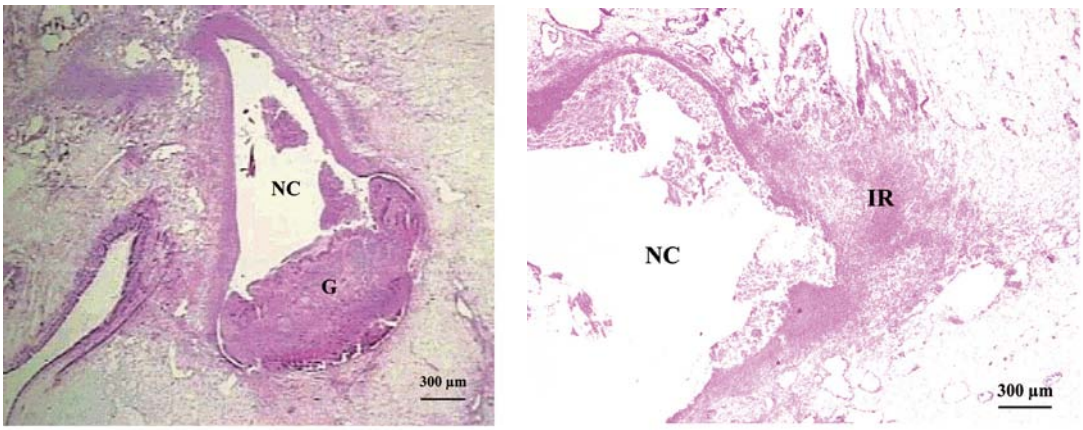


Fig. 8. Pattern of the different stages in pearl sac formation. (1) The nucleus is inserted into the pearl pouch with the graft (G). (2) After 15 days, the graft (G) (arrow) has either become fused with the connective tissue of the receiving oyster (CT) (A) or trapped in gametes (B). (3) After 30 days, a complete pearl sac can be observed with a homogeneous structure (A3 - arrow) or with structural deformation due to a large amount of cellular debris (arrow – B3). NC: nucleus cavity. The diagrams in the middle represent the ideal evolution of the graft and its development into pearl sac during the post-grafting period.

the development of a pearl sac in cases where grafting was successful. Histological analysis allowed us to suggest some explanations for nucleus rejection phenomena.

All histological examinations of oysters that had rejected their nuclei revealed lesions of the connective pearl pouch (gonad) tissue. Haemocytes were particularly numerous in the incised zones. These observations suggest that the main causes of nucleus rejection could be an inflammatory reaction following the transplant operation.

Furthermore, histological observations suggested that the graft location inside the pearl pouch was associated with the homogeneity of pearl sac development. The location was often chosen empirically by the grafter who introduced the graft either above or below the nucleus, assuming that graft can "move" in the pouch pearl after its insertion. Whatever the location, it was necessary to have maximum contact between the outer edge of the graft and the nucleus. Indeed, it was from this graft epithelium that the pearl sac develops (Aoki 1966;



A B

Fig. 9. Photomicrographs showing histological sections of the pearl pouch in oysters that have rejected the nucleus. (A) Graft degeneration in the nucleus cavity. (B) Massive inflammation of "receiving" oyster connective tissue without the graft. G: graft; IR: inflammation reaction; NC: nucleus cavity.

Dix 1973; Herbaut et al. 2000; Hui 2001; Arnaud-Haond 2007; Wada and Komaru 1996).

A graft located on the side and bottom of the pearl pouch seemed to facilitate the development of a complete pearl sac with a homogeneous structure, which would guarantee good pearl quality. However, a graft located near the incision site seemed to delay or hamper pearl sac formation. These results suggested that the more debris there was around the graft, the more time it will take for formation and growth of the pearl sac. Moreover, this location seemed to induce a structural deformation of the pearl sac that bore a certain resemblance to the outgrowths observed on some "pear-shaped" or "baroque" pearls (Caseiro 1993).

Our results support the empirical practice of grafters who take care about the rearing and preparation of "receiving" oysters, particularly the deliberate use of non mature oysters. As a whole, this study provides grafters with scientific arguments to support such empirical techniques and those of graft placement.

In conclusion, the observations made in this study show that the causes of failure in the grafting process (nucleus rejection and mortality) are multiple and that there are many factors that seem to act alone or in synergy. The quality of the preparation of the graft, the surgery and healing of the receiving oyster are all essential parameters for grafting success. Results of this study provide the basis for significant benefits to the cultured pearl industry, for example, to propose "standardized" practices to improve grafting technique and reduce the unpredictability of pearl formation process, which is still too widespread in Polynesia.

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