

## Histological Development of Pearl-Sac Formation in Thai Freshwater Mussels

Kannika Chatchavalanich<sup>1\*</sup>, Aurapa Nagachinda<sup>2</sup>, Uthaiwan Kovitvadhi<sup>1</sup>,  
Satit Kovitvadhi<sup>3</sup>, Amara Thongpan<sup>4</sup> and Oamduen Meejui<sup>5</sup>

### ABSTRACT

Seven species of freshwater pearl mussels collected from three different basins in Thailand were mantle-transplanted and returned to culture in their natural habitats. The microscopic structure of the forming pearl sacs on days 7, 15, 30, and 60 was described. The outer mantle epithelium became a continuous sheet of complete pearl sac, having a small amount of material at day 7. An increasing amount of secreted materials was seen on day 15 and had developed into layers of dense materials by day 30, when the pearl sac epithelium changed from a columnar to squamous epithelium. At day 60, calcium was first seen at the central part of the pearl and later in peripheral parts. Pearls inside the sac were variable in shape. Occasionally, several sacs formed from a single mantle-transplanted piece. Four species of pearl mussel giving superior pearl characters and containing calcium deposits by day 60 were *Hyriopsis (Limnoscapha) myersiana*, *Chamberlainia hainesiana*, *H. (L.) desowitzi* and *Pseudodon* sp.

**Key words:** pearl sac formation, histological development, mantle transplantation, freshwater mussel

### INTRODUCTION

Pearls are an important material used in traditional Chinese medicine and some cosmetics. The People's Republic of China (China) is the largest producer of freshwater pearls, accounting for 99% of the world's total (Li *et al.*, 2007). Freshwater pearls differ from other cultured pearls, in that the great majority of them are not bead-nucleated. For the medicinal and cosmetic industries, however, pearl materials are mainly

obtained through culturing of non-nucleated pearls by mantle transplantation onto the same species (mantle allograft). This process of culturing closely resembles natural pearl formation (Kafuku and Ikenoue, 1983; Binhe, 1984) giving high quality pearls, and therefore, is preferred for ornamentation and jewelry use.

Aside from China, Thailand is one of the countries with high potential for freshwater pearl culturing. The wide variety of water resources and suitable atmospheric conditions contribute to the

<sup>1</sup> Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

<sup>2</sup> Aquatic Biodiversity Research Group, Inland Fisheries Resources Research and Development Institute, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand.

<sup>3</sup> Department of Agriculture, Faculty of Science and Technology, Bansomdejchaopraya Rajabhat University, Bangkok 10600, Thailand.

<sup>4</sup> Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

<sup>5</sup> Kanchanaburi Inland Fisheries Research and Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand.

\* Corresponding author, e-mail: fsciknc@ku.ac.th

numerous species of freshwater pearl mussel found in all regions of the country (Brandt, 1974; Kovitvadhi *et al.*, 2005). Based on shell thickness and the shining intensity of the inner shells, there are at least seven species of pearl mussel with high potential, namely, *Chamberlainia hainesiana* and *Hyriopsis (Limnoscapha) myersiana* (Mae Khlong Basin), *C. hainesiana*, *Hyriopsis (Hyriopsis) bialatus*, *H. (Limnoscapha) desowitzi*, *H. (L.) myersiana*, *Pseudodon vondembuschianus ellipticus* and *Pseudodon* sp. (Chao Phraya Basin), *H. (H.) bialatus* and *Pseudodon inoscularis cumingi* (Moon Basin) (Kovitvadhi *et al.*, 2005). All seven species from the three basins are used for non-nucleated pearl formation by mantle transplantation. The process of pearl-sac formation in cultured pearl mussels was closely observed and recorded at the microscopic level to be used in further considerations regarding commercial pearl production.

## MATERIALS AND METHODS

### Mantle transplantation

Seven adult freshwater pearl mussels, *Chamberlainia hainesiana*, *Hyriopsis (Limnoscapha) myersiana*, *H. (H.) bialatus*, *Pseudodon inoscularis cumingi*, *H. (L.) dezowitzi*, *Pseudodon vondembuschianus ellipticus* and *Pseudodon* sp. were collected from three different river basins, in Thailand, namely, the Mae Khlong River, Chao Phraya River and Moon River basins. Samples that contained the preferred characteristic for pearl culturing of luster of the inner shell, as described by Kovitvadhi *et al.* (2005), were selected for transplantation. Non-nucleated mantle tissue transplantation was performed by inserting four square-shape mantles (0.4 × 0.4cm) from donor mussels into each operation mussel (recipient mussel). The transfer tissue was specifically removed from the central parts of the outer regions of donor tissue to obtain medium thickness in the transplanted mantles. The four

mantles were positioned at the left and right posterior ends (two on each side). Eighty operation mussels were prepared for each species.

### Culturing of operation mussels

Operation mussels were transferred to culture baskets, 30 cm in diameter and 15 cm high, hanging at a water depth of 2 m in the three different natural basins previously mentioned. Each basket contained both sexes of all species collected from each basin; forty baskets per basin were maintained for histological observation.

### Pearl sac collection for histological observation

Two-to-four pearl sacs were removed from four operation mussels of each species on days 7, 15, 30 and 60 after mantle insertion at each culturing site (basin). The sacs were fixed with 10% neutral buffered formalin for 24 h. After standard paraffin methods, sections 6 µm thick were cut and stained with hematoxylin and eosin (H&E) for light microscopy. To observe calcium deposition, Alizarin Red S staining was used.

## RESULTS

The progressive development of pearl sacs was microscopically observed and recorded over the period of the experiment.

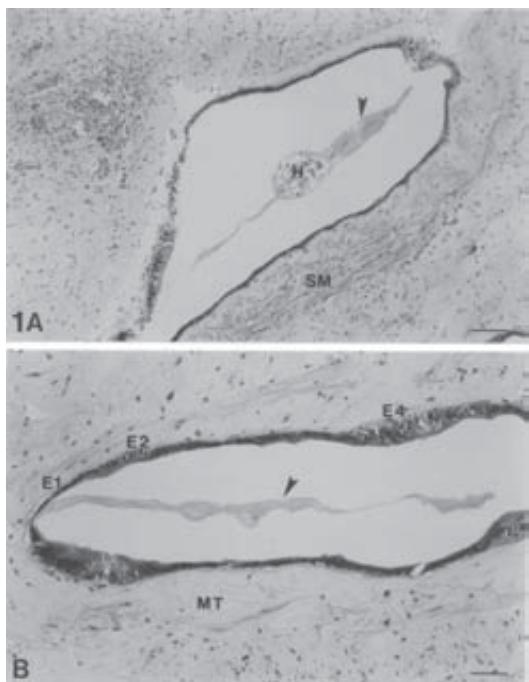
### Pearl sac after 7 d

The pearl sac was completely formed, showing a continuous sheet of outer mantle epithelium. Some secreted materials were found in the sac. The materials were homogeneous and stained purplish pink with H&E staining. Some haematocytes were also observed in the sac (Figure 1A). Various types and different heights of pearl sac epithelium were observed (Figure 1B), ranging from a simple cuboidal epithelium, to a simple columnar epithelium with mucous cells (Figure 2A). Some epithelial linings were composed of simple high columnar epithelia with striated

borders at the apical end, and mucous cells were interspersed among the epithelial cells (Figure 2B), while others were pseudostratified columnar epithelia with striated borders and mucous cells (Figure 2C). Underneath the epithelium was mesodermal tissue, which contained connective tissue, numerous acidophilic granular cells, blood vessels and smooth muscle cells.

#### Pearl sac after 15 d

The accumulation of purplish pink

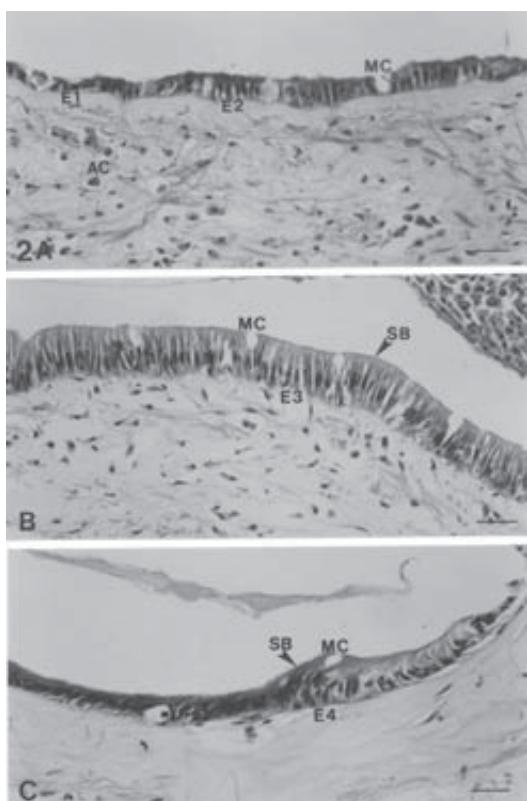


**Figure 1** Pearl sac of *Chamberlainia hainesiana* reared in Mae Khlong River Basin after 7 d: (A) secreted materials (arrowhead) within a completed pearl sac; (B) different types of pearl-sac epithelia, simple cuboidal epithelium (E1) simple columnar epithelium (E2) and pseudostratified columnar epithelium (E4); H, haemocytes; MT, mesodermal tissue; SM, smooth muscle. H&E stain. Scale bar length: (A) = 100  $\mu$ m; (B) = 50  $\mu$ m.

secreted materials in layers was clearly observed. Numerous haematocytes were found in the pearl sac of some mussel species (Figure 3A, B).

#### Pearl sac after 30 d

Highly accumulated pearl substances, aligned as thick homogeneous layers, were observed. The middle part was faintly stained purplish blue with hematoxylin, while the outer region was darkly stained (Figures 4A and C). The central region of the pearl contained some



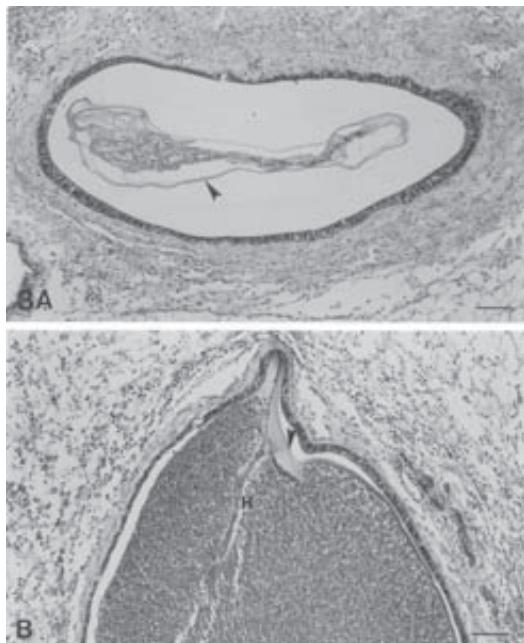
**Figure 2** Various types of pearl sac epithelia: (A) simple cuboidal epithelium (E1) and simple columnar epithelium (E2); (B) simple high columnar epithelium (E3); (C) pseudostratified columnar epithelium (E4). AC, acidophilic granular cell; H, haemocytes; MC, mucous cells; SB, striated border. H&E stain. All scale bars = 25  $\mu$ m.

haematocytes. Calcium was not accumulated at this stage of pearl formation as shown from the negative staining with Alizarin Red S (Figure 4B). Interestingly, simple cuboidal and squamous epithelia without cilia were found in the advanced stage of pearl-sac formation (Figure 4C).

#### Pearl sac after 60 d

Four out of the seven species of mussels sampled, namely, *Hyriopsis (L.) myersiana*, *H. (L.) dezowitzi*, *Pseudodon* sp and *Chamberlainia hainesiana*, had accumulated calcium in the form of large globules in the pearl sac (Table 1). The calcium-deposited regions were positively-stained with Alizarin Red S (Figures 5B and 6A-D). However, the outer region of the pearl itself was devoid of calcium, but in the central part calcium accumulation was darkly-stained with hematoxylin (Figure 5A). The remaining three

species, namely, *H. (H.) bialatus*, *Pseudodon inoscularis cumingi* and *Pseudodon vondembuschianus ellipticus* had no calcium deposition in the 60-d pearl sac. However, they could produce pearl substance in a thick homogeneous layer that stained purple, similar to those having calcium in the pearl sacs (Figure 7A and B). The shape of the pearl sacs at this stage was not definite, but some were oval in shape. The number of sacs also varied (Figure 7A) and numerous haematocytes were occasionally found in these sacs (Figure 7B).



**Figure 3** Pearl sac of *Hyriopsis (H.) bialatus* after 15 d, showing secreted material (arrowhead), H, haematocytes. H&E stain. Scale bar = 100  $\mu$ m: (A) reared in Chao Phraya River Basin; and (B) reared in Mun River Basin.



**Figure 4** Pearl sac of *Hyriopsis (L.) myersiana* reared in Chao Phraya River Basin after 30 d: (A) Pearl substances stained violet with H&E; (B) Pearl substances having no calcium deposition when stained with Alizarin Red S; and (C) Higher magnification of (A) showing pearl substances (arrowhead), haematocytes (H) and simple squamous epithelium (E5). Scale bar (A), (B) = 100  $\mu$ m; (C) 50 =  $\mu$ m.

## DISCUSSION

The process of pearl-sac formation starts with the proliferation of the outer mantle epithelial cells to make a continuous sheet in the mesodermal tissue, where the mantle allograft (transplantation) was implanted. Subsequent secretion of shell materials (i.e., periostracum, prismatic and nacreous) causes the accumulation of pearl substance inside the pearl sac itself (Kawakami, 1952; Binhe, 1984).

The current study results indicated that all seven species of pearl mussel studied completed pearl-sac formation by day 7, which was close to the time taken by the freshwater pearl mussel *Hyriopsis schlegelii* (Machii, 1962), but quite different from the longer time (30 d) required to form the pearl sac in *Hyriopsis cumingii* (Shi *et al.*, 1985). Panha and Kosavittkul (1997) studied mantle transplantations in three species of freshwater pearl mussel, *Hyriopsis (Limnoscapha) myersiana*, *H. (L.) desowitzi* and *Chamberlainia hainesiana*, and reported that the pearl sac was completely formed within 15 d after an allograft.

It should be noted here that the types and

heights of pearl sac epithelial cells depended largely on the selected mantle regions where transplantation took place. Mantle epithelia of the bivalved mussels located in different regions are not the same in thickness and type and, therefore, produced different types of pearl sac (Beedham 1958; Tsuji, 1960; Hillman and Shuster, 1962; Dix, 1973). The functions of each type of epithelial cell are also different, with tall, stratified, columnar epithelium secreting substances that made periostracum, while simple cuboidal and squamous epithelium without cilia produced pearl substances for the nacreous layer (Dix, 1973; Norton *et al.*, 2000). On the other hand, mucous cells infiltrating between epithelial cells were responsible for mucosubstances controlling the production of organic matter and at the same time absorbing the calcium from the surrounding water into the mussels. These mucous substances were eventually combined with calcium and became a part of the pearl layer (Neff, 1972).

Epithelial types changed (from high to low) with the advanced stages of pearl sac formation in *Pinctada imbricata* (Kawakami, 1952; Aoki, 1966). Tall, columnar epithelia at the

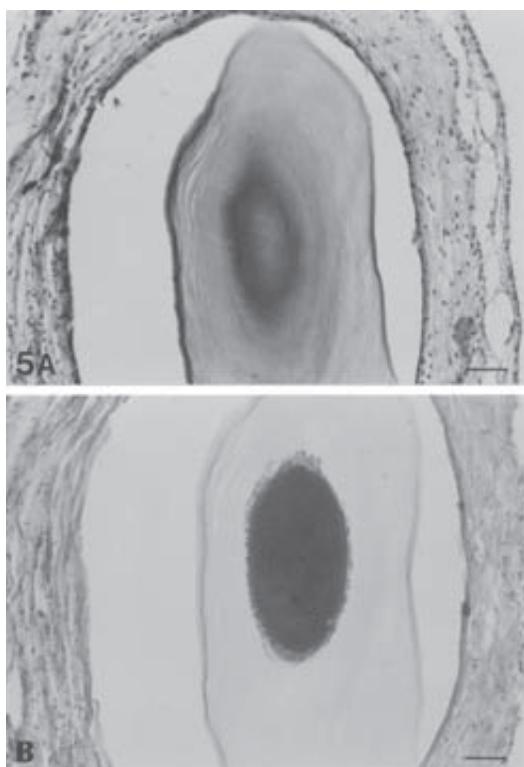
**Table 1** Calcium deposition found in the pearl sacs of cultured mussels after 60 d.

Basin /Mussel species	Calcium deposition (days)			
	7	15	30	60
Mae Khlong River Basin				
<i>Chamberlainia hainesiana</i>	-	-	-	-
<i>Hyriopsis (Limnoscapha) myersiana</i>	-	-	-	+
Mun River Basin				
<i>Hyriopsis (Hyriopsis) bialatus</i>	-	-	-	-
<i>Pseudodon inoscularis cumingi</i>	-	-	-	-
Chao Phraya River Basin				
<i>Chamberlainia hainesiana</i>	-	-	-	+
<i>Hyriopsis (Hyriopsis) bialatus</i>	-	-	-	-
<i>Hyriopsis (Limnoscapha) dezowitzi</i>	-	-	-	+
<i>Hyriopsis (Limnoscapha) myersiana</i>	-	-	-	+
<i>Pseudodon vondembuschianus ellipticus</i>	-	-	-	-
<i>Pseudodon</i> sp.	-	-	-	+

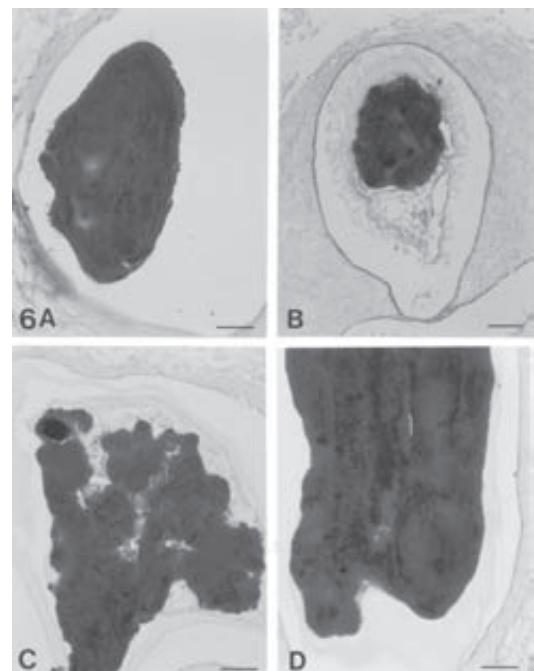
+ = calcium deposition; - = no calcium deposition.

early stage were responsible for periostracum production. Medium height epithelia, on the other hand, produced substances for the prismatic layer, while nacreous substances were secreted from the squamous epithelium at approximately 40 d after mantle transplantation (Kawakami, 1952; Aoki, 1966). The current results suggested that pearl sac substances found on day 7 and day 15, which stained purplish pink with H&E, could be conchiolin of the periostracum. This was indicated by X-rays according to Wada (1958 a, b), followed by day-30 secretion, which stained purple with H&E, indicating prismatic and nacreous substances, after which the epithelial cells of the pearl sac became flat. However, it should be noted

here that the earliest secretion of nacreous substance deposits was at the innermost part of the pearl, while the later ones were densely accumulated at the outer layers, as seen from the dark staining of hematoxylin. Calcium, on the other hand, was the latest deposit in both prismatic and nacreous layers (Kawakami, 1952; Wada, 1958a, b), starting also from the central region of the pearl and progressing to fill up the whole pearl, as seen from the staining of hematoxylin and eosin, and Alizarin Red S in the middle region only of the 60-d pearl. However, calcium deposition could be stalled by environmental conditions (Wada, 1959a, b). Aside from the interruption of pearl



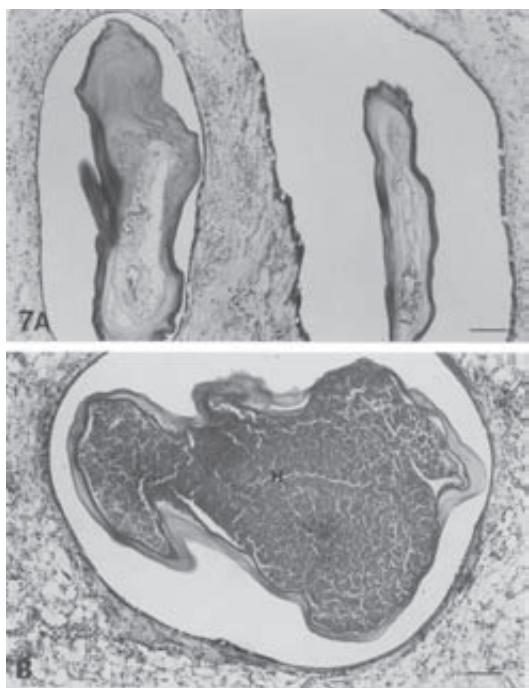
**Figure 5** Pearl sac of *Hyriopsis (L.) myersiana* after 60 d, reared in Chao Phraya River Basin showing calcium deposition in the center of pearl and stained with: (A) H&E; and (B) Alizarin Red S. Scale bar = 50  $\mu$ m.



**Figure 6** Pearl sac at 60 d of: (A) *Hyriopsis (L.) myersiana* reared in Mae Khlong River Basin; (B) *Pseudodon* sp. reared in Chainat province; (C) *Hyriopsis (L.) desowitzi* reared in Chao Phraya River Basin; and (D) *Chamberlainia hainesiana* reared in Chao Phraya River Basin. Stain = Alizarin Red S. Scale bar = 100  $\mu$ m.

formation, haematocytes found in the pearl also indicated that the mussels had responded to the irritation caused by foreign substances, similar to the report on *Pinctada maxima* (Scoones, 1996), as well as by means of infection or tissue damage, which resulted in the malformation of pearl (Norton *et al.*, 2000). The observed multiformaltion of pearl sacs from one mantle piece also indicated that several pearls could be produced instead of one pearl at one specific transplanted region.

The current results confirmed previous findings that the rate of calcium accumulation in pearls depended largely on the species and ages of the mussels, as well as on the physical and biological environment of the mussel's habitat.



**Figure 7** Pearl sac at 60 d of: (A) *Chamberlainia hainesiana* reared in Mae Khlong River Basin, showing two pearl sacs forming from one mantle transplantation; and (B) *Hyriopsis (H.) bialatus* reared in Mun River Basin, showing numerous haematocytes (H) in a pearl sac. Stain = H&E. Scale bar = 100  $\mu$ m.

*Chamberlainia hainesiana* cultured in the Mae Khlong River basin contained no calcium deposition on the pearls at day 60. On the contrary, *C. hainesiana* cultured in the Chao Phraya River basin for the same period showed a distinctive amount of calcium. From observations in the current study, the pearl sacs having calcium deposition at an earlier time produced larger pearl sizes than those at later times, as noted by Nagajinda *et al.* (2005).

## CONCLUSION

All seven species of freshwater pearl mussel cultured in three different natural habitats had completely formed pearl sacs by day 7. Sequential development of pearl sac tissues surrounding the transplanted mantle were observed, while the secretion of pearl substances forming the pearl were also recorded. However, the species that produced superior pearls having calcium accumulation by day 60 were *Hyriopsis (Limnoscapha) myersiana*, cultured in the Mae Khlong River basin and in the Chao Phraya River basin, and *Chamberlainia hainesiana*, *Hyriopsis (Limnoscapha) desowitzi* and *Pseudodon sp.* cultured in the Chao Phraya River basin. The species and ages of freshwater pearl mussels, as well as the conditions of the culturing water, contributed to the quality and process of pearl formation.

## ACKNOWLEDGEMENT

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