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Pigment Distribution and Secretion in the Mantle of the Pacific Oyster (*Crassostrea gigas*)

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Abstract The color of Mollusca shells is one of the most important attributes to consumers. At the cellular level, black color is mainly from the melanin produced by melanocytes. The melanosome is a specialized membrane-bound organelle that is involved in melanin synthesis, storage, and transportation. How the complex pigmentation process in the *Crassostrea gigas* is established remains an open question. The objectives of this studies are to examine the morphological characteristics of melanosomes or melanin of mantle pigmentation in the Pacific oyster, thereby investigating its contribution to shell color. The results show that pigmented granules of the mantles vary among the three lobes, and the melanosomes at different stages are enriched in distinct cargo molecules, which indicate the remarkable difference between the marginal mantle and central mantle. Examination of mantle histology reveals that the mantle margin of the oyster is characterized by three different folds, including the outer secretory, middle sensory, and inner muscular fold. Ferrous ion chelating assays against the tyrosine hydroxylase indicate that a large amount of melanin is localized in the inner surface of the middle fold. Transmission electron microscopy analyses show that the mantle edge is composed of tall columnar and cuboidal epidermal cells and some pigmented melanocytes intersperse among these cells. The numbers of melanosomes among the three lobes are different. In the inner fold and the middle fold of the mantle, some single dispersion, or aggregation of melanosomes with different degrees of melanization are found in the outer surface. Numerous melanosomes are distributed in the epithelium of the outer fold of the mantle, and mainly are at the apical microvillar surface near the lumen. However, melanosomes are occasionally observed in the central mantle, and they are relatively less. This work provides new insights into the process of melanin deposit in the mantle and shell pigmentation in *C. gigas*.

Key words *Crassostrea gigas*; mantle; melanosome; melanin; pigmentation; cells

1 Introduction

The diversity and complexity of pigmentation patterns on mollusk shells have been an active research field. The color patterns on their shells make the mollusca fascinating models to study morphogenesis. The generation of a mollusk shell pattern is, in fundamental terms, a symmetry-breaking process. Such a process can be efficiently modeled by one-dimensional reaction diffusion (Kondo and Miura, 2010). It requires an autonomous line of secretory cells experiencing local and lateral interactions along the mantle edge to control pigmentation (Budd *et al*., 2014). Mollusk shell formation is controlled by an evolutionarily homologous organ, *i.e*., mantle, which covers the visceral mass and directly contact with shell (Jabbour-Zahab *et al*., 1992; Budd *et al*., 2014). The mantle is divided into distal, central, and proximal zones, and they are responsible for the secretion of structurally different shell layers (Jolly *et al*., 2004; Marin and Luquet, 2004; McDougall *et al*., 2011). Shells grow in a linear way by the addition of new material to the growing edge that contacts with the mantle (Williams, 2017; Feng *et al*., 2019). It is here that new pigments produced by the mantle are established in the periostracum (Gantsevich *et al*., 2005; Li *et al*., 2014; Williams, 2017).

Melanin of Mollusca is the most widespread pigment in nature (Williams, 2017). In vertebrates, melanocytes are the most common type of chromatophores and are responsible for most of the dorsal pigmentation. Each melanocyte contains thousands of melanin-containing melanosomes. Melanosomes are morphologically and functionally unique organelles within which melanin pigments are synthesized and stored (Marks and Seabra, 2001). The size and maturation of melanosomes can cause different skin colors (Miyamura *et al*., 2007). Several studies have focused on the distribution and formation of melanin or melanocytes in invertebrates. It has been reported that the number of melanosomes in the melanocytes of albino *Apostichopus japonicus* is less than it in the normal ones (Zhao *et al*., 2012). Moreover, in Cephalopods, the biochemical steps in melanin production have been linked to melanosomes in the melanin-producing cells in the ink gland (Derby, 2014). In *Pinctada persica*, the mantle edge stained with haematoxylin and eosin is lined by cuboidal and columnar epithe-

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lia, and some cells containing brown granules were distributed among these epidermal cells (Parvizi *et al*., 2018). In addition, brown pigmented cells were sparsely distributed in the outer fold and middle fold in *Pinctada margaritifera* (Jabbour-Zahab *et al*., 1992). The presence of pigment in mantle epithelia showed their possible role in black calcitic prism deposition.

The Pacific oyster is a shellfish with high commercial value and possesses multiple traits, such as fast growth rate, high reproductive performance, and rapid adaptation, making it desirable for aquaculture (Ge *et al*., 2014). The complex pigment patterns (black, golden, orange and white) on *C. gigas* shells makes it a fascinating model to study morphogenesis of pigmentation. The shell colors have been considered as a quantitative trait which lays a foundation for future marker-assisted breeding (Ge *et al*., 2014; Xu *et al*., 2019). Recently, genome-wide expression profiling compared *C. gigas* with different shell colors, and the results indicated that several candidate genes, such as *Rab7* and *Tyrp1*, might be involved in shell pigmentation (Feng *et al*., 2015). Among these candidates, tyrosinase and peroxidase have been identified to play key roles in shell matrices and melanin pigmentation in *C. gigas* shells (Feng *et al*., 2019; Zhu *et al*., 2021). Previous morphological studies on the mantle histology and ultrastructure of various oyster species have reported the presence of pigments found in mantle edges. (Jabbour-Zahab *et al*., 1992; Fang *et al*., 2008; Parvizi *et al*., 2018). Additionally, Han *et al.* (2022) confirmed the presence of melanin in oysters using three melanin staining methods, including DOPA, Masson-Fontana and ferrous sulfate staining methods. Besides, melanocytes containing melanosomes with electron-density components were found below the membrane, indicating the melanosomes exist in the mantle tissues of the Pacific oyster (Han *et al*., 2022). It is believed that the mantle edges have three distinct folds, including outer secretory, middle sensory, and inner muscular folds in most advanced mollusks. Moreover, the epithelia of the mantle edges that secrete different components of the shell show several distinctive features. However, little is known about patterning mechanisms of pigmentation among the three distinct lobes in the mollusks.

In this study, the fine structures of three distinct lobes were investigated to identify the cellular basis of the pigmentation patterns in *C. gigas*. Data from this study will provide functional morphology of the mollusks integument information to understand the mechanisms of the shell coloration, which will bring new insights into the genetic basis for the selective breeding the *C. gigas* with different shell colors.

2 Materials and Methods

2.1 Pigmentation Mapping

Wild populations of juvenile *C. gigas* (shell height: 48.13 mm ± 4.69 mm; shell length: 31.08 mm ± 4.07 mm; shell width: 11.13 mm ± 1.86 mm) were collected from the sea along Weihai City of China in November 2021. The *C. gigas* were cultured in filtered seawater with in 1 mol L^{-1} $MgCl_2$ for 8 h. Then the mantles were dissected and mounted on slides. Image acquisitions were performed with Olympus DP80 microscope.

2.2 Electron Microscopy

The oysters were anesthetized in 1 mol L^{-1} $MgCl_2$ in 50% seawater and 50% fresh water for 8 h. Mantle samples were fixed in 2.5% glutaraldehyde for 2 h. They were postfixed in osmium tetroxide, dehydrated in acetone, and embedded in EPON 812 resin. Then the sections were cut at 5 μm and then stained with toluidine blue to identify different mantle regions under the light microscope. Ultrathin sections of 60 nm were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (JEM-1200EX) at 80.0 kV.

2.3 Histological Observation of the Mantles

Mantle tissues were fixed in buffered formalin following the method of Carson *et al*. (1973). All samples were dehydrated through a graded series of EtOH (70%, 80%, 90%, and 100%), cleared in xylene, and then embedded in paraffin. The sections were cut at 5 μm using a Leica RM 2016 microtome (Leica). The paraffin-embedded microsections were stained for morphological studies with hematoxylin and eosin (H&E).

The chelation of ferrous ions by the melanin pigment in the mantles was estimated by the method of Jabbour-Zahab *et al.* (1992). Briefly, the sections were dewaxed with xylene and then dehydrated in gradient alcohol. After the sections were stained in ferrous solution for 1 h and then washed for 12 min four times in ultrapure water. Sections were immersed in acid potassium ferricyanide solution for 30 min. After washed for four times, the sections were then placed in nuclear fast red solution for 10 min. Images of stained mantle tissues were captured using an Olympus DP80 microscope.

3 Results

3.1 Histological Observation of the Mantle

The mantle tissue of the *C. gigas* is divided into two zones, namely the marginal mantle and central mantle. The mantle with pigmented granules that can be clearly viewed in the light microscopy (Fig.1). The degree of pigment deposition within the marginal and central mantles is different, which indicates the pigmentation and scattering properties vary among mantle areas.

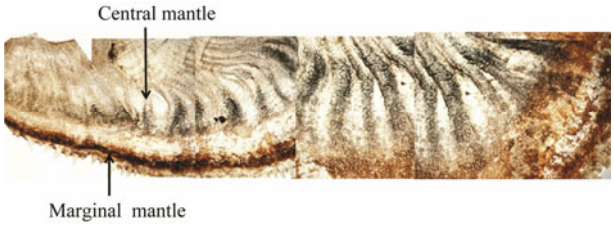

Fig.1 Mantle tissue of *C. gigas*.

To further study the structures of the mantles in the oyster, we performed H&E stain. The *C. gigas* marginal mantle is a thick and muscular membrane, and is characterized by three different folds, including the outer secretory, middle sensory, and inner muscular folds, as shown in Fig.2A. These folds are composed of the outer epithelium, inner epithelium, and connective tissues. Close to the shell is the outer mantle fold, and the periostracum is formed within the periostracal groove between the outer fold and the middle fold. It is clear that the middle fold has finger-like ciliated tentacles on its outer surface (Fig.2B). Moreover, a large rounded ganglion is located at the convergent boundaries of the three folds (shown in Fig.2C). Central mantle is a transparent and epithelial membrane, which consists of the epithelial tissue that is attached to irregular connective tissues. Hemocytes and muscle fibers are observed within the central mantle (Fig.2D). Ferrous ion chelating assays against the tyrosine hydroxylase showed that a large amount of melanin was present in the epithelium of marginal mantle (Fig.2E), particularly in the inner fold and the inner surface of the middle fold, as shown in Fig.2F. However, melanin granules are more abundant in the inner surface of the middle fold than those of the inner fold and outer fold.

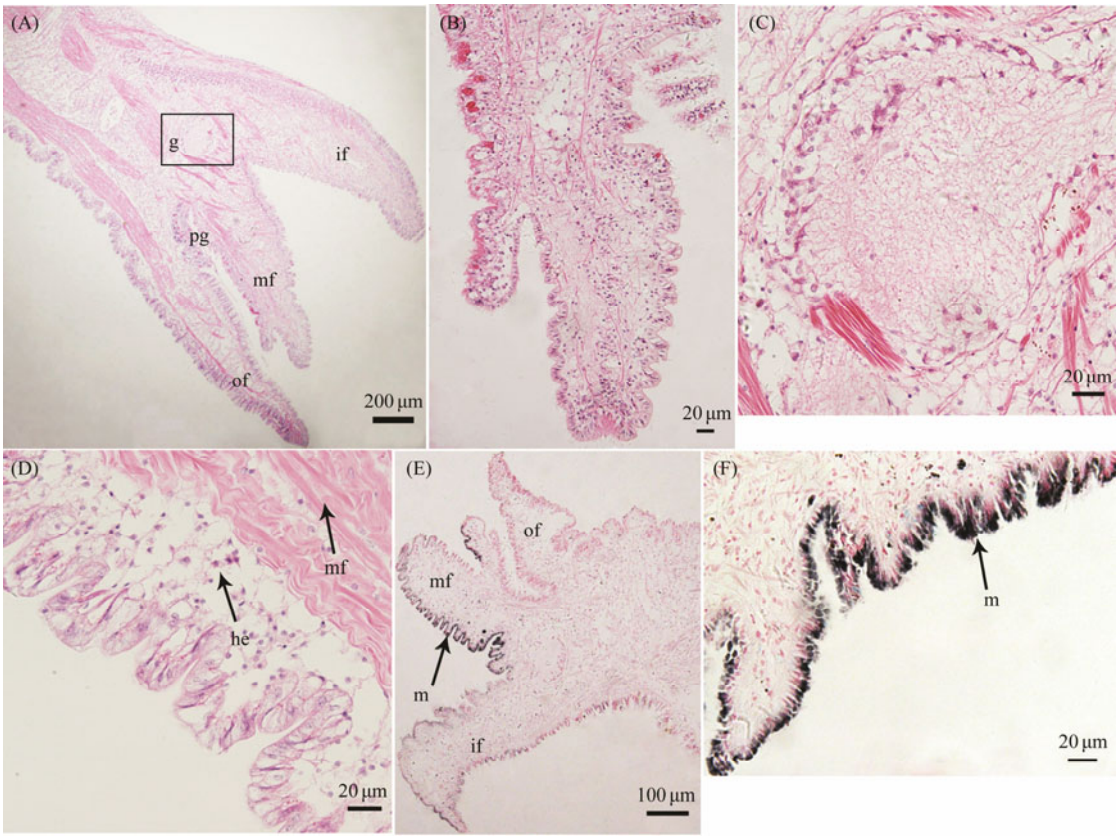

Fig.2 Histological sections of the *C. gigas* mantle. A, marginal mantle and central mantle. B, middle fold of the marginal mantle. C, ganglion in the marginal mantle. D, epithelium of the central mantle. E, distribution of melanin of the mantle. F, distribution of melanin in the middle fold. of, outer fold; mf, middle fold; if, inner fold; pg, periostracal groove; g, ganglion; mf, muscle fiber; he, hemocytes; m, melanin.

3.2 Transmission Electron Microscopy of the Mantle

By taking advantage of the high spatial resolution of a transmission electron microscope, we compared the difference of the ultrastructure among the marginal and central mantle of *C. gigas*. The apical plasma membrane of the epithelia is arranged in an array of microvilli in all cases (Figs.3A, 3E, 4A, 4E, 5A, 5E, 6A). Most of the cells in the epidermis are cuboidal epidermal cells, lying on a membrane. The epithelium is separated with its neighbour by an intercellular junction. Apparently, melanocytes are distributed in the marginal and central mantle, which is a characteristic of melanin-containing melanosomes, varying from 0.3 to 0.6 μm in size (Figs.3D, 3H, 4C, 4D, 4G, 5D, 5G, 6C, 6D). In the inner (Figs.3A–D) and outer (Figs.3E–H) surfaces of the inner fold, cells located below the membrane possess more melanosomes than those in the epidermis (Figs.3A, B, E, F). Only several melanosomes are found within the inner epithelium of the inner fold (black arrowhead) (Fig.3C). Interestingly, non-pigmented vesicles also appear in the basal portion of the inner epithelium of the inner fold (white arrowheads) (Fig.3D). However, in the outer surface, the number of melanosome is higher than that in the inner surface. Some single dispersion, or aggregation of melanosomes with different degrees of melanization are found in this region (Fig.3H). In the middle fold, it is evident that the melanosome within the outer surface (Figs.4E, F) are concentrated in the apical region compared with inner surface (Figs.4A, B). Notably, the number of melanosome within the inner surface (Figs.4A–D) is sig-

nificantly higher than that in the outer surface (Figs.4E−H). Additionally, some melanosomes with partially pigmented granules were also observed on the outer surface (white arrowheads) (Figs.4G, H). In the outer fold, there are numerous single and large melanosomes within the inner (Figs.5A−D) and outer (Figs.5E−H) surfaces, characterized by ellipsoidal and intensely melanotic. Overall, the melanosome count in the outer fold is higher than that in the inner fold and middle fold. The vesicles loaded with multiple melanosomes (black arrowhead) (Fig.5B) were concentrated towards the apical microvillar surface near the lumen (shell-abutting). The majority of melanosomes reside along the membrane within the inner epithelium and there are usually aggregations of glycogen rosettes in this region (Fig.5D). Typically, granular endoplasmic reticulum are present in the peripheral region of melanosomes (Figs. 5D, G). Surprisingly, melanosomes are occasionally observed in the central mantle, but they are relatively less (Fig.6). There appear to be an increase in the amount of glycogen when compared with those in the marginal mantle.

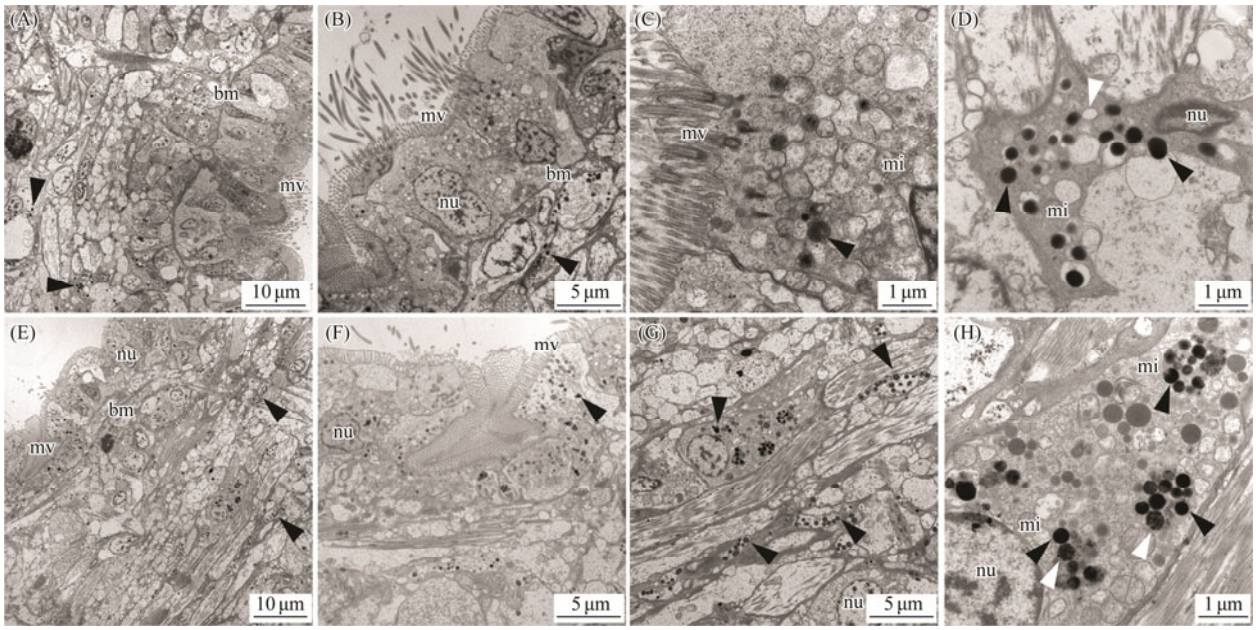

Fig.3 Transmission electron microphotographs of the inner fold of mantle epithelia. A, B, C and D: Ultrastructure of the inner surface of the inner fold. Black arrowheads indicate melanosomes. White arrowheads indicate non-pigmented vesicles. E, F, G and H: Ultrastructure of the outer surface of the inner fold. Black arrowheads indicate melanosomes. White arrowheads indicate melanosomes with partially pigmented granules. mv, microvilli; bm, basal membrane; nu, nuclei; mi, mitochondria.

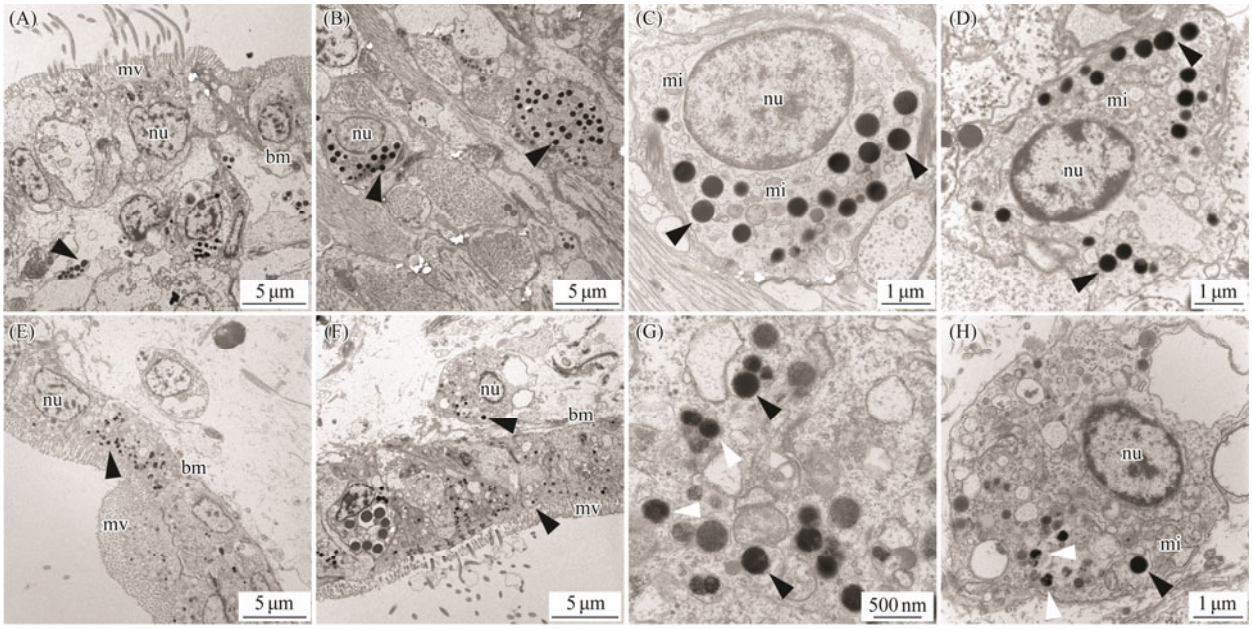

Fig.4 Transmission electron microphotographs of the middle fold of mantle epithelia. A, B, C and D: Ultrastructure of the inner surface of the middle fold. E, F, G and H: Ultrastructure of the outer surface of the middle fold. Black arrowheads indicate melanosomes. White arrowheads indicate melanosomes with partially pigmented granules. mv, microvilli; bm, basal membrane; nu, nuclei; mi, mitochondria.

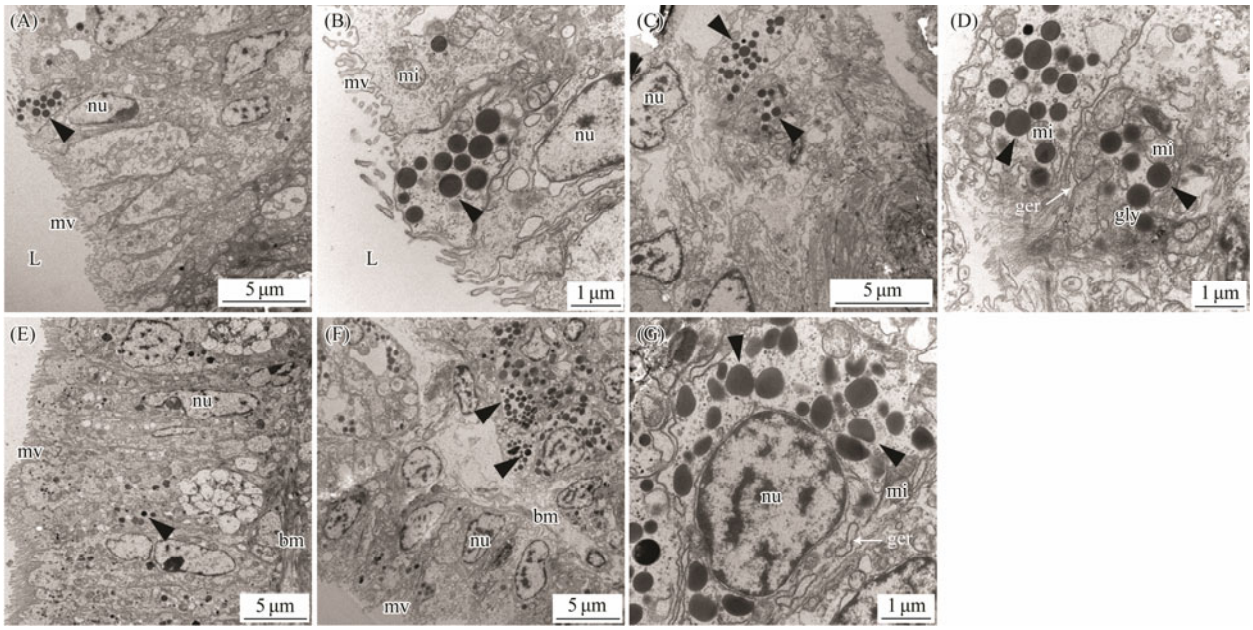

Fig.5 Transmission electron microphotographs of the outer fold of mantle epithelia. A, B, C and D: Ultrastructure of the inner surface of the outer fold. E, F, G and H: Ultrastructure of the outer surface of the outer fold. Black arrowheads indicate melanosomes. mv, microvilli; bm, basal membrane; nu, nuclei; mi, mitochondria; L, luman; ger, granular endoplasmie retieulum; gly, glycogen.

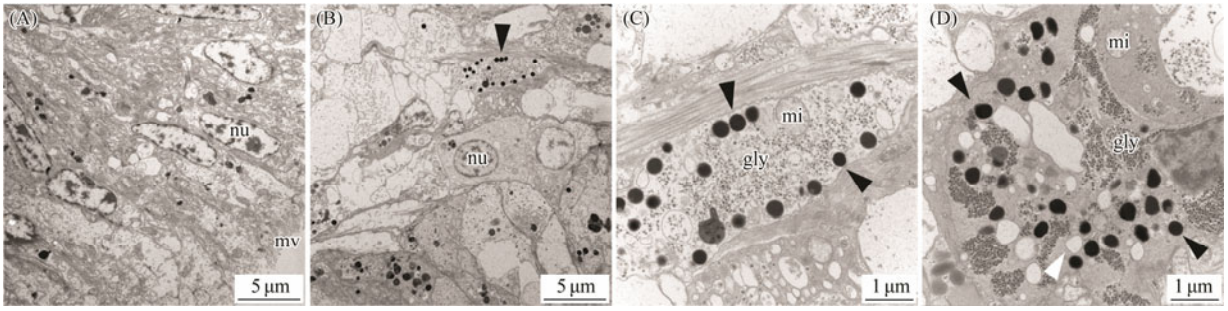

Fig.6 Transmission electron microphotographs of central mantle epithelia. Black arrowheads indicate melanosomes. White arrowheads indicate non-pigmented vesicles. mv, microvilli; nu, nuclei; mi, mitochondria; gly, glycogen.

4 Discussion

The various mantle edge morphology may affect shell characteristics, including shape, colour, size and thickness (Parvizi *et al*., 2018). In the Yesso Scallop, the middle fold of mantle was composed of several protuberances with abundant nerve fibers, whereas the inner fold was highly muscular and was much larger than the other folds (Mao *et al*., 2019). It is conceived that the muscular curtain of the inner fold is used to regulate water flow into and out of the mantle cavity (Audino *et al*., 2015). Meanwhile, in contrast to other folds, the inner fold was taller and bigger, and contained pleated epithelium in the pearl oysters (Parvizi *et al*., 2018). However, the outer fold was longer than the inner fold in *Velesunio ambiguus* and *Hyridella depressa*, and it was divided into two or more lobes (Richardson *et al*., 1981; Colville and Lim, 2003). In some species, such as *Pulvinites exempla*, the mantle edge was composed of four folds, including two inner folds and two outer folds (Tëmkin, 2006). In *C.gigas*, the mantle edge was divided into three folds, they were similarly unfused along the mantle edge and free from attachment to the inner surface of mantles to allow deep movement by pallial retractor muscles either three or four folds (Yonge, 1977; Tëmkin, 2006). Altogether, the mantle has specific and different morphology in each species of bivalves. It is believed that the mantle edges have three distinct folds, including outer secretory, middle sensory, and inner muscular folds in most advanced mollusks. Moreover, the epithelia of the mantle edges concerned with the secretion of the different components of the shell show several distinctive features.

Indeed, outer fold of *P. exempla* contains amounts of pigmented granules, while a very low concentration of pigmented cells was present in the inner fold and the adjacent portion of the inner mantle surface, suggesting their role in secretion (Tëmkin, 2006). A recent study conducted in specimens of *C. gigas* through staining with Masson-Fontana and ferrous ion showed a distribution of melanin in the mantle tissues, suggesting the presence of melanin granules (Han *et al*., 2022). In this study, the melanin was mainly found in the inner fold and the inner surface of the middle fold using the ferrous ion staining. Fibres of the

muscles run along the inner and outer surfaces of mantle tissue, penetrating into inner and outer mantle folds, suggesting the possibility of gradual transfer of melanin from the inner and middle folds to the outer fold along actin filaments directly, as reported in the fish melanophores previously (Aspengren *et al*., 2008). In addition, the cells of the outer fold mantle secrete organic matrix into the extrapallial space, the matrix can combine with inorganic ions to form calcium carbonate, and then deposit on the shell (Ballarini and Heuer, 2007). These observations led to the conclusion that the melanin granules could be involved in the shell coloration. Similar results were found in the *Pinctada maritlza*, in that species melanins were observed in the middle and inner fold of the mantle epithelia (Jabbour-Zahab *et al*., 1992).

Melanin, a black or brown biological polymer formed from tyrosine, was founded in the body wall of sea cucumbers (Zhao *et al*., 2012). Meanwhile, melanin was also histologically distributed in the epithelium of *Haliotis tuberculata* side foot (Bravo Portela *et al*., 2012). In these species, the pigment was in the form of melanosomes. Pigmented granules were commonly observed in bivalve's mantles, and most appeared as secretory vesicles. Secretory vesicles associate with the Golgi complex and will change size and electron density when they move to the cell apex region (Álvarez Nogal and Molist García, 2015). These vesicles can contact with the plasma membrane (Colville and Lim, 2003), and then they can take up or release materials. The epidermal cells of the mantle were associated with pigmentation of the shell and soft tissues. Recently, melanocytes containing melanosomes were identified below the mantle epidermis in *C. gigas* (Han *et al*., 2022). In this study, ultrastructures of the marginal and central mantle of *C. gigas* were observed. A large number of melanocytes which contained melanosomes were distributed on the mantle surface. Melanosomes with many electron dense particles known as the melanin granules become more and larger from the epidermis of the inner fold to the outer fold. The increase of number and size of melanosome may be ascribed to function of specific mantle region. In general, pigment granules of epidermal cells of the outer mantle contribute to the formation and structure of the shell through chitin (Álvarez Nogal and Molist García, 2015). The growth process of a bivalve shell was controlled by the material that was secreted by the epithelium of the outer mantle fold (Checa, 2000). It was indicated that the difference of human skin color was mainly due to the melanocytic activity in each melanocyte, as well as the size and maturation of melanosomes (Aspengren *et al*., 2008). Thus, it can be hypothesized that melanosomes within the outer mantle may release the melanin to control shell color as these cells secrete materials to form the shell.

Regarding the variations in the distribution of melanosomes within epithelium in mantle, three distinct stages were defined. Stage I represents vesicles with non-pigmented, stage II represents melanosomes with partially pigmented granules, stage III represents melanosomes with completely pigmented granules. According to Derby (2014), non-pigmented melanosome vesicles represent premelanosomes in which melanins were not yet deposited. The change from stage II to stage III was a progress from early stage melanosomes to mature melanosomes. Similar studies were reported in *H. tuberculata*, pigmented cells on a groove of foot containing mature and immature melanosomes (Bravo Portela *et al*., 2012). Formation of the periostracum takes place over the basal third of the inner face of the outer fold epithelium (Bubel, 1973). Our observations indicate that the inner surface of the outer fold epithelium is with one cell type, characterized by the presence of numerous melanosomes. It is likely that these melanosomes take part in the formation of periostracum *via* extrapallial space (luman). Furthermore, in the inner surface of the outer fold, there are a large number of mitochondria within the melanocyte and a highly developed rough endoplasmic reticulum. The presence of mitochondria suggests high metabolic activity in this area. Moreover, it is generally accepted that rough endoplasmic reticulum can form early premelanosomes (Palumbo, 2003). However, the process of melanin synthesis in *C. gigas* is complex and is involved in various melanogenic enzymes. Whether the melanogenic enzymes co-localize and are functionally interactive in the melanogenic compartments of mantle cells is not clear. With this issue, large amounts of active tyrosinase were found in the mantle edge in our previous studies, and the localization of tyrosinase gene (*Tyr*) was located in the inner surface of the outer fold by ISH analysis (Zhu *et al*., 2021). These evidences suggest that the tyrosinase can take part in the melanization. Another important factor related to the melanin formation is microphthalmia-associated transcription factor (*Mitf*), which not only regulates the survival and proliferation of melanocytes, but also promotes the transcription of tyrosinase gene (Noguchi *et al*., 2014). It is probable that the rough endoplasmic reticulum formed vesicles, then, melanization occurred at the outside of the vesicles with the catalytic action of melanogenic enzymes, forming particulate melanosomes. Finally, melanosomes fuse with the cell membrane at the apical pole, releasing the melanin into the extrapallial space (Fig.7). Similar study can be found in melanin-producing systems of ink gland of *Sepia officinalis* (Derby, 2014).

What's more, there is a tendency of reduction in the protein synthetic apparatus of the cells from the edge mantle to the central mantle, whereas the quantity of glycogen increased. Early studies showed that this may be associated with a change of secretory activity from periostracum formation to calcium secretion (Kniprath, 1972). In addition, it may be related to shell calcification, since carbon dioxide generated as a result of glycogen metabolism can provide a carbonate source for the calcification process (Bubel, 1973).

In conclusion, the pigmented granules of the mantles vary among the three lobes and melanosomes at different stages, and are enriched in distinct cargo molecules. It causes a remarkable difference between the marginal mantle and central mantle. Most importantly, melanosomes within the epidermal cells of the outer mantle might be associated with shell pigmentation. In particular, the maturation of melanosomes probably affects the growth and proliferation

of melanosomes. This study sheds new light on the cellular basis for mantle melanin formation, which can contribute to understand better the shell pigmentation in the *C. gigas*.

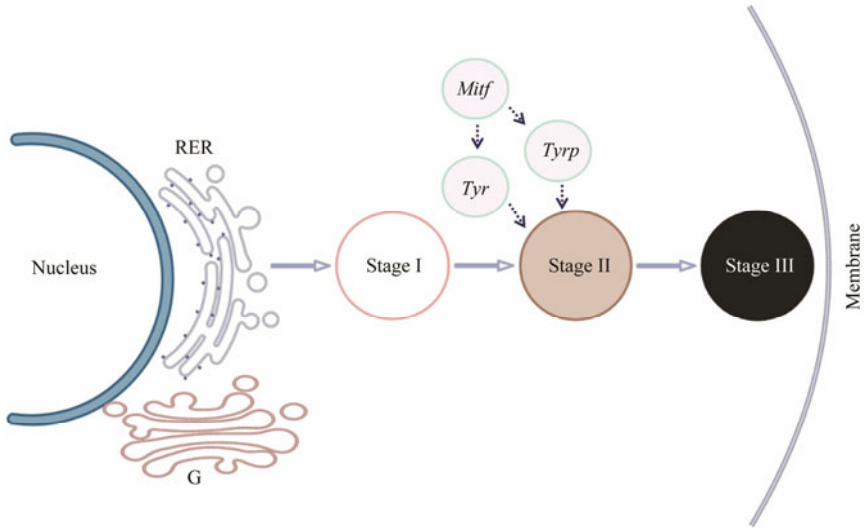

Fig.7 Overview of melanin formation in the mantle of *C. gigas*. Stage I represent non-pigmented vesicles. Stage II represent melanosomes with partially pigmented granules. Stage III represent melanosomes with completely pigmented granules. *Tyr*, tyrosinase gene; *Tyrp*, tyrosinase-like protein gene; *Mitf*, microphthalmia-associated transcription factor; RER, rough endoplasmic reticulum; G, Golgi.

Acknowledgements

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