

Comparison of pigment composition and melanin content among white, light-green, dark-green, and purple morphs of sea cucumber, *Apostichopus japonicus*

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Received 21 August 2016; accepted 14 February 2017

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Abstract

Sea cucumber, *Apostichopus japonicus* (Selenka), is a commercially important marine species in China. Among the differently colored varieties sold in China, white and purple sea cucumbers have the greatest appeal to consumers. Identification of the pigments that may contribute to the formation of different color morphs of sea cucumbers will provide a scientific basis for improving the cultivability of desirable color morphs. In this study, sea cucumbers were divided into four categories according to their body color: white, light green, dark green, and purple. The pigment composition and contents in the four groups were analyzed by high performance liquid chromatography (HPLC). The results show that the pigment contents differed significantly among the white, light-green, dark-green, and purple sea cucumbers, and there were fewer types of pigments in white sea cucumber than in the other color morphs. The only pigments detected in white sea cucumbers were guanine and pterioic acid. Guanine and pterioic acid are structural colors, and they were also detected in light-green, dark-green, and purple sea cucumbers. Every pigment detected, except for pterioic acid, was present at a higher concentration in purple morphs than in the other color morphs. The biological color pigments melanin, astaxanthin, β -carotene, and lutein were detected in light-green, dark-green, and purple sea cucumbers. While progesterone and lycopene, which are also biological color pigments, were not detected in any of the color morphs. Melanin was the major pigment contributing to body color, and its concentration increased with deepening color of the sea cucumber body. Transmission electron microscopy analyses revealed that white sea cucumbers had the fewest epidermal melanocytes in the body wall, and their melanocytes contained fewer melanosomes as well as non-pigmented pre-melanosomes. Sea cucumbers with deeper body colors contained more melanin granules. In the body wall of dark-green and purple sea cucumbers, melanin granules were secreted out of the cell. The results of this study provide evidence for the main factors responsible for differences in coloration among white, light-green, dark-green, and purple sea cucumbers, and also provide the foundation for further research on the formation of body color in sea cucumber, *A. japonicus*.

Key words: *Apostichopus japonicus*, pigment composition, color morphs, melanin, pigment content

Citation: Xing Lili, Sun Lina, Liu Shilin, Li Xiaoni, Miao Ting, Zhang Libin, Yang Hongsheng. 2017. Comparison of pigment composition and melanin content among white, light-green, dark-green, and purple morphs of sea cucumber, *Apostichopus japonicus*. Acta Oceanologica Sinica, 36(12): 45–51, doi: 10.1007/s13131-017-1056-5

1 Introduction

Sea cucumber has been used as a traditional medicine and consumed as a tonic for hundreds of years in China (Chen, 2003). Today, color is one of the most important traits affecting the price of sea cucumber products (Kanno et al., 2006). In recent years, white and purple sea cucumbers have been farmed at many coastal sites in China, such as Weihai, Yantai and Qingdao, to meet the increasing market demands for this organism as an ornamental and edible commodity. In Japan, sea cucumbers are divided into three forms according to their body color: red, green and black (Choe and Ohshima, 1961). However, the relationship between the body color of the various morphs and their taxonomic status is still controversial. It had been suggested that red sea cucumber is a different species from the green and black sea

cucumbers (Sang, 1990). What's more, the result showed that red sea cucumber had obvious genetic differences with green and black sea cucumber through isozyme markers and microsatellite technology demonstration (Kanno and Kijima, 2002; Kanno et al., 2005). And there was no significant difference between green and black sea cucumber (Kan-No and Kijima, 2003). Another suggestion was that the three color morphs are a single species of *A. japonicus* that differ in their body color (Zhang et al., 2015). This view challenged the justification for previous taxonomic studies. In China, there are three color variants (white, green and purple) of sea cucumber. In this study, green sea cucumbers were divided into light green and dark green, because of the substantial difference in their color depth (Fig. 1).

There are two types of body coloration in animals: biological

Foundation item: The Agricultural Seed Project of Shandong Province.

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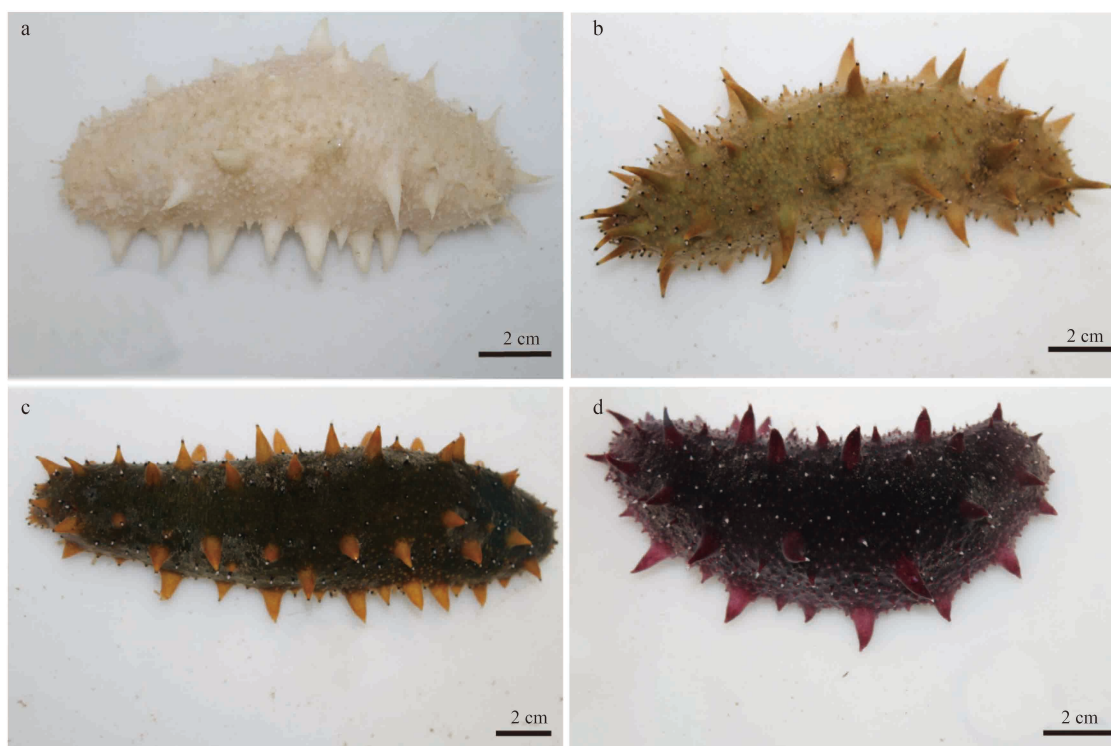


Fig. 1. Four color morphs of sea cucumber. a. White, b. light green, c. dark green, and d. purple.

coloration and structural coloration (Parker, 2000). Biological coloration is the reflection of pigments produced in the pigment cells on the body surface. Pigment cells are derived from specialized cells known as neural crest cells in the ectoderm. Pigment cells produce different types of pigments, and are classified into different types (melanocyte, xanthophore, erythrophore, iridocyte, and leucophore) depending on the type of pigment they produce (Streelman et al., 2007). Pigment cells can produce one or several kinds of pigment particles, which can form different colors when they are dissolved in the cytoplasm (Liu and Chen, 2008) (Table 1). Melanin is found naturally in a wide range of species from microbes to humans and has two forms: the black-brown eumelanin and the yellow-red pheomelanin (Wakamatsu and Ito, 2002). Pigments play critical roles as antioxidants, anti-viral agents, and photo-protective agents, as well as being involved in camouflage, mimicry, and social communication (Nielsen et al., 2006; Protá and Thomson, 1976; Slominski et al., 2004). Inside pigment cells, melanin is synthesized and stored

in a tissue-specific, lysosome-related, specialized organelle known as the melanosome (Raposo and Marks, 2007). The formation of structural color relies on submicroscopic structures that reflect the light to show different colors. Some animals can change their own body color rapidly to adapt the environment, as a result of rapid movement of pigmented particles. However, the types of pigment cells and the concentrations of pigment particles are the main factors in color differences among individuals (Yu, 1996).

To date, very few studies have focused on color differences in echinoderms, especially the origin and differentiation of their pigment cells and pigments. Here, we investigated the pigment composition and melanin content in white, light-green, dark-green, and purple sea cucumbers. The results provide evidence for the main factors responsible for differences in coloration among the four color morphs, and also provide the foundation for further research on the formation of body color in sea cucumber, *A. japonicas*.

Table 1. Pigments produced in five types of pigment cells and their colors (Liu and Chen, 2008)

| Cell | Pigment | Pigment particle | Pigmentary color |
|--------------|-------------|---------------------|------------------------|
| Melanocyte | melanin | melanin granule | black-brown |
| Erythrophore | carotenoids | pteridine granule | red-orange |
| Erythrophore | pteridine | carotenoid vesicle | red-orange |
| Xanthophore | carotenoids | pteridine granule | yellow-orange |
| Xanthophore | pteridine | carotenoid vesicle | yellow-orange |
| Iridocyst | guanine | reflecting platelet | silver, reddish-purple |
| Leucophore | purine | reflecting platelet | white |

2 Materials and methods

2.1 Animal and sample treatment

All four color morphs (white, light-green, dark-green, and

purple) of *A. japonicus* used in this experiment were collected from Shandong Oriental Ocean Sci-Tech Co. Ltd. (Yantai City, Shandong Province, China). The animals were transferred to the laboratory and held in natural seawater (water temperature 14°C,

salinity 30) for 2 d. All sea cucumbers used in this study were one year old. Thirty individuals of each color morph were selected after acclimatization. The body wall was sampled and immediately frozen in liquid nitrogen.

2.2 Tissue sections for transmission electron microscopy analysis

Anterior body wall samples were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.2–7.4), and the tissues were washed in 0.1 mol/L PBS overnight. The tissues were fixed in 1% (w/v) osmium tetroxide for 1 h at 4°C, washed in 0.1 mol/L PBS, and then dehydrated with graded ethyl alcohol and propylene oxide. Dehydrated tissues were embedded in araldite blocks. Ultrathin sections were cut using an ultramicrotome and collected on copper grids for uranyl acetate staining. Stained sections were observed under a Hitachi 7650 transmission electron microscope (TEM; Hitachi, Tokyo, Japan) (Xu, et al., 2015).

2.3 Melanin analysis

Sea cucumber body wall samples (2 g) were ground into a powder, and then the powder was transferred to a 40-mL glass bottle. Then, 5 mL 1 mol/L K_2CO_3 , 1 mL 5% H_2O_2 , and 5 mL distilled water were added. The mixture was ultrasonically extracted at 80°C for 20 min. After cooling in water, 1 mL 10% (w/v) Na_2SO_3 was added to terminate the reaction, and then the pH of the mixture was adjusted to 1 with 5 mol/L HCl. The supernatant was filtered and extracted twice with 50 mL diethyl ether. The organic phases were combined, vacuum dried, and then redissolved in the mobile phase to a final volume of 40 mL. An aliquot of the sample was filtered through a 0.45- μ m organic membrane filter and then analyzed by HPLC (Brand: THERMO; Model: Ultimate 3000, Place of production: America; Configuration: U3000 automatic injector, four flow high pressure pump, C18 reversed-phase column, pH 2.5–7.5, UV detector). The mobile phase was water and methanol (70:30, v/v) and the flow rate was 1 mL/min. The detection wavelength was 280 nm, and the column temperature was 25°C.

2.4 Astaxanthin analysis

Sea cucumber body wall samples (3 g) were ground into a powder, and then the powder was transferred to a 20-mL glass bottle. Then, 10 mL extractant (trichloromethane–methanol, 1:1, v/v) was added, and the mixture was extracted ultrasonically at 20°C in the dark for 2 h. The mixture was filtered, and then 5 mL extractant was added to the residue and the mixture was re-extracted ultrasonically at 20°C in the dark for 1 h. The mixture was filtered through filter paper, and the residue was washed twice with extractant. Then, the filtrates were combined and diluted with methanol to a final volume of 20 mL. An aliquot of the sample was filtered through a 0.45- μ m organic membrane filter before HPLC analysis. The mobile phase was water–methanol (5:95, v/v) and the flow rate was 1 mL/min. The detection wavelength was 480 nm, and the column temperature was 25°C.

2.5 β -carotene analysis

Sea cucumber body wall samples (1 g) were ground to a powder, and then the powder was transferred to a 40-mL glass bottle. Then, 20 mL extractant (acetone–petroleum ether, 1:1, v/v, containing 0.05% BHT) was added. The mixture was ultrasonically extracted at 40°C in the dark for 2 h, and then filtered. The residue was re-extracted in 10 mL extractant ultrasonically at 20°C in the dark for 1 h, and then filtered through filter paper. The residue was washed three times with extractant. The filtrates

were combined and diluted with acetone to 40 mL. An aliquot of the sample was filtered through a 0.45- μ m organic membrane filter before HPLC analysis. The mobile phase was ethyl acetate–acetonitrile (1:9, v/v) and the flow rate was 1 mL/min. The detection wavelength was 450 nm and the column temperature was 25°C.

2.6 Guanine analysis

Sea cucumber body wall samples (3 g) were ground to a powder and then the powder was transferred to a 40-mL glass bottle. Then, 10 mL extractant (methanol–water, 1:9, v/v) was added and the sample was ultrasonically extracted at 40°C in the dark for 2 h, and then filtered. A further 10 mL extractant was added to the residue, and the sample was re-extracted ultrasonically at 40°C in the dark for 2 h. The sample was then filtered, and the residue was washed twice with the extractant. The filtrates were combined and then diluted with methanol to a final volume of 20 mL. An aliquot of the sample was filtered through a 0.45- μ m organic membrane filter before HPLC analysis. The mobile phase was water and the flow rate was 1 mL/min. The detection wavelength was 250 nm and the column temperature was 25°C.

2.7 Pteric acid analysis

Sea cucumber body wall samples (3 g) were ground to a powder and then the powder was transferred to a 40-mL glass bottle. Then, 10 mL extractant (distilled water) was added, and the pH was adjusted to 3 using 1 mol/L HCl. The sample was then heated to 80–90°C and ultrasonically hydrolyzed for 3 h. After cooling, 10% ammonia was added to adjust the pH to 10, and then the mixture was diluted to 20 mL. After ultrasonic dissolution for 30 min, an aliquot of the sample was filtered through a 0.45- μ m organic membrane filter before HPLC analysis. The mobile phase was water and methanol (95:5, v/v) and the flow rate was 0.6 mL/min. The detection wavelength was 285 nm and the column temperature was 25°C.

2.8 Zeaxanthin and lutein analyses

Sea cucumber body wall samples (3 g) were ground to a powder and then the powder was transferred to a 40-mL glass bottle. Then, 10 mL extractant (methanol) was added, and the sample was ultrasonically extracted at 40°C in the dark for 2 h. After ultrasonic extraction, the sample was filtered, and then 10 mL methanol was added to the residue. The sample was re-extracted ultrasonically at 40°C in the dark for 1 h, and then filtered through filter paper. The residue was washed twice with extractant. Then, the filtrates were combined and diluted with methanol to a final volume of 40 mL. An aliquot of the sample was filtered through a 0.45- μ m organic membrane filter before HPLC analysis. The mobile phase was water and methanol (5:95, v/v) and the flow rate was 1 mL/min. The detection wavelength was 450 nm and the column temperature was 25°C.

2.9 Progesterone analysis

Sea cucumber body wall samples (2 g) were ground to a powder and then the powder was transferred to a 20-mL glass bottle. Then, 10 mL extractant (diethyl ether–methanol, 1:1, v/v) was added, and the mixture was extracted ultrasonically at 40°C in the dark for 2 h. The mixture was then filtered, and 5 mL extractant was added to the residue. The mixture was re-extracted ultrasonically at 40°C in the dark for 2 h, and then filtered through filter paper. The residue was washed twice with extractant. The filtrates were combined and diluted with methanol to a final volume of 20 mL. An aliquot of the sample was filtered

through a 0.45- μm organic membrane filter before HPLC analysis. The mobile phase was water–methanol (30:70, v/v) and the flow rate was 1 mL/min. The detection wavelength was 241 nm and the column temperature was 25°C.

2.10 Lycopene analysis

Sea cucumber body wall samples (1 g) were ground into a powder, and then the powder was transferred to a 40-mL glass bottle. Then, 20 mL extractant (acetone–petroleum ether (1:1 v/v, containing 0.05% BHT) was added. The mixture was extracted ultrasonically at 40°C in the dark for 2 h, and then filtered. Another 10 mL extractant was added to the residue and the mixture was re-extracted ultrasonically at 40°C in the dark for 1 h. The mixture was filtered through filter paper and the residue was washed three times with extractant. The filtrates were combined and diluted to 40 mL with acetone. An aliquot of the sample was filtered through a 0.45- μm organic membrane filter and then analyzed by HPLC. The mobile phase was ethyl acetate–acetonitrile (1:9, v/v) and the flow rate was 1 mL/min. The detection wavelength was 450 nm, and the column temperature was 25°C.

2.11 Statistical analysis

Differences in pigment composition among the colored

groups were detected by one-way analysis of variance (ANOVA), followed by post-hoc analysis using Duncan's multiple range test. Statistical analyses were conducted using SPSS v. 11 (SPSS Inc., Chicago, IL, USA). Differences were considered significant (*) or highly significant (**) at the probability levels of $P < 0.05$ and $P < 0.01$, respectively.

3 Results

3.1 White sea cucumbers contain fewer pigments than other color morphs do

In total, seven biological pigments (melanin, astaxanthin, β -carotene, zeaxanthin, lutein, progesterone, and lycopene) and two structural pigments (guanine and pteric acid) were analyzed in the four color morphs of sea cucumber (Table 2). Only guanine and pteric acid were detected in white sea cucumber. These structural pigments were also present in light-green, dark-green, and purple sea cucumbers. Melanin, astaxanthin, β -carotene, and lutein were detected in all color morphs except for white sea cucumber. Progesterone and lycopene, which are also biological pigments, were not detected in any of the color morphs.

Table 2. Pigment composition and content (mean \pm SD; $\mu\text{g/g}$) in white, light-green, dark-green, and purple sea cucumber

| Pigment | White <i>A. japonicus</i> | Light-green <i>A. japonicus</i> | Dark-green <i>A. japonicus</i> | Purple <i>A. japonicus</i> |
|---------------------------|---------------------------|---------------------------------|--------------------------------|----------------------------|
| Melanin ¹ | not detected | 15.10 \pm 0.70 | 19.96 \pm 0.30 | 44.84 \pm 0.07 |
| Astaxanthin ² | not detected | 10.29 \pm 0.08 | 8.34 \pm 0.16 | 17.20 \pm 0.24 |
| -carotene ³ | not detected | 1.73 \pm 0.03 | 2.43 \pm 0.09 | 3.77 \pm 0.06 |
| Guanine | 39.58 \pm 0.94 | 145.49 \pm 2.33 | 153.31 \pm 0.52 | 184.09 \pm 0.79 |
| Pteric acid | 1.45 \pm 0.04 | 0.33 \pm 0.02 | 0.58 \pm 0.02 | 0.86 \pm 0.03 |
| Zeaxanthin ⁴ | not detected | 5.45 \pm 0.20 | 3.78 \pm 0.02 | 12.79 \pm 0.13 |
| Lutein ⁵ | not detected | 4.90 \pm 0.08 | 5.14 \pm 0.03 | 7.88 \pm 0.06 |
| Progesterone ⁶ | not detected | not detected | not detected | not detected |
| Lycopene ⁷ | not detected | not detected | not detected | not detected |

Note: Limit of detection (LOD) in $\mu\text{g/g}$: 1.08 (1), 0.07 (2), 0.73 (3), 0.46 (4), 0.38 (5), 0.32 (6), and 0.61 (7).

3.2 Pigment concentrations are higher in deeply colored sea cucumbers

Five biological pigments were detected in the body wall of the sea cucumbers. Among them, melanin played the main role in body color formation in the light-green, dark-green, and purple sea cucumbers (15.10, 19.96, and 44.84 $\mu\text{g/g}$, respectively; Table 2, Fig. 2). The melanin content in the purple sea cucumbers was 196.95% higher than that in the light-green ones ($P < 0.05$), and 124.65% higher than that in the dark-green ones ($P < 0.05$). The melanin content also differed significantly between the light-green and dark-green sea cucumbers. Astaxanthin also played an important role in body color formation in the light-green, dark-green, and purple sea cucumbers (10.29, 8.34, and 17.20 $\mu\text{g/g}$, respectively; Table 2, Fig. 2).

Of the two structural pigments, guanine showed the highest concentrations in sea cucumbers (39.58, 145.49, 153.31, and 184.09 $\mu\text{g/g}$ in white, light-green, dark-green, and purple color morphs, respectively; Fig. 3, Table 2).

3.3 Amount of melanin granules secreted by melanosomes outside cell

The TEM observations revealed that white sea cucumbers had the lowest density of epidermal melanocytes in the body

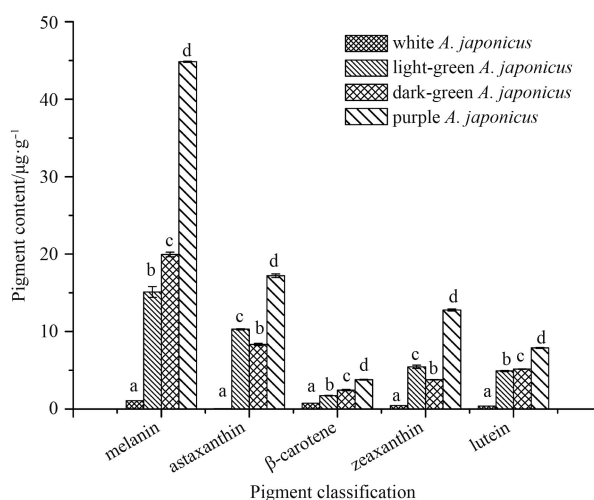


Fig. 2. Concentrations of five main biological colors in white, light-green, dark-green, and purple sea cucumbers. Though melanin, astaxanthin, β -carotene, zeaxanthin, and lutein were not detected in white sea cucumber, the values of limit detection rather than zero were used in making this graph for better comparison.

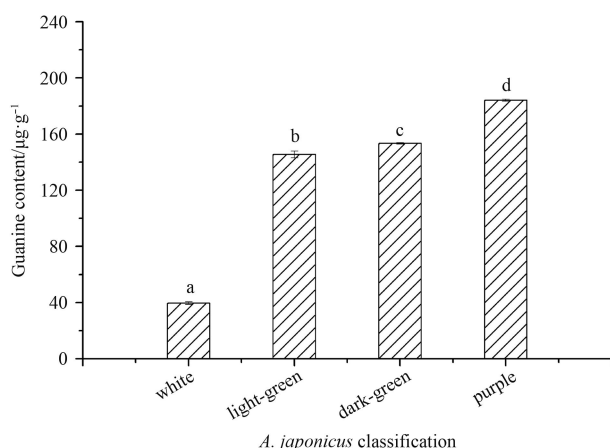


Fig. 3. Guanine content in white, light-green, dark-green, and purple sea cucumbers.

wall, and their melanocytes contained non-pigmented pre-melanosomes. As the body color deepened among the four color morphs, the density of melanosomes and melanin granules increased. In the body wall of dark-green and purple sea cucumbers, melanin granules were secreted out of the cells, especially in the purple morph. Compared with white sea cucumber, light-green sea cucumber had more mature melanosomes, although no melanin granules were secreted outside the cells (Fig. 4).

4 Discussion

4.1 Pigments are the decisive factor in body color

Melanin is believed to play a photoprotective role, and has also been shown to reduce lipoperoxidation in pigmented tissue (Sarna et al., 2003; Scalia et al., 1990). In a variety of model systems, melanin has been shown to scavenge reactive free radicals (Rózanowska et al., 1999), quench electronically excited states (Sarna et al., 1985), and sequester redox-active metal ions (Zaręba et al., 1995). Astaxanthin is a strong coloring agent and a potent antioxidant. Its strong antioxidant activity points to its po-

tential to treat several health conditions (Guerin et al., 2003). β -carotene is an important source of vitamin A and it also has antioxidant properties, enhancing immunity (Zhu et al., 2005). Guanine is a structural color that is produced in iridocytes. It forms crystal plates that reflect light (Levy-Lior et al., 2010). Zeaxanthin and lutein are carotenoids that are distributed widely in nature, and these pigments are attracting increasing attention because of their antioxidant activities and physiological functions (Lu and Yao, 2003).

Differently colored sea cucumbers may have different nutrient composition and growth, even when they are grown under the same conditions. The purple *A. japonicas* may be a suitable type to culture under high-salinity conditions, whereas the white morph requires more stable salinity (Bai et al., 2015). Furthermore, growth and composition analyses showed that the mean body wall production rate and ash content is higher in the red morph than in the green morph, while the crude protein, crude fat, and carbohydrate contents do not differ between the red and green morphs (Jiang et al., 2013). Differences in pigment composition and contents can explain the different color morphs of sea cucumber. Our results indicated that the concentrations of all of the analyzed pigments, except for pterioic acid, are higher in the purple morph than in the other color morphs. These high pigment concentrations may be a factor in the greater adaptability of the purple sea cucumbers. Similarly, the low concentrations of pigments in white sea cucumber may explain its sensitivity to adverse environmental conditions.

4.2 Melanin is the key pigment in the formation of body color

Red sea cucumbers were reported to inhabit offshore gravel beds, while the green and black ones were reported to inhabit the sand muddy bottom inshore (Nishimura, 1995). Sea cucumbers are divided into black, green and red morphs in Japan, but into green, white and purple morphs in China. The color of purple sea cucumber is similar to that of red sea cucumber. In the process of breeding, we have observed that purple sea cucumbers are more sensitive to light. This may be because the purple and red sea cucumbers contain more melanin than do the other color morphs.

Melanosomes are lysosome-related organelles in which

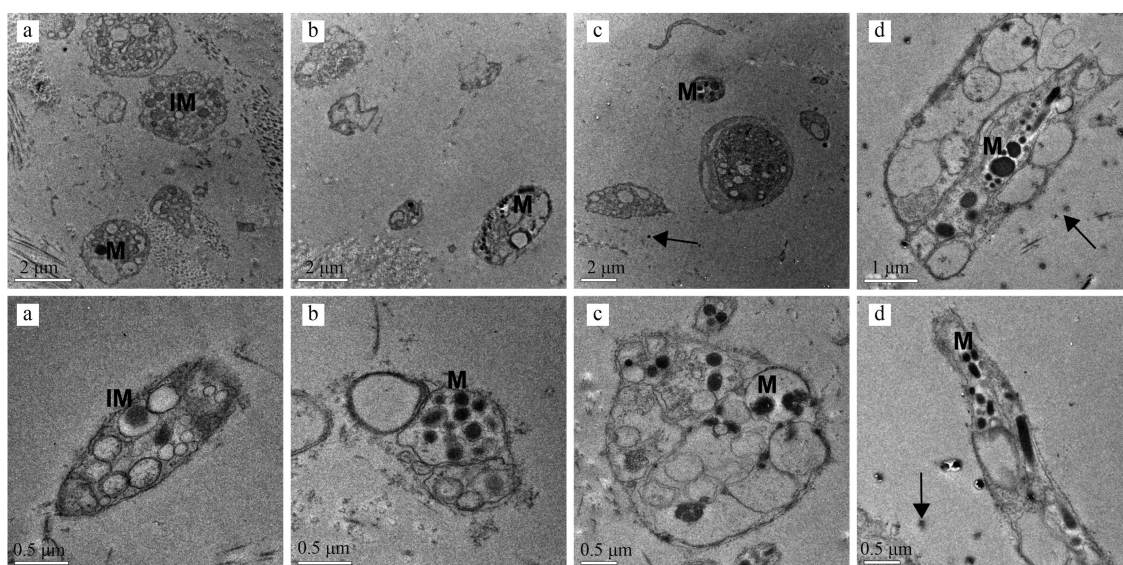


Fig. 4. Transmission electron micrographs of white (a), light-green (b), dark-green (c), and purple (d) sea cucumbers. IM represents immature melanosomes, M melanosomes, and arrows melanins secreted by melanosomes.

melanins are synthesized and stored. Early stage melanosomes are characterized morphologically by intraluminal fibrils, upon which melanins are deposited in later stages (Hurbain et al., 2008). Melanosomes function in secretion and phagocytosis (Hurbain et al., 2008; Schiaffino, 2010). During formation, melanosomes go through a series of morphologically defined stages. Mature electron-dense Stage IV melanosomes contain tightly packed melanins, and develop from specialized nonpigmented immature Stages I and II melanosomes and partially pigmented Stage III melanosomes. Each stage represents a distinct biogenetic intermediate (Seiji et al., 1963). Melanosome development involves four steps. Stage I corresponds to the early matrix organization. Stage II eumelanosomes contain an organized matrix but lack melanin, while Stage II pheomelanosomes contain melanin. Melanin deposition occurs at Stage III. In Stage IV, melanosomes are fully melanized (Slominski et al., 2004). Reductions in the number, structure, and/or function of melanosomes in melanocytes and in the retinal pigment epithelium (RPE) lead to albinism. This term comprises a heterogeneous group of diseases characterized by variable hypopigmentation of the skin (evidently affected in oculocutaneous albinism, but not or only mildly affected in ocular albinism) and severe developmental defects of the eyes, including foveal hypoplasia and misrouting of the optic tracts at the chiasm, which are secondary to RPE hypopigmentation (King et al., 2001). In our study, we found that white sea cucumbers had the lowest density of melanosomes, contained non-pigmented pre-melanosomes, and did not secrete melanin granules outside of cells. Consequently, melanin could not be detected in body wall of white sea cucumbers. The light-green sea cucumbers contained mature melanosomes, but they did not secrete melanin granules. The dark-green sea cucumbers contained mature melanosomes that secreted melanin granules into the body wall. Among all of the color morphs, purple sea cucumbers contained the most mature melanosomes and had the largest amount of melanin granules secreted out of melanosomes. These results indicate that the color of sea cucumber is mainly related to the density of mature melanosomes and the amount of melanin granules secreted out of melanosomes.

Acknowledgements

The authors thank all members of the research department of Shandong Oriental Ocean Sci-Tech Co., Ltd. The authors also thank the two anonymous reviewers for their professional revision of the manuscript.

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