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Original research article

The Pearl Sac Formation in Male and Female *Pinctada maxima* Host Oysters Implanted With Allograft Saibo

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ABSTRACT

An experiment was conducted to study the effect of male and female host oysters on the pearl sac formation in *Pinctada maxima* oyster. One hundred sixty oysters were used in a completely randomized design with 2 × 4 factorial arrangement and 20 replications. The 1st factor was that sex of host oyster consisted of two levels that is males and females. The 2nd factor was week after nucleus implantation with four levels that is 1, 2, 3, and 4 weeks. The parameters observed were the percentage of successful oysters to form the pearl sac, the speed of pearl sac formation, the percentage of nucleus coverage by the pearl sac, histology of the pearl sac growth and development, and haemolymph glucose, calcium and phosphorus concentrations. Our results showed that the percentages of host oysters that succeeded in forming a pearl sac were 80% and 75% in female and male host oysters, respectively. There was no statistical difference in nucleus rejection and mortality in male and female host oysters but the results indicated that male host oysters showed a numerically higher nucleus rejection. The speed of pearl sac growth and the percentage of nucleus coverage by the pearl sac in female host oysters were better than those in male host oysters. Haemolymph calcium, phosphorus and glucose concentrations, oxygen consumption, and histological development of the pearl sac were not different between male and female host oysters. Pearl sac formation in the female host oysters was better than that in male host oysters.

1. Introduction

Naturally, the formation and development of pearl sac and pearl synthesis in oyster are started from the entry of core particle inside the body tissue of an oyster and the irritation caused by it (Victor *et al.* 2000). The modification adopted in culture pearl production is the use of a pallial mantle or saibo from the donor oyster and the implantation of the saibo into the organ in the body of the host oysters. Therefore, the success of pearl production in culture pearl industry is determined by the success of nucleus implantation and the formation of pearl sac around the implanted nucleus (Awaji & Suzuki 1995; Cochennec-Laureau *et al.* 2010; Kawakami 1954; Machii 1968; Masaoka *et al.* 2013) by the proliferation of graft tissue to form a layer of secretory epithelium that synthesizes and deposits successive layers of organic matrix (Aoki 1966; Masaoka *et al.* 2013). The synthesis and deposition of organic matrix around the implanted nucleus is the beginning of pearl synthesis and formation (Cochennec-Laureau *et al.* 2010).

The success of pearl production in culture pearl industry is affected by the number or the percentage of rejection of implanted nucleus by the host oysters and the mortality of the implanted host oysters during the pearl sac formation and pearl synthesis until the harvesting of the pearl. The quality of pearl produced is greatly determined by the genotype of the donor oyster, including shell colour (McGinty *et al.* 2010; McGinty *et al.* 2011; McGinty *et al.* 2012). However, the biological process of providing organic and inorganic materials and precursors for pearl synthesis is determined by the biological and physiological conditions of the host oysters and the histological conditions of tissue at the site of nucleus implantation. The physiological and biological conditions of male and female oysters are different due to the effects of sex hormones produced in certain sex (Chávez-Villalba *et al.* 2013) and the site of nucleus implantation in male or female gonads are different histologically (Eckelbarger & Davis 1996a, 1996b). In general, the sexes

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of the host oysters are not considered as a determinant factor in pearl industry. Implantation is conducted randomly without selecting sex of the host oysters. However, in practice, implantation is conducted in gonadally mature host oysters. Therefore, the physiological and biological conditions of gonadally mature male and female host oysters are greatly different. This difference would affect the implantation success and pearl sac formation. In addition, different cells and tissues in male and female gonads of host oysters could affect the interaction of the grafted tissue to form a pearl sac during the early phase of pearl sac formation.

The difference in hormone secretions and histological base of gonad between male and female host oysters would affect the biological and physiological conditions of the host oysters that could eventually affect the histological responses of the host oysters to the implanted saibo that could eventually affect pearl sac growth and development and pearl synthesis. The study on the effect of sex of host oysters on pearl sac formation and pearl synthesis is not available in the literature. It was reported that female oysters require a greater energy for the growth and development of gametes (Chávez-Villalba *et al.* 2011). This experiment was designed to study the effect of male and female host oysters on histological and physiological changes during pearl sac formation in *Pinctada maxima* oyster. This is the 1st study to report the histological and physiological changes in male and female host oysters during pearl sac formation.

2. Materials and Methods

2.1. Experimental materials and design

This study was conducted from February 2012 to June 2012 at the commercial pearl farm of C.V. Aru Duta Indah in the Garaga, Obi Island (01°25'S, 127°20'E), North Moluccas Province, Indonesia. Host and donor oysters used in this experiment were *Pinctada maxima* oysters that were cultured by the commercial pearl farm. A total of 160 oysters were assigned into a completely randomized design with a 2 × 4 factorial arrangement with 20 replications. The 1st factor was the sex of host oyster consisted of two levels that is female and male. The 2nd factor was week of measurement after implantation of nucleus that consisted of four levels that is 1, 2, 3 and 4 weeks.

Forty oysters were used for the measurement of the percentage of successful oysters forming a pearl sac during 4 weeks of experiment (2 groups × 20 replications, 20 male oysters and 20 female oysters). Seventy two oysters were used for the measurement of the nucleus rejection and oyster mortality. Twenty four oysters (12 female host oysters and 12 male host oysters) that succeeded in implantation were used for the measurement of haemolymph glucose, calcium and phosphorus concentrations, the speed of pearl sac growth and development and percentage of nucleus coverage by pearls sac (2 × 4 with 3 replications). Twenty four oysters succeeded in implantation were used for the measurement of histological growth and development of the pearl sac.

The parameters measured were oxygen consumption, haemolymph glucose, calcium and phosphorus concentrations, the speed of pearl sac formation and the percentage of nucleus coverage by the pearl sac and histological growth and development of the pearl sac. The total number of oysters used in the experiment was 160 oysters. *Pinctada maxima* oysters used in the experiment as host oysters were selected by criteria of normal morphology (without shell malformation), the same level of gonad maturity (gonadal development phase), with similar dorso-ventral margin of 12 cm and anterior–posterior margin of 11 cm, body weight ranged 180–210 g, and 18 months of age for male and female host oysters.

The saibo used in the experiment was taken from normal male and female *Pinctada maxima* oysters and selection of *Pinctada maxima* oyster as a donor oyster was based on the same criteria used in selecting the host oyster. The sex of the host and the donor oysters were same (allograft). The saibo was prepared from the pallial mantle of the donor oysters. The pallial mantle obtained was then cut into a piece of 3 × 3 × 1 mm and then soaked in a physiological solution and ready for use for implantation in the host oysters. The diameter of nucleus used in the experiment was 6.4 mm. Before implantation, the saibo was attached to the nucleus with the inner mantle that had a direct contact with the nucleus.

During implantation, the host oysters were placed in a standing position so that the oysters experienced oxygen deficiency that stimulated the opening of the oyster shells. The shell opener was used to keep the shell open during implantation. When the shells were open, a spatula was used to separate the gill covering the gonads. After making a small incision (6.6 mm) in the sites of implantation on ventral gonad, the nucleus that was attached with a saibo was inserted. After implantation, the experimental oysters were reared in marine pearl oysters culture system (according to the standard operation in the pearl company) by the dorsal position at the top at a depth of 3 m under water. Observations and measurements were conducted every week for a month. All this process was done carefully so that the oysters did not experience stress. For histological observation of pearl sac development, the gonad of the oyster that succeeded to form pearl sac was cut and isolated and was saved in buffer normal formalin for future histological preparation in the laboratory.

2.2. Parameters measured

The parameters measured were the percentage of successful oyster to form the pearl sac, the speed of pearl sac growth and development, the percentage of nucleus coverage by the pearl sac, histological development of the pearl sac, and haemolymph glucose, calcium and phosphorus concentrations.

The percentage of successful oysters to form pearl sac (PSPS) was calculated by dividing the number of oysters that form pearl sac (PS) by the total number of treated oysters (TO).

$$\mathrm{PSPS} = \frac{\mathrm{PS}}{\mathrm{TO}} \times 100\%$$

The number of oysters that form PS was calculated by subtracting the total number of treated oysters by the number of dead oysters and oysters experiencing nucleus rejection.

The speed of pearl sac growth (SPSG) (mm/day) was calculated by measuring the length of a pearl sac formed (PSF) during 1 week and was divided by 7 days according to the equation:

$$\mathrm{SPSG} = \frac{\mathrm{PSF}}{7\ \mathrm{days}}$$

PSF was determined by the circumference of a circle using the formula $2 \times \pi \times r$ and then was reduced by the length of the nucleus that was not covered by the pearl sac.

Measurement of the percentage of nucleus coverage by pearls sac (PNCP) was done by measuring the area of pearl sac formed (PSF) divided by nucleus area (NA) multiplied by a 100% according to the equation:

$$\mathrm{PNCP} = \frac{\mathrm{PSF}}{\mathrm{NA}} \times\ 100\%$$

NA was calculated by measuring the radius of the nucleus covered by the pearl sac with formula $4 \times \pi \times r^2$ and PSF values were obtained by using the graphical methods square (mm graph paper).

Oxygen consumption was measured by method used by Bayne (1971). The haemolymph was taken from the heart ventricle and

auricle of pearl oysters with a syringe and put into an Eppendorf tube. The haemolymph samples were added three drops of 3.8% sodium citrate to prevent clotting. The samples were stored frozen until analysis of haemolymph glucose, calcium and phosphorus concentrations. Haemolymph glucose concentration was analysed by Glucose liquicolor-GOD-PAP method (CE Human, Germany).

Haemolymph calcium concentration was determined by method explained by Reitz *et al.* (1960) and haemolymph phosphorus concentration was determined by method reported by Taussky & Shorr (1953). Salinity and pH of the water in the culture system were measured weekly during the experiment. Temperature of the marine water media was measured daily.

For histological analysis, the gonad organs used for nucleus implantation were isolated for histological preparation. The histological preparation of the developing pearl sac used the haematoxylin-eosin staining technique.

2.3. Data analysis

The data collected were analysed by using analysis of variance by testing the effect of main factor that is different sex (female and male) and weeks after implantation (1, 2, 3 and 4 weeks) and the interactions between different sex and weeks after implantation.

3. Results

3.1. The percentage of oysters succeeded in forming pearl sac, nucleus rejection and mortality in female and male *Pinctada Maxima* host oysters

In general, there was no difference between male and female host oysters in the percentages of oysters that succeeded in forming pearl sac. The percentages of oysters that succeeded in forming pearls sac in the female and male *Pinctada maxima* host oysters were 80% and 75%, respectively. The percentages of oysters that died in female and male host oysters were same that is, 8.3%. The number of male host oysters died was only found during the 1st week after nucleus implantation. However, in female host oysters, the mortality was only found in week 2 after nucleus implantation. The percentages of nucleus rejection in female and male *Pinctada maxima* host oysters were 11.7% and 16.7%, respectively. In general, the higher number of nucleus rejection and oysters died were found 2 weeks after nucleus implantation and then decreased and reached the lowest level 4 weeks after nucleus implantation.

3.2. The speed of pearl sac growth and the percentage of nucleus coverage by pearl sac in female and male *Pinctada Maxima* host oysters

The speed of pearl sac growth and the percentages of nucleus coverage by the pearl sac in female and male *Pinctada maxima* host oysters during 4 weeks after nucleus implantation are presented in Table 1. The results of this study clearly showed that the speed of pearl sac growth in female host oyster was faster (around 7%–13%) as compared to that in male host oysters during 4 weeks of observation after nucleus implantation. The percentage of nucleus coverage by the pearl sac in female host oyster was also consistently higher (around 2%–12%) as compared to that in male host oysters during 4 weeks of observation after nucleus implantation.

Table 1. The speed of pearl sac growth and the percentage of nucleus coverage by the pearl sac female and male *Pinctada maxima* host oysters 4 weeks after nucleus implantation

Sex	Weeks after implantation			
	1	2	3	4
The speed of pearl sac growth (mm/d)				
Male	1.46 ± 0.08^{b}	1.93 ± 0.09^{a}	2.18 ± 0.08^{b}	2.51 ± 0.08^{b}
Female	1.65 ± 0.08^{a}	2.08 ± 0.08^{a}	2.51 ± 0.08^{a}	2.80 ± 0.08^{a}
The percentage of nucleus coverage (%)				
Male	48.06 ± 1.55^{a}	65.63 ± 1.79^{b}	81.40 ± 2.05^{b}	94.57 ± 1.55^{a}
Female	49.10 ± 2.37^{a}	70.54 ± 2.05^{a}	93.02 ± 1.55^{a}	97.16 ± 0.90^{a}

[a,b]Numbers followed by different letters in the same column show a significant difference ($p < 0.05$)

3.3. Histology of haemocyte infiltration during the development of the pearl sac 4 weeks after nucleus implantation in female and male *Pinctada Maxima* host oysters

In general, the patterns of haemocyte infiltration in female (Figure 1) and male (Figure 2) *Pinctada maxima* host oysters in forming pearl sac were similar. The pattern of histological changes during 4 weeks of pearl sac formation was similar in both male and female host oysters. One week after nucleus implantation (Figure 1A and Figure 2A), the infiltrations of haemocyte and inflammatory cells were high that were associated with the injury and incision during implantation process. Two weeks after nucleus implantation (Figure 1B and Figure 2B), haemocytes and inflammatory cell infiltrations decreased and 3 weeks after nucleus implantation (Figure 1C and Figure 2C), the haemocytes and inflammatory cell infiltrations were very low and the injury began to recover. Four weeks after nucleus implantation (Figure 1D and Figure 2D), there was no haemocyte and inflammatory cell found and the host oysters were recovered from implantation-related injury.

3.4. Histology of the pearl sac growth and development 4 weeks after nucleus implantation in female and male *Pinctada Maxima* host oysters

The results of this study showed that pearl sac growth in female and male *Pinctada maxima* host oysters were relatively similar. There was no significant difference in histological changes during 4-week pearl sac growth and development 4 weeks after nucleus implantation. In detail, histology of pearl sac growth and development during 4 weeks of observation in female and male *Pinctada maxima* host oysters are presented in Figures 3 and 4, respectively. One week after nucleus implantation (Figures 3A and 4A), the inner mantle was degraded and only the outer mantle was visible. Epithelial mucosal layers had one to two layers of cuboidal epithelium cells that experienced necrosis (pyknosis of nuclei) and degeneration. The cuboidal epithelial cells underwent necrosis during formation of pearls sac. Between mucosal and sub-mucosal layers there was a basement membrane that functioned as a base for the attachment of the epithelial cells during pearl sac formation. Sub-mucosal layer showed the vacuoles containing liquid and nutrients required by the epithelial cells during degeneration and the vacuoles were formed from degeneration of epithelial cells. In this period, sub-mucosal layer started to dilate. The tunica muscularis was found as a base for the attachment of sub-mucosal layers. The presence of haemocytes and dilation of sub-mucosal layer were found. Two weeks after nucleus implantation (Figures 3B and 4B), epithelial mucosal layers had one to two layers of cuboidal epithelial cells but largely had monolayer of epithelial cell. The presence of haemocytes was reduced and sub-mucosal layers showed dilation. Three weeks after nucleus implantation (Figures 3C and 4C), monolayer of epithelial cells was surrounding the nucleus but it was not perfect since some vacuoles were found and sub-mucosal layer was dilated. Four weeks after nucleus implantation (Figures 3D and 4D), monolayer of epithelial cells was surrounding the nucleus that formed a complete pearl sac and some vacuoles were found that indicated the presence of degeneration.

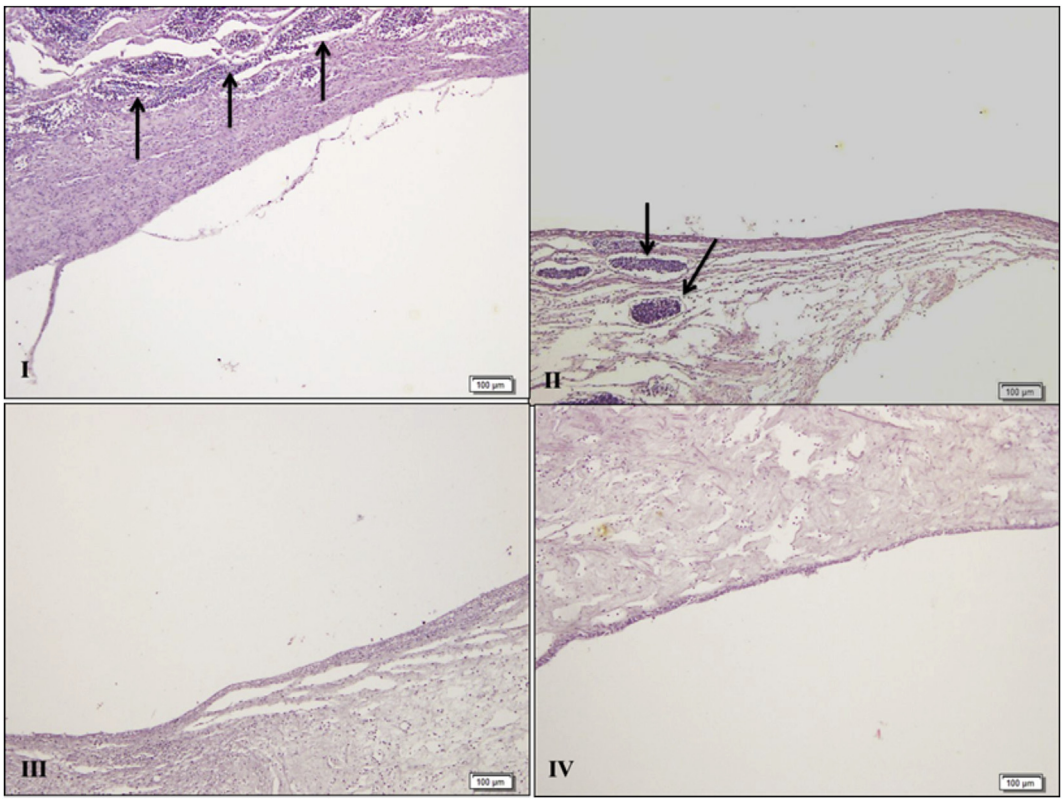

Figure 1. Histology of haemocyte infiltration during pearl sac development in the *Pinctada maxima* female host oysters. Arrows indicate haemocytes.

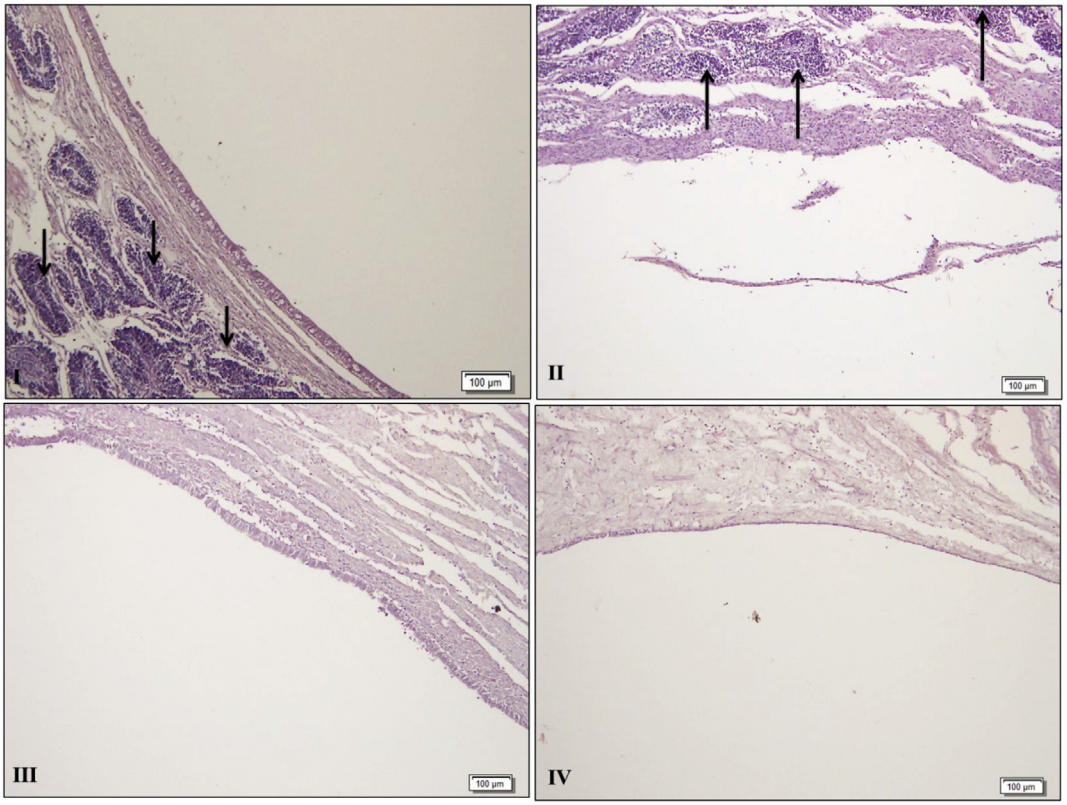

Figure 2. Histology of haemocyte infiltration during pearl sac development in the *Pinctada maxima* male host oysters. Arrows indicate haemocytes.

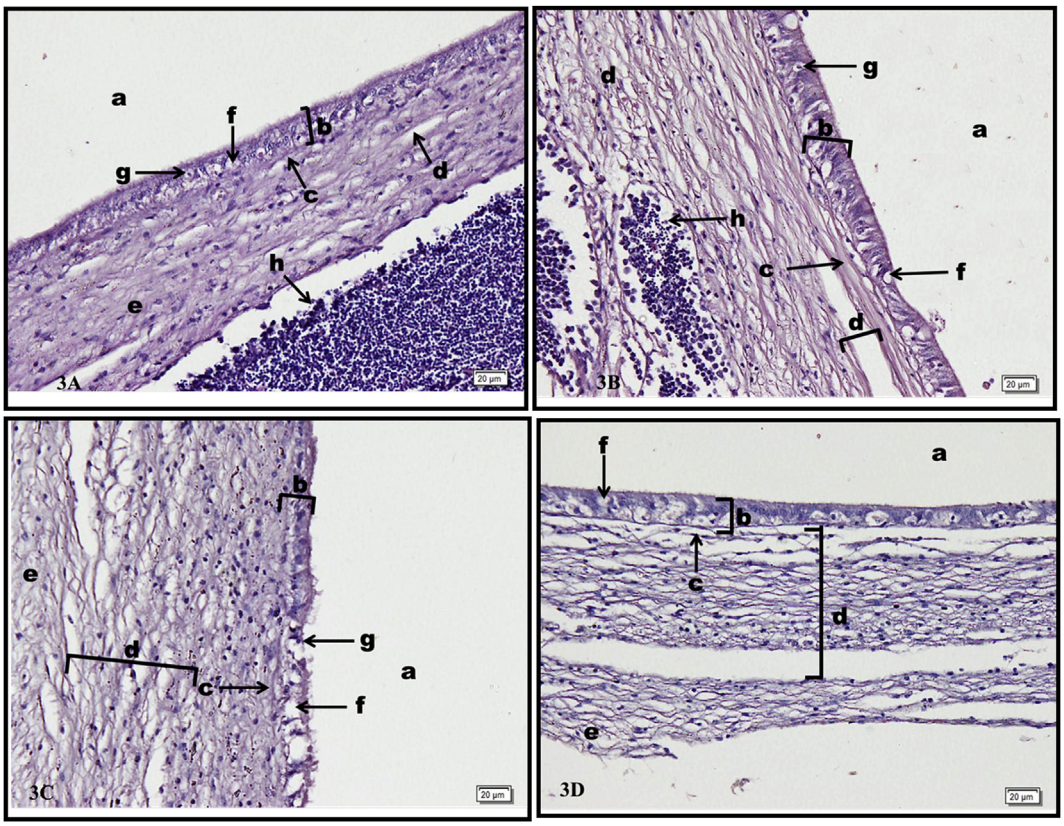

Figure 3. Histology of pearl sac development in *Pinctada maxima* female host oysters 4 weeks after nucleus implantation. (a) Nucleus, (b) epithelial mucosal layers having one to two layers of cuboidal epithelial cells that would undergo necrosis, (c) basement membrane, (d) sub-mucosal layers, (e) tunica muscularis, (f) vacuole, (g) pyknosis and (h) haemocyte.

3.5. Oxygen consumption, haemolymph glucose concentrations, haemolymph calcium and phosphorus concentrations in female and male *Pinctada Maxima* host oysters

Oxygen consumption, haemolymph glucose concentrations, haemolymph calcium and phosphorus concentrations in female and male *Pinctada maxima* host oysters during 4 weeks of observation are presented in Table 2. Metabolic rate as indicated by the averages of oxygen consumption in female and male *Pinctada maxima* host oysters was not different significantly even though male host oysters had higher oxygen consumption as compared to female host oysters. With the advance of pearl sac growth after nucleus implantation, the rate of oxygen consumptions was relatively stable in male and female host oysters. Marine water temperature fluctuated around 26.2–30.4 °C and salinity was 32 ppt. Marine water temperature during the measurement of oxygen consumption rate ranged 27.5–28 °C and salinity was 32 ppt.

Haemolymph glucose concentrations in male host oysters were higher and consistently higher during 4-week measurement after nucleus implantation as compared to those in female host oysters. Haemolymph glucose concentration was the highest 1 week after nucleus implantation and decreased and reached the lowest concentration 4 weeks after nucleus implantation.

The patterns of haemolymph calcium and phosphorus concentrations in female and male host oysters were similar. There was no significant difference in haemolymph calcium concentrations between male and female host oysters. During 4 weeks of observation after nucleus implantation, haemolymph calcium concentrations in male host oysters were consistently higher as compared to those in female host oysters and the pattern increased with the advance of pearl sac growth and development.

Haemolymph phosphorus concentrations were similar in male and female host oysters 1–3 weeks after nucleus implantation. There was a tendency that haemolymph phosphorus concentration increased with the advance of pearl sac growth and development after nucleus implantation. However, 4 weeks after nucleus implantation, female host oysters had higher haemolymph phosphorus concentration as compared to male host oysters.

4. Discussion

The observation in this experiment clearly showed that female host oysters had faster speed of pearl sac growth and higher percentage of nucleus coverage by the pearl sac as compared to male host oysters without any difference in haemocyte infiltration, histological development of pearl sac and oxygen consumption, haemolymph glucose, calcium and phosphorus concentrations. The success of implantation was higher and the number of nucleus rejection was lower and oyster mortality was similar during 4 weeks after nucleus implantation in male and female host oysters. Pearl sac development in female host oysters was better when compared to male host oysters. In female host oysters, the percentage of dead oysters and nucleus rejection was 20%, whereas in the male host oyster it was 25%. To reduce the number of oysters that reject nucleus it is advised to use anaesthesia at the time of implantation (Mamangkey *et al.* 2009; Norton *et al.* 1996; Norton *et al.* 2000). Norton *et al.* (2000) also reported that the pearl oyster *Pinctada margaritifera's* death was 24% and 16% rejection

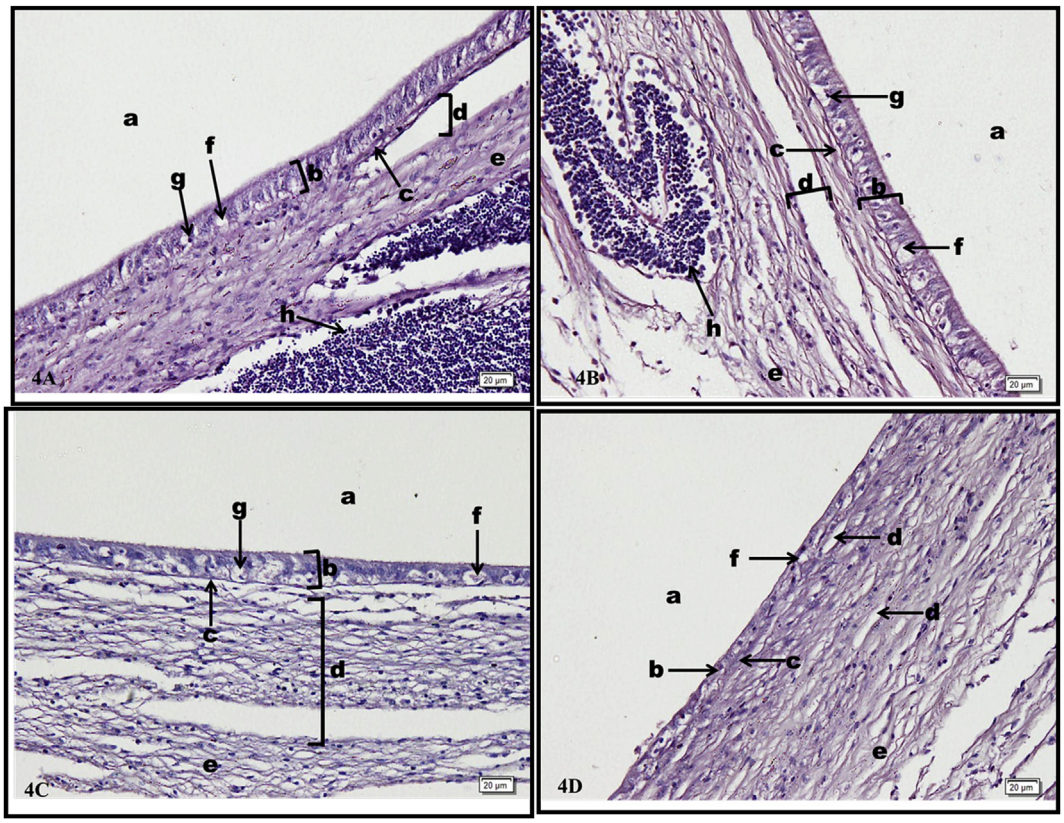

Figure 4. Histology of pearl sac development in *Pinctada maxima* male host oysters 4 weeks after implantation. (a) nucleus, (b) epithelial mucosal layers having one to two layers of cuboidal epithelial cells that would undergo necrosis, (c) basement membrane, (d) sub-mucosal layers, (e) tunica muscularis, (f) vacuole, (g) pyknosis and (h) haemocyte.

Table 2. The average of oxygen consumption, haemolymph glucose concentrations, haemolymph calcium and phosphorus concentrations in female and male *Pinctada maxima* host oysters 4 weeks after nucleus implantation

Sex	Weeks after implantation				
	1	2	3	4	
Oxygen consumption ($\mu gO_2^{-1}\ g^{-1}\ h^{-1}$)					
Male	3.6 ± 0.3^a	3.6 ± 0.1^a	3.6 ± 0.1^a		3.7 ± 0.1^a
Female	3.5 ± 0.4^a	3.4 ± 0.3^a	3.6 ± 0.1^a		3.5 ± 0.2^a
Haemolymph glucose concentrations (mg/dL)					
Male	2.72 ± 0.24^a	2.46 ± 0.33^a	1.41 ± 0.16^a	0.75 ± 0.19^a	
Female	1.92 ± 0.80^a	1.64 ± 0.46^a	1.25 ± 0.41^a	0.19 ± 0.003^b	
Haemolymph calcium concentrations (ppm)					
Male	278.93 ± 3.45^a	276.36 ± 1.40^a	281.45 ± 0.64^b	283.74 ± 0.89^a	
Female	283.60 ± 1.38^a	274.99 ± 0.70^a	289.41 ± 0.68^a	288.40 ± 0.53^a	
Haemolymph phosphorus concentrations (ppm)					
Male	6.66 ± 1.00^a	7.37 ± 0.80^a	8.37 ± 1.06^b	7.91 ± 1.23^a	
Female	7.07 ± 0.41^a	7.33 ± 0.73^a	10.89 ± 0.57^a	8.04 ± 1.93^a	

[a,b]Numbers followed by different letters in the same column show a significant different ($p < 0.05$)

nucleus. To reduce the number of deaths and nucleus rejection after implantation it is suggested to use anaesthesia during surgery.

The difference in the success of implantation in female host oysters could be related to the histological condition of male gonad as compared to female gonad. Even though there was no study on the difference between male and female host oysters gonad, the difference is clear and would affect the interaction of implanted saibo with the host tissues in the female and male gonads. The difference in the pearl sac growth and development in male and female host oysters could be explained by the effect of sex-related hormone and histological conditions of the implantation site in male and female host oysters. These hormonal and histological differences could have strong effects on the biological and physiological response of the host oysters to the grafted tissue. Because during implantation, the stage of gonadal maturity is in the stage of development, the activity of sex-related hormones in different sexes of host oysters is different (Arjarasirikoon *et al.* 2004). Female host oysters were under the dominance of oestrogen and male host oysters were under the dominance of testosterone (Andrew *et al.* 2008; Eckelbarger & Davis 1996a, 1996b; Gauthier-Clerc *et al.* 2006). The different hormonal conditions in male and female host oysters could affect the fusion between the graft tissues and the connective tissue of the host oysters that eventually affect the success of nucleus implantation and pearl sac formation.

The histology of male and female gonads were different that could affect the success of implantation and the growth of pearl sac.

The difference in histological condition of male and female gonad could affect the fusion between the graft tissues and the connective tissue of the host oysters. The data indicated that the gonad of male host oysters had lower success in receiving the implanted nucleus. The lack of fusion between the graft tissue and the receiving oyster connective tissues is the main reason for rejection of nucleus. The maximum contact between the outer edge of the graft tissues and the nucleus is required to increase the success of implantation. This lack of fusion could be caused by the distension of the receiving oyster connective tissue associated with the presence of haemocytes all around the incision zone and the nucleus and degenerative lesions of the transplanted graft within the pearl sac (Cochennec-Laureau *et al.* 2010).

Histological observation showed that there was a high infiltration of haemocytes around gonadal connective tissue. Haemocyte function in wound repair, digestion and transport of nutrients, excretion and immunity (Cheng *et al.* 2004). Implantation process caused the oyster stress as indicated by the increased haemolymph glucose concentrations and infiltrations of haemocytes. Stress increases the total haemocytes on the *Crassostrea gigas* oyster. Stress affects several hormones' activities such as corticotrophin releasing hormone, adrenocorticotrophic hormone (ACTH), cytokines, noradrenaline, adrenaline, dopamine, and cortisol (Lacoste *et al.* 2002). Stress activates the endocrine system such as corticotrophin releasing hormone, which stimulates the release of ACTH. The presence of ACTH stimulates the release of biogenic amino acids, which eventually lead to secondary effects on oysters (Hooper *et al.* 2007).

The similar rate of oyster that died in male and female host oysters indicated that the hormonal condition and histology of male and female gonads did not affect the process causing the host oysters' death during nucleus implantation. The common cause of death during nucleus implantation in pearl oysters is infection of the wounds inflicted at the time of the implantation operation. However, diseases, biofouling, shell boring and pollution may also be responsible for oyster mortality. Generally, the average oyster mortality rate is below 10% (Chellam *et al.* 1991) and the mortality rate observed in the oysters implanted in this experiment was similar either in male or female host oysters. Observation in *Pinctada margaritifera* reported that the majority of dead oysters showed irreversible injuries of digestive tract and such accidental damage, made during the grafting operation, which was accompanied by a strong inflammatory reaction (Cochennec-Laureau *et al.* 2010).

The higher rate of pearl sac growth and development and the percentage of nucleus coverage by the pearl sac in female host oysters could be related to the hormonal dominance of oestrogen or testosterone in female or male host oysters. Regardless of the lower rate of implantation success and pearl sac formation due to the high rate of nucleus rejection and oyster mortality in male host oysters, when implantation was successful and pearl sac was formed, the histological observation indicated a similar pattern of pearl sac development. In the oysters' success in forming pearls sac during 4-week observation, the intensity of haemocytes in the implanted tissues was similar in both male and female host oysters.

However, the speed of pearl sac growth and the percentage of nucleus coverage by the pearl sac in female host oysters were higher as compared to those in male host oysters. The different rate of pearl sac growth and development in male and female host oysters was probably associated with the contribution and interactions of the graft cells with the cells at the site of implantation and the availability of substrates as precursors of cell proliferation of pearl sac cells in the site of nucleus implantation. The cells in the male gonad probably had lower capacity to support the supply of nutrients for the growth and development of pearls sac as good as female gonad. In addition, female hormonal condition could support the supply of nutrients for the growth and development of pearls sac. Oestrogen dominance in female host oysters could have higher mitotic effect on pearl sac cell itself as compared to testosterone dominance in male host oysters. In female organisms, during gonadal development, oestrogen secretion increases with the increased gonad maturity. In contrast, in male organism, during sexual maturity, testosterone is not automatically higher during the development phase of gonadal organ (Gauthier-Clerc *et al.* 2006).

In addition, the difference in the speed of pearl sac formation observed in this study was not related to external environment of the host oysters such as salinity and temperature that would affect the physiological changes in the body of the host oysters. The experiment was conducted in the same water environmental condition. Water temperature was reported to affect the speed of pearl sac formation (Aoki 1956; Aoki 1966; Machii & Nakahara 1957) through the effect of water temperature on the mitotic activity of pearl sac epithelial cells (Awaji & Machii 2011).

The observation in this experiment showed that male host oysters had higher metabolic rates as indicated by the higher oxygen consumption, even though statistically it was not different. Haemolymph glucose concentrations that could be related to stress condition showed that male host oysters had higher stress condition as compared to female host oysters. Male-related hormone such as testosterone is related to the increased metabolism and physical activities. However, previous reports showed that female oysters had higher metabolic rate in relation to the growth and development of gametes in the developing gonad (Chávez-Villalba *et al.* 2011; Chávez-Villalba *et al.* 2013). These data indicated that the female gonads facilitated the nutrition availability for the development of the pearl sac and to support mineralization process during the synthesis and formation of the pearls.

Haemolymph glucose concentrations of male and female host oysters were the highest during the 1st week after nucleus implantation and decreased to the lowest level 4 weeks after nucleus implantation and male host oysters had higher haemolymph glucose concentrations. These data indicated that male host oysters had higher stress response to implantation as compared to female host oysters. This stress response could be contributed to the lower success of implantation and higher nucleus rejection in male host oysters. Stress stimulates gluconeogenesis and the mobilization of glucose from glycogen deposit that resulted in the increased haemolymph glucose concentration (Veldhuijzen & Cuperus 1975; Veldhuijzen & Van BeeK 1975). Hamano *et al.* (2005) showed that insulin-like substrate played an important role in maintaining glucose concentration in oyster.

The glucose concentrations in the haemolymph during 4 weeks after nucleus implantation showed a similar pattern with the wound healing and inflammatory response to graft implantation. The degree of implantation was the highest during the 1st 2 weeks after implantation and reached the lowest level 4 weeks after implantation. The same pattern of glucose concentrations in the haemolymph was observed. The increased glucose concentration in the haemolymph might have an association with the high stress during early implantation due to inflammatory response of the host oysters (Lacoste *et al.* 2002). During stress, cortisol was reported to increase (Hooper *et al.* 2007), that was associated with the increased glucose concentrations. Increased stress during early implantation increased haemocyte infiltration and haemolymph glucose concentration. When the implantation injury was cured, haemocytes was low and haemolymph glucose concentration reached the lowest levels. The decreased haemolymph glucose concentration with the advancement of pearl sac growth after implantation could indicate the possibility of increased glucose uptake without increased in glucose mobilization or uptake to the

haemolymph. Glucose is required as an energy source for basal metabolism and for supporting synthetic activities and for synthesis of material build up from glucose, such as conchiolin. Conchiolin is organic in nature and consists of mucopolysaccarides (Chellam *et al.* 1991). However, there were no available data to compare the haemolymph glucose concentration in the oysters during pearl sac growth and development. Machii *et al.* have analysed salts, heavy metals, and free amino acids in the hemolymph of various mollusks, including *P. fucata* (Kawai *et al.* 1981) but these data were not related to the pearl sac growing phase.

Parallel with the higher implantation success and pearl sac growth and development and nucleus coverage by the pearl sac, the haemolymph calcium and phosphorus concentrations increased with the advance of pearl sac growth and development and female host oysters had higher calcium and phosphorus concentrations. The consistent higher haemolymph calcium and phosphorus concentrations in female host oysters as compared to male host oysters indicated the mineral availability to support pearl sac growth and development and synthesis of organic matrix during pearl sac formation. It was reported that after the pearl sac formation, pearl sac epithelial cells start to secrete shell matrices together with active transport of calcium and bicarbonate ions (Wilbur & Saleuddin 1983) that could affect the calcium concentration in the haemolymph. The cells of the pearl sac derive their nourishment from the surrounding tissues (haemolymph) (Chellam *et al.* 1991). There is a possibility that oestrogen could stimulate mineral mobilization from their storage in the tissue in preparation of calcium and phosphorus requirement for pearl sac formation and pearl synthesis. However, how the female host oysters had higher calcium and phosphorus concentrations as compared to male host oyster is not clear. Even though the synthesis of pearl was not started during these 4 weeks of observation after nucleus implantation, the increased haemolymph concentrations of calcium and phosphorus indicate the preparation of calcium, and probably phosphorus, for pearl production.

Conflict of interest

There is no conflict of interest related to the research and the publication.

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