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Epidermal Melanophores of a Freshwater Goby, *Odontobutis obscura* : Regulation of Movements and Ultrastructure

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In the integument of a freshwater goby, *Odontobutis obscura*, there exist two kinds of melanophores : epidermal and dermal melanophores. The epidermal melanophores are located in the intermediate layer of the epidermis and possess thin perikarya containing round nuclei, and slender, ramified processes that frequently connect with adjacent processes to form a network. The epidermal melanophores are innervated by the adrenergic nerves. The nervous stimulation induced aggregation of melanosomes in the melanophores through alpha adrenoceptors on their cell membranes. The mechanisms were the same as those of the dermal melanophores. The fine structural features of these cells were very similar to those of the dermal melanopores described previously in various fish species. The epidermal cells loaded with melanin granules were not observed ; the integument of this species could not function as an epidermal melanin unit. Macrophages may be responsible for the catabolism of the pigment since phagocytized melanosomes and melanosome-containing lysosomes were found frequently in neighborhood of the epidermal melanophores.

INTRODUCTION

Although dermal melanophores are almost always evident in poikilotherms, the occurrence of epidermal melanophores among the animals is highly variable. In reptiles, the presence of epidermal melanophores seems the rule than the exception. Among amphibians, these cells are almost always found at some stage of embryonic, larval or adult development. While, their occurrence in fishes is rather infrequent, literature dealing with the epidermal pigment cells is scanty, as compared with the much greater amount of dermal ones (Bagnara and Hadley, 1973).

On fish epidermal melanophores, in addition to light microscopic observations (cf. Parker, 1948 ; Khokhar, 1971 ; Burton, 1975, 1978), some ultrastructural observations with special consideration with their functions have been reported (Imaki and Chavin, 1975 a, b ; Lamer and Chavin, 1975 ; Miyashita and Fujii, 1980). Their physiological researches are restricted to those of the siluroid catfish, *Parasilurus asotus* (Fujii and Miyashita, 1976, 1978, 1982).

Thus, on fish epidermal melanophores, much important problems concerning the significance or functions in relation to their ultrastructure, the regulation mechanisms and the development remain to be settled. At present, much accumulation on the occurrence of epidermal melanophores in various species of fishes also will be essential.

During the course of physiological studies on fish chromatophores, we have found that the integument of a freshwater goby, *Odontobutis obscura*, contains epidermal melanophores which are motile (Iga and Matsuno, 1984 unpublished, 1986). In the present work, therefore,

regulation of the movements and the ultrastructure on the epidermal malanophores of this fish species were examined and their possible function was discussed.

MATERIALS AND METHODS

A freshwater goby, *Odontobutis obscura*, of body length 6 to 11 cm, was used for this study. Scales isolated from the dorso-lateral trunk of the fish were employed as experimental preparations. In some experiments, scales from chemically sympathectomized fish were employed as denervated preparations. The denervation was achieved by intraperitoneal injection of 80 μg/g body wt. of 6-hydroxydopamine hydrobromide (Sigma Chemical, St. Louis), as described elsewhere (Iga et al., 1978).

For light microscopic observations, an isolated scale was attached, epidermal side down, under a cover slip, which was mounted in a small chamber filled with a physiological saline solution for freshwater teleosts, which had the following composition (mM) : NaCl, 128 ; KCl, 1.8 : $CaCl_2$, 2.8 ; Tris-HCl buffer, 5.0 (pH, 7.2).

Responses of the dermal melanophores were recorded by the photoelectric method described previously (Iga, 1983), while, in the epidermal melanophores, the magnitude of each response was calculated as the change of the visible length of a given process of a cell, because in the electrical recording method responses of the epidermal melanophores were evidently affected by those of the dermal ones located below the epidermal melanophores.

For electron microscopic observations,the scales were fixed in a solution that contained 1.5% paraformaldehyde and 1.5% gultaraldehyde in 100 mM cacodylate buffer (pH 7.3) for 90 min at room temperature, rinsed with 100 mM cacodylate buffer for 30 min, and then postfixed with 1% OsO_4 in 100 mM phosphate buffer (pH 7.3) for 2 hr at 4°C. Samples were dehydrated through a graded series of ethanol, immersed in n-butyl-glycidyl-ether, and embedded in epoxy resin. Sections were cut with a glass or a diamond knife. After staining with uranyl acetate and lead citrate, the sections were observed under a JEM 100C electron microscope.

All the experiments were carried out at a room temperature between 20 and 25°C.

RESULTS AND DISCUSSION

Gross morphology of epidermal melanophores

Scales removed from the living animals were observed in physiological solution. Two morphologically distinct types of melanophores were identified in different focus planes (Fig. 1). One occurs in a superficial layer and the other more deeply below the superficial layer. As will be described in the latter section, electron microscopy disclosed that these melanophores were epidermal and dermal melanophores, respectively. The dermis contained xanthophores and iridophores in addition to the melanophores as a chromatophore component (Iga and Matsuno, 1986).

In younger fish the frequency of the epidermal melanophores was low. The cells increased

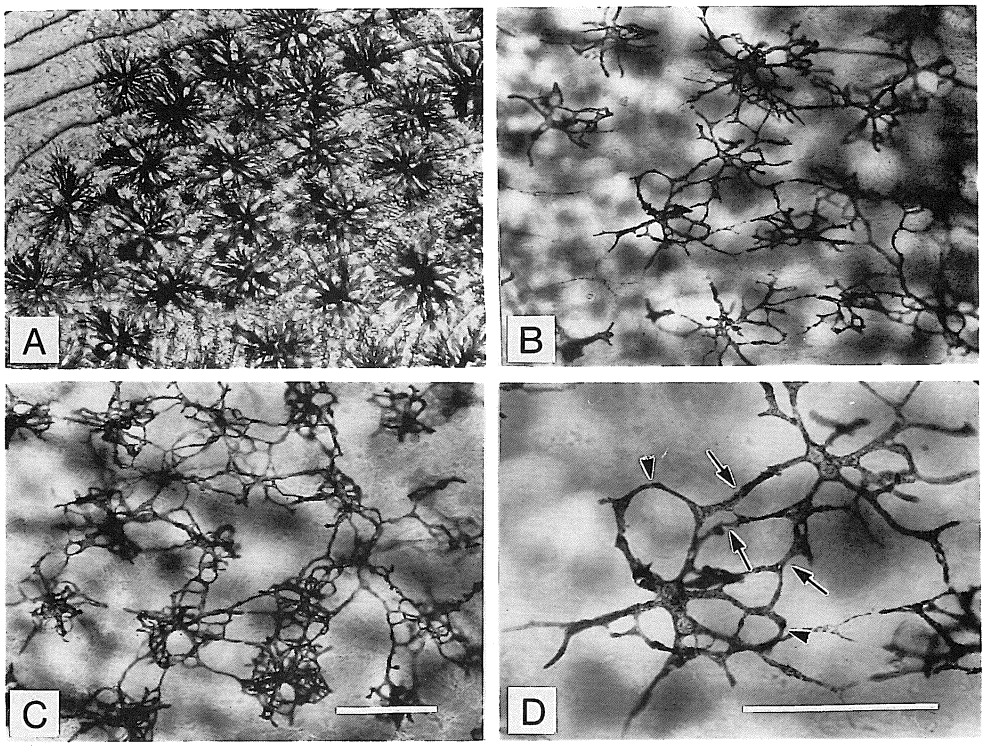


Fig.1. Photomicrogarphs of melanophores in scales isolated from a freshwater goby, *Odontobutis obscura*. A : Dermal melanophores, B and C : epidermal melanophores of morphologically two different shapes, D : High magnification view of epidermal melanophores. Arrows indicate the connection between epidermal melanophores with their cellular processes. Arrow heads indicate the connection between adjacent processes of an epidermal melanophore. Scale bars=100 μm.

gradually in number as fish grew larger, with a concomitant increase in the size of the cells and the number of dendritic processes, as reported in a catfish, *P. asotus*, by Miyashita and Fujii (1980).

The epidermal melanophores are larger than the dermal ones in diameter. They possess thin cell centers where the nuclei are located. Relatively few, long fine processes arise from the centers to ramify in all directions (Fig.1B). The cellular processes extend frequently both horizontally and vertically to anastomose, as the result these melanophores assume basket-like shapes (Fig.1C). Furthermore, the processes between neighboring epidermal melanophores appear to join with one another to form a lace-work indicating a possible continuity as those described in *Ictalurus melas* (Khokhar, 1971) (Fig.1D).

The processes of the dermal melanophores, on the other hand, are thick and short and mostly confined to one plane, i. e., parallel to the skin surface and the processes of adjoining melanophores remain separate from one another (Fig.1A).

Responses of epidermal melanophores

It is well known that K^+ ion causes an aggregation of melanosomes of fish melanophores by acting on the chromatic nerve varicosities to release the transmitter. To inspect a possibility of the nervous regulation of the epidermal melanophores, responses of the melanophores to K^+ ion were examined.

The epidermal melanophores remained dispersed their melanosomes in the saline solution. When immersed in a high-K solution (a mixture of equal volumes of an isotonic solution of KCl and physiological saline), the melanophores responded with a rapid aggregation of melanosomes (Fig.2), and returned to the initial dispersed state following reimmersion into the physiological saline. Figure 3 illustrates the responses of the dermal

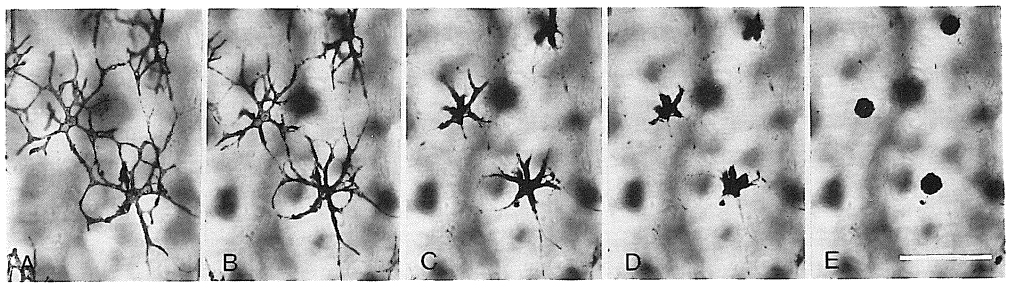


Fig.2. Photomicrogarphs showing the responeses of epidermal melanophores to high-K^+ solution. A : in physiological saline, B, C, D and E : 1, 2, 3 and 5 min after the application of high-K^+ solution, respectively. Being out of focus, dermal melanophores are also visible in these prints. Scale bar= 100 μm.

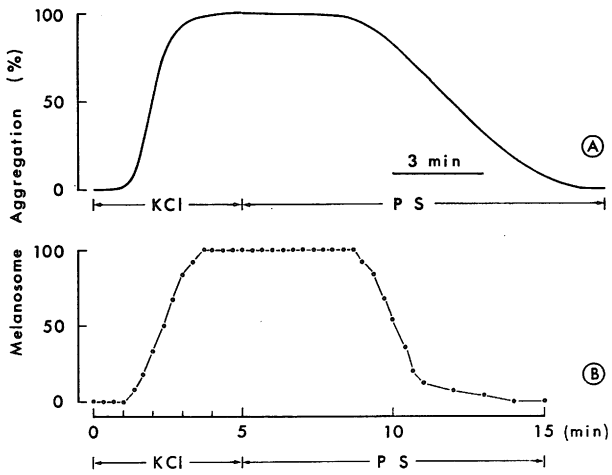


Fig.3. Typical recordings of the responses of dermal (A) and epidermal (B) melanophores to high-K^+ solution. The responeses of the dermal melanophores are photoelectrically recorded, while those of the epidermal melanophores are shown by changes of visible length of a given process of an epidermal melanophore.

(A) and epidermal (B) melanophores to K^+ ion. As shown in this figure, the rate of motile response was similar between the epidermal and dermal melanophores, although the methods recording in this figure were different in two kinds of melanophores.

The K-induced response suggested that the epidermal melanophores were regulated by the nerves. This was confirmed by the denervation experiment in which scales from 6-OH-dopaminized animals were used. The aggregation response to K^+ ion did not occur in both epidermal and dermal melanophores in scales from these animals. Thus, it was concluded that the epidermal melanophores also were regulated by the nerves as the dermal melanophores were done.

Next, effects of adrenergic antagonists to the K^+-induced response were examined in the epidermal melanophores. Phenoxybenzamine effectively blocked the aggregation response induced by K^+ ion, while propranolol was not effective (data not shown). The same results were true in the dermal melanophores. These results show that the nerves controlling the epidermal melanophores are adrenergic and the transmitter released from the nerves, supposed as noradrenaline, acts on alpha type of adrenoceptors on the cell membranes of epidermal melanophores to arouse pigment aggregation. These mechanisms were the same as those in the dermal melanophores.

A lillte is known about receptor mechanisms of epidermal melanophores. Epidermal melanophores of a catfish, *P. asotus*, responded to electrical stimulation or MSH as the same as dermal melanophores did (Fujii and Miyashita, 1976, 1982). Burton and O'Doriscoll (1992) stated the epidermal melanophores in the winter flounder *Pseudopleuronectes americanus* were more sensitive than dermal melanophores to noradrenaline. Epidermal melanophores in the lungfish, *Lepidosiren paradoxa*, which did not possess the nerve supply, were more responsive to MCH and melatonin than dermal cells (Visconti and Castrucci, 1993).

Ultrastructure of epidermal melanophores

Figure 4 is an electron micrograph showing the general structural composition of the integument of the freshwater goby, *Odontobutis obscura*, which consists of two main compositions, the epidermis and dermis bordered by the basement membrane from the epidermis.

The epidermis, in the present material used, is approximately 40 μm thick and consists of 5 to 6 layers of epidermal cells. In the upper layer of the epidermis, there exist mucous cells which contain basally located nuclei and are characterized with their inclusion, electron dense granules, or sometimes electron opaque vacuoles, in the cytoplasm. The epidermal melanophores occur in the middle layer of the epidermis, just over the columnar cells which occupy the basal layer of the epidermis. Frequently, cells appearing to be the epidermal macrophages as judged by their heterogenous cytoplasmic contents occur among the epidermal cells.

The main cellular compositions of the dermis are dermal chromatophores and blood vessels. The dermal chromatophores, that is, melanophores, xanthophores and iridophores which are motile, are abundant. Their ultrastructure has previously been described (Iga and

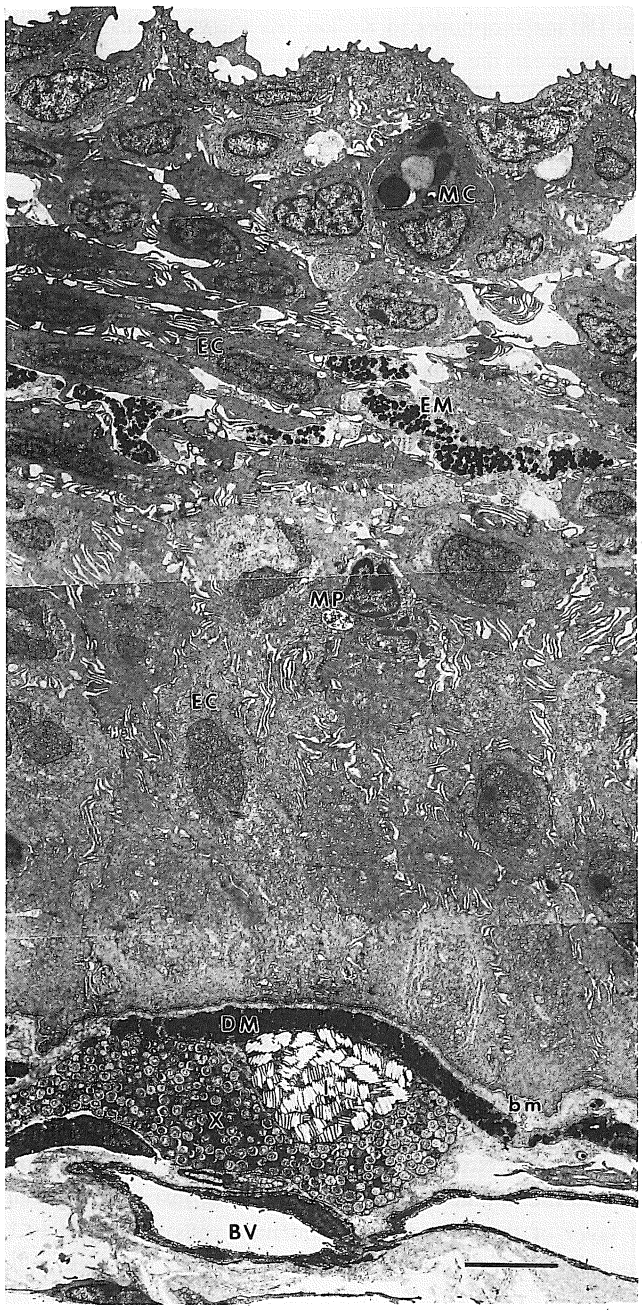


Fig.4. Electron micrograph of vertical section through the integument of a freshwater goby, *Odontobutis obscura*. The epidermis contains epidermal cells (EC), epidermal melanophores (EM), mucous cells (NC) and macrophages (MP), separated from the dermis by the basement membrane (bm). The dermal chromatophores, a melanophore (DM), an iridophore (I) and a xanthophore (X), lie under the basement membrane. BV : blood vessel. Scale bar=5 μm.

Matsuno, 1986 ; Matsuno and Iga, 1989).

Two kinds of epidermal melanophores were observed. The cells of the first type contain electron-dense, spherical or oval pigment granules, melanosomes. In addition to melanosomes, the cells include nuclei, endoplasmic reticulum, in particular smooth surface ones, ribosomes, Golgi apparatus and centrioles (Figs 5 and 6). Microtubules are also common inclusion (Fig.7). The cytoplasm is bounded by a rather smooth cell membrane devoid of specialized structure such as desmosomes, which are prominently observed on the cell membranes of the epidermal cells (Fig.5). These ultrastructural features of the epidermal melanophores are foundamentally similar to those of the dermal ones (Iga and Matsuno, 1986 ; Matsuno and Iga, 1989) and also those of epidermal melanophores from three lungfish, *Lepidosiren, Protopterus* and *Neoceratodus* (Imaki and Chavin, 1975 a, b) a coelacanth, *Latimeria* (Lamer and Chavin, 1975) and a catfish, *Parasilurus* (Miyashita and Fujii, 1980).

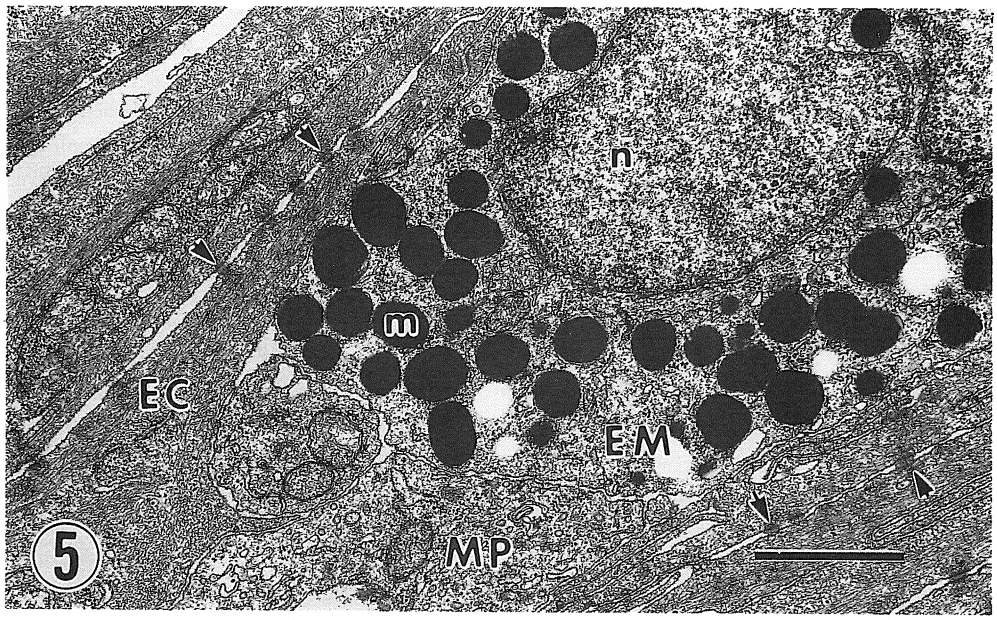
The melanophores of the other type include predominantly premature forms of melanosomes, i. e., premelanosomes. These premelanosomes exhibit a particular internal structure that consists of numerous fibrillar elements composed of a series of fine granules oriented parallel to the major axis of the granule in longitudinal section (Fig.8). Frequently, the cells containing premelanosomes appeared in neighborhood of the matured epidermal melanophores, as reported in *Parasilurus* catfish by Miyashita and Fujii (1980).

In the present observation, the process of melanosome formation was not determined, the detail remains unsettled, especially in relation to the problem on occurrence of the melanophores in the epidermis.

We could not observe the epidermal cells, keratinocytes, loaded with melanosomes, suggesting that the epidermal melanophores could not possess cytocrine activity. Thus, the epidermal melanin unit known in higher vertebrates (Hadley and Quevedo, 1966) is absent in the integument of the present material. The similar observations have been made in the integument of two lungfish, *Lepidosiren* and *Protopterus* (Imaki and Chavin, 1975 a, b) and a catfish *Parasilurus* (Miyashita and Fujii, 1980). While, in a lungfish, *Neoceratodus* (Imaki and Chavin, 1975 a) and a coelacanth, *Latimeria* (Lamer and Chavin, 1975), the existence of the epidermal melanin unit was confirmed.

Macrophages were often found in the epidermis, mostly in the neighborhood of the epidermal melanophores (Fig.5). Phagocytized melanosomes, phagosomes, or melanin-including secondary lysosomes were frequently observed in their cytopasm (Fig.9). These observations suggest that these phagocytes are apparently responsible for the degradation of the pigmentary material.

In the light microscopic observation, we could always detect close connections with the cellular processes between the epidermal melanophores, or anastomosis of the processes in a single cell. Figure 10 is an electron micrograph showing the structure like intracellular bridge (arrows). Although the cell membranes are very closely attached, there is no evidence that indicates the actual cytoplasmic connection between the epidermal melanophores. Observations of repeated responeses of the melanophores to K^+ ion revealed that the direction of melanosome-movement was constant around the sites where close connection with the cellular



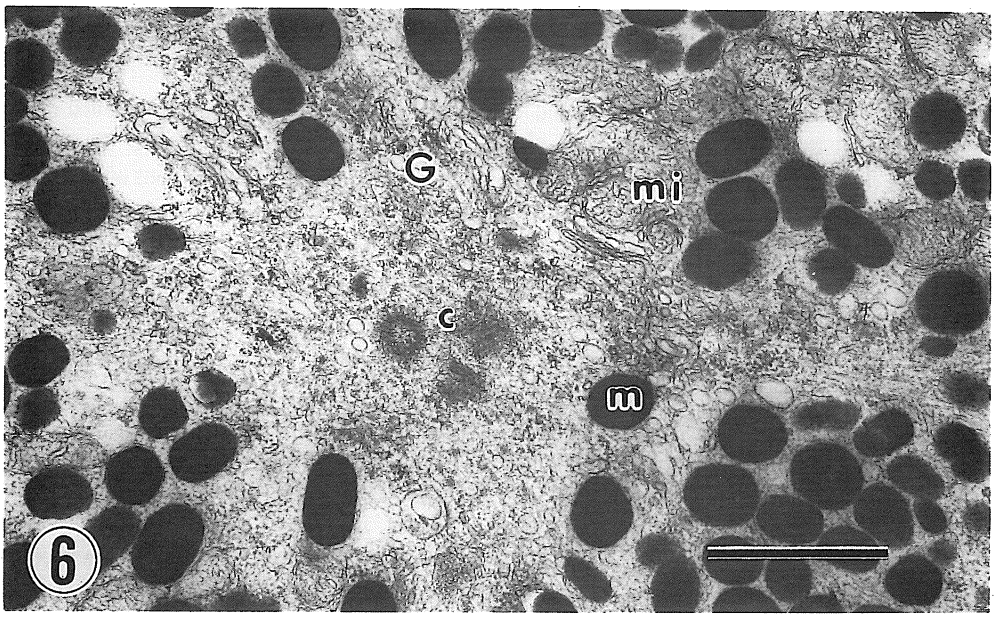
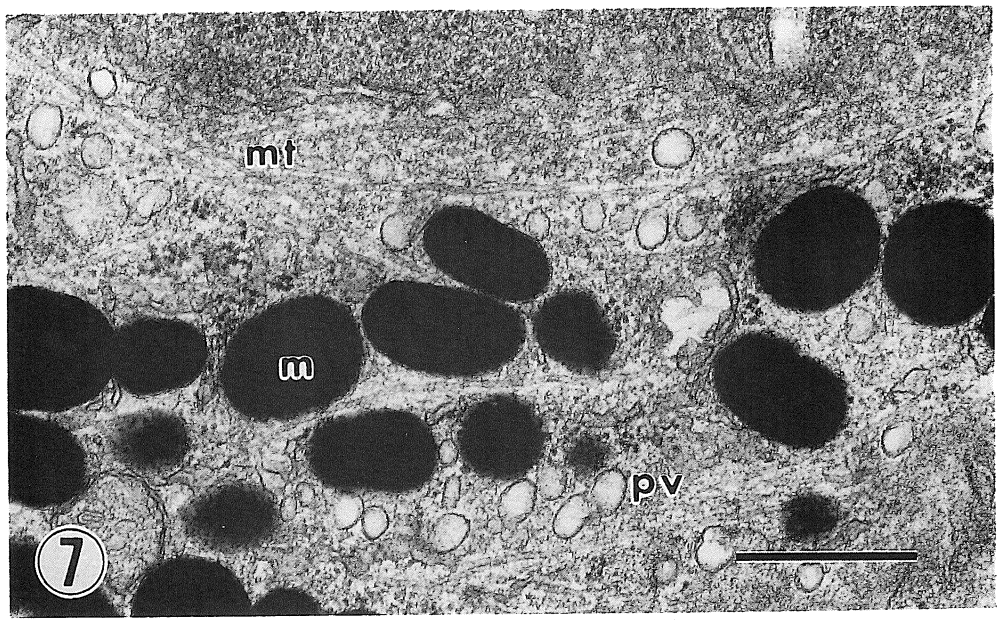


Fig.5. Electron micrograph showing an epidermal melanophore (EM) surrounded by epidermal cells (keratinocytes) (EC). MP : macrophage, n : nucleus, m : melanosome. Arrow heads indicate desmosomes between epidermal cells. Scale bar=1 μm.

Fig.6. Electron micrograph showing the central part of an epidermal melanophore. c : centriole, G : Golgi apparatus, m : melanosome, mi : mitochondria: Scale bar=1 μm.



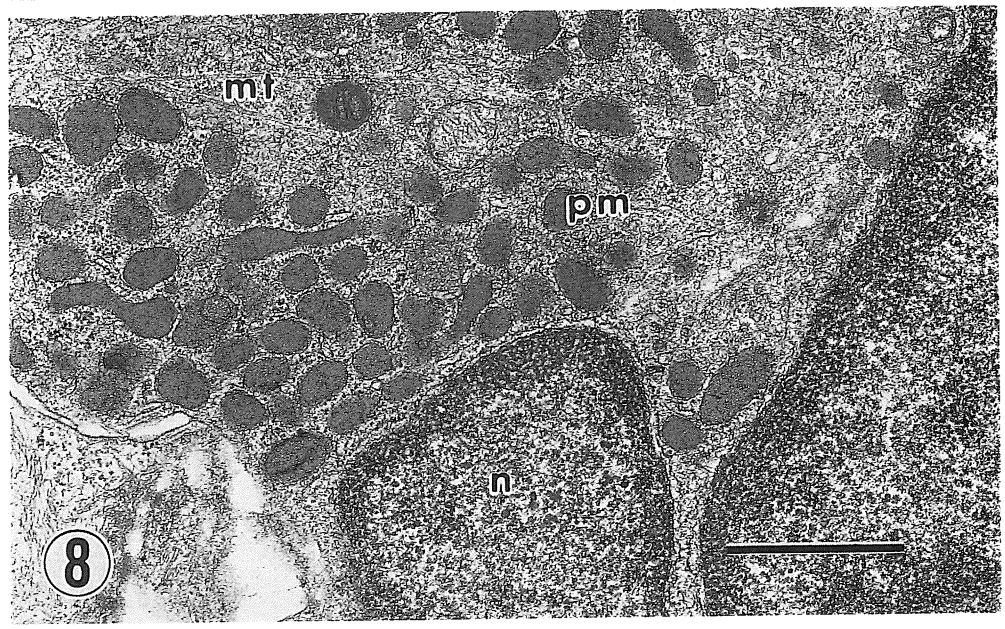


Fig.7. Electron micrograph showing the basal part of a process of an epidermal melanophore. m : melanosome, mt : microtubule, pv : pinocytotic vesicle. Scale bar=0.5 μm.

Fig.8. Electron micrograph showing premelanosome-containing melanophore. mt : microtubule, n : nucleus, pm : premelanosome, Scale bar=1 μm.

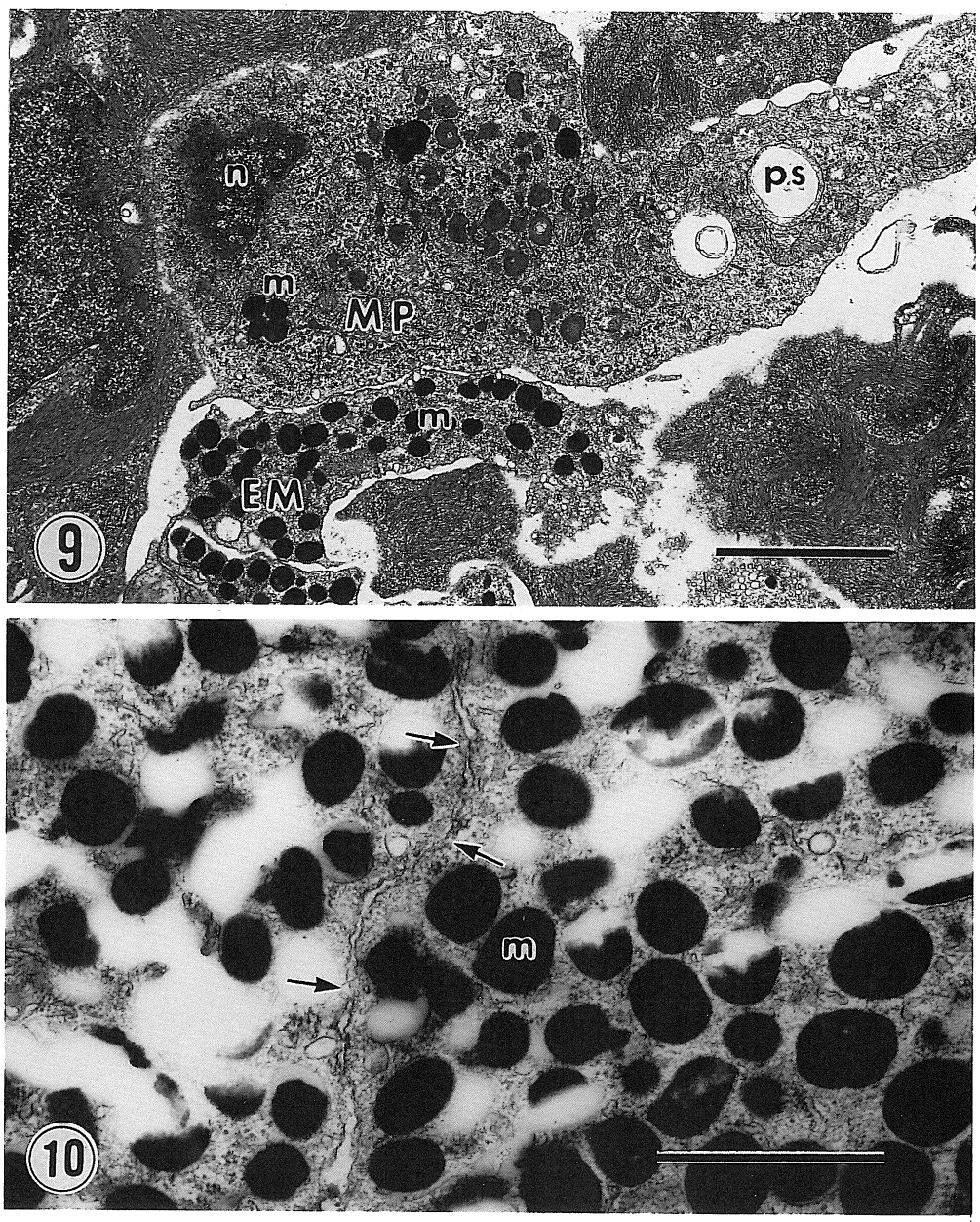


Fig.9. Electron micrograph showing a macrophage in neighborhood of an epidermal melanophore. ME : epidermal melanophore, MP : macrophage, m : melanosome, n : nucleus, ps : phagosome. Scale bar=3 μm.

Fig.10. Electron micrograph showing close connections between epidermal melanophores. m : melanosome. Arrows indicated the sites where the cellular processes are closely attached. Scale bar=1 μm.

processes were seen.

In the present work, it was shown that *Odontobutis* epidermal melanophores were capable of responding rapidly to nervous stimulation and their response characteristics were comparable with those of the dermal melanophores. The epidermal melanophores in this fish species may contribute complementally to physiological color changes, which are mainly due to the activity of dermal melanophores.

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