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Process of Pigment Cell Differentiation in Skin on the Left and Right Sides of the Japanese Flounder, *Paralichthys olivaceus*, during Metamorphosis

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Abstract Two groups of larvae of the Japanese flounder, *Paralichthys olivaceus*, were reared in the laboratory. The survivors of the first group (normal) showed normal pigmentation, and the second group (albinic) exhibited nearly complete pseudoalbinism after metamorphosis. The process of pigment cell differentiation on the left and right sides was observed mainly by transmission electron microscope (TEM) in relation to metamorphosis.

In the normal group, chromatoblasts in the left side skin differentiated successfully, but those in the right side skin showed shrinkage and collapse during metamorphosis. Mucus cells are known as typical cells of ocular side skin in flatfish. The ratio of mucus cell density (left side/right side) increased from the onset of metamorphosis. These results suggest, some components of skin changed asymmetrically in process of metamorphosis before differences in fine structures of chromatoblasts were detected between the left and right sides of the normal group. However, in the albinic group, the same process of chromatoblast collapse occurred on the left and right sides, and there was no change in the ratio of mucus cell density during metamorphosis.

Flatfish larvae have bilateral symmetry, but after metamorphosis juveniles have many asymmetrical features such as eye position, shape of scales, and body color. The pattern formation of pigment cells has recently become an important aspect of pigment cell research. Several hypotheses have been proposed to explain these phenomena (MacMillan, 1976; Tucker and Erickson, 1986; Fukuzawa and Ide, 1986; Yasutomi, 1987). However there are many inexplicable things in the actual phenomena. Moreover, there is currently little knowledge about the asymmetrical pattern of formation of pigment cells in flatfish.

Pigment cells are known to differentiate from neural crest cells, which have multidifferentiational abilities through chromatoblasts (Weston, 1970). Larvae of the Japanese flounder, *Paralichthys olivaceus*, have large melanophores (larval type melanophores) symmetrically on both sides. At the climax of metamorphosis, small (adult type) melanophores appear only in the skin of the left (ocular) side. Xanthophores and iridophores also are rapidly formed on the ocular side at this time. As a result of these changes, the ocular side has a distinctive pigmentation pattern. No difference in melanoblast distribution has been detected between on the left and right sides at the onset of metamorphosis (Seikai et al., 1987). These results strongly suggest that differentiation and proliferation of melanoblasts occurs on the left (future ocular) side, and that inhibition of these processes occurs on the right (future blind) side during metamorphosis.

In contrast to natural populations of flatfish, hatchery-reared flatfish frequently show a high proportion of pseudoalbinism lacking pigmentation on the ocular side (Shelbourn, 1974). This abnormality is one of the major problems in many hatcheries in Japan. Pigment cell distribution in pseudoalbinic skin resembles that of the blind side of normal flatfish, therefore investigation into the process of asymmetrical body color formation should be undertaken to resolve this problem.

The objects of this study are to record the process of differentiation of pigment cells in the skin on the left and right sides during larval metamorphosis, and to investigate the process of pigment cell differentiation in cases of pseudoalbinism.

Materials and methods

Determination of larval development

Minami (1982) defined the developmental stages

of flounder larvae and juveniles as stages A to I. In addition to this classification, metamorphosis of flounder larvae was appraised by morphological characters, especially eye migration and swimming behavior. The developmental stages A-C, D-E, F-H, and I represented pre-metamorphosis, onset of metamorphosis, mid-metamorphosis, and climax metamorphosis, respectively. During pre-metamorphosis, the swimming posture of larvae was as usual for fishes. From the onset of metamorphosis, larvae began to swim with a slight incline to the right side. During metamorphosis, right side eye movement became to be obvious.

Rearing and sampling of flounder larvae

Brood stocks were reared in captivity and spawned naturally at the Miyazu Station of Japan Sea-Farming Association, Miyazu City, Kyoto Prefecture. Fertilized eggs were transported to the Fisheries Research Station of Kyoto University, and then hatched in a 500 *l* plastic tank. Stocking densities of eggs were 20,000 per tank, and hatching rate in excess of 90%. Larval flounder were fed exclusively on the rotifer, *Brachionus plicatilis*, for 10 days after hatching. Two thousand 10 day larvae (B stage: pre-metamorphosis, about 5.5 mm TL) were divided into two equal groups and placed in 100 *l* tanks. One group was labeled "normal", and another "albinic". Survivors of the normal group showed normal pigmentation at the climax metamorphosis, whereas those of the albinic showed nearly complete pseudo-albinism at the climax metamorphosis. The former *group* was fed on wild zooplankton (Seikai, 1985b), and the latter group on newly hatched *Artemia* nauplii of Brazilian strain and rotifers (Seiaki, 1985a). Rearing conditions were otherwise identical (Seikai, 1985a). Several larvae at each developmental stage later than B-stage were selected from the two groups for this study. Larval rearing continued for 40–45 days, most of the survivors having completed metamorphosis during this period.

Larger juveniles (30–50 mm TL), from different rearing stock or wild sources, which showed normal pigmentation, were also used in the present study.

Electron microscopic study

Three to five larvae at each developmental stage from the two groups were used for electron microscopic study. The larvae were fixed at 4°C for 2–4 hours in 2.5% glutaraldehyde with 0.1 M Na-cacodilate buffer containing 2.3 g/*l* NaCl. After completely rinsing with 0.1 M Na-cacodylate buffer, a second fixation was made with 1% OsO_4 in 0.1 M Na-cacodilate buffer for 1–2 hours. The fixed samples were dehydrated with ethanol and propylene oxide, and then embedded in epoxy resine (Epon 812 or Supper) (Luft, 1961). Transverse ultra thin sections of dorsal skin, at the posterior part of the trunk, were taken from both left and right sides by ultramicrotome (LKB 8800). These sections, which were stained with uranyl acetate (Stempak and Ward, 1964) and lead citrate (Karnovsky, 1961), were observed by transmission electron microscope (TEM) (JEOL, 100 SX-1). Some of the larvae examined were treated with dopa assay (Laidlaw, 1932) before the first fixation, and then processed following the same procedure.

Identification of melanoblasts was made by finding cells which contained dopa assay positive vehicles. The relative positions between the melanoblasts and other surrounding cells, and the fine structures of the melanoblasts were confirmed. In samples without treatment with Dopa assay, melanoblasts were identified by their relative positions and fine structures. In conjunction with TEM observations, light microscopic observations were also made after fixation with Bouin's solution, paraffin embedding, and hematoxylin-eosin staining.

Quantitative analysis of mucus cell density in larval skin

Three to five individuals of each stage from pre-metamorphosis until climax metamorphosis were sampled from normal and albinic groups. A normally pigmented wild Japanese flounder (326 mm TL) was also used to observe the distribution of mucus cells. Continuous transverse sections of 4 μm thickness were made, and then stained with hematoxylin and eosin. In each individual, ten sections from the posterior part of the trunk were chosen, and the total number of mucus cells on each side skin of ten sections was counted. Finally, the ratios of mucus cell densities between the left and right sides were found, and the mean values and standard deviations calculated for three selected individuals with good sections at each developmental stage for both normal and albinic groups.

Results

Larval rearing and pigmentation

The supply of wild zooplankton fluctuated daily in composition and quantity during larval rearing, occasionally being insufficient for the metabolic requirements of the larvae. Hence, survival rates of

the normal group were around 28%, significantly lower than those of the albinic group (70–80%). At the end of rearing, all surviving juveniles in the normal group showed normal pigmentation, and those in the albinic group exhibited nearly complete pseudoalbinism. Therefore, larvae in each group were considered to be on the paths expected for normal pigmentation and nearly complete pseudoalbinism before the climax metamorphosis.

Larval skin structure and pigment cell differentiation in the normal group

The structural changes of larval and juvenile skin in the normal group with photomicroscopic and TEM observation were as follows. The epidermis of the left and right sides at pre-metamorphosis consisted of an epidermal layer of one or two cells. The dermis had underveloped collagenous lamella. No differences in construction were observed between the skin of the left and right sides.

At the onset of metamorphosis (D-E stages), collagenous lamella became densely packed. During second half of mid-metamorphosis, larval type melanophores on the left side dispersed more than those on the right side.

After climax metamorphosis, the skin became thicker, and scales began to form in the collagenous layer in the dermis on both sides. On the left side, dense melanophores began to be observed just under the epidermis along with those already present under the collagenous layer. However, the upper melanophores were not observed in the skin on the right side. These features are shown in Fig. 1, a photomicrograph of juvenile skin just after climax metamorphosis.

TEM observation revealed that larval epidermis consisted of epidermal cells, mucus cells, and chloride cells which were filled with mitochondria. The epidermis contacted the dermis through a basement membrane. The sequential construction of dermis, subepidermal collagenous lamellae, fibroblasts, and then melanophores and xanthophores was observed. During pre-metamorphosis, collagenous lamellae were constructed loosely. Immature melanophores (melanoblasts), which had a rod-like appearance, lacking dendritic processes associated with numerous melanosomes of maturation stages 2–3 (Toda and Fitzpatrick, 1971) and typical Golgi's apparatus were observed in the larval skin (Fig. 2). Subepidermal collagenous lamellae under which fibroblasts and chromatophores were stratified in order, was packed precisely later than the onset of metamorphosis. At mid-metamorphosis (G stage), the left side skin contained many melanoblasts which were identified by dopa assay. They were regarded as being on the path to differentiation (Fig. 3-1). During the climax of metamorphosis, melanophores with mature melanosomes appeared, and many fibroblasts invaded the subepidermal collagenous lamella (Fig. 3-3). In contrast, from the middle of metamorphosis, chromatoblasts on the right side lost their cytoplasm (Fig. 3-2). Subsequently, cytolysis of the chromatoblasts increased with the progress of metamorphosis, until the cells were pressed into a sheet-like structure at the climax of metamorphosis (Fig. 3-4). Xanthophores, which had already differentiated during the larval period, showed no major change.

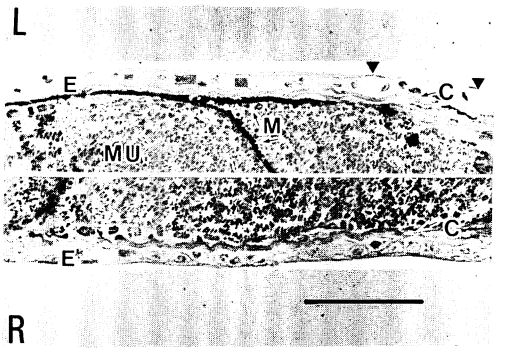

Fig. 1. Cross sections of dorsal skin through a posterior part of the trunk of normally pigmented juvenile flounder *P. olivaceus* (20 mm TL). L: left (ocular) side, R: right (blind) side, E: epidermis, C: subepidermal collagenous lamella, M: melanophore, MU: muscle, ▼: mucus cell, scale bar: 100 μm.

Normally pigmented juveniles of Japanese flounder (45 mm TL) at approximately one month after metamorphosis from both reared and wild sources had complex structures including melanophores, xanthophores and iridophores just under the basement membrane on the left side, but had only iridophores in the same position on the right side.

Mucus cells were reported as typical cells of the ocular side skin in plaice, *Pleuronectes platessa* (Roberts et al., 1972). In the present study, normally pigmented skin of wild Japanese flounder (326 mm TL) was examined. Larger mucus cells in higher densities were found in the ocular side skin rather than in the blind side skin (Fig. 4). Mucus cells were ascertained to be the typical cells of the

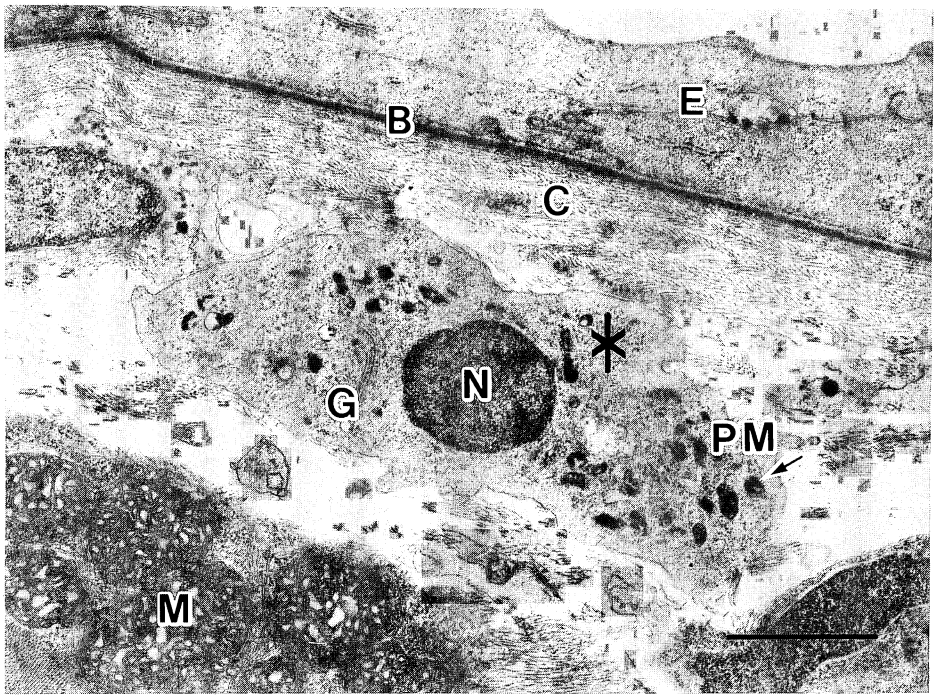

Fig. 2. Electron micrograph of larval skin at the pre-metamorphic stage (C stage). Note the chromatoblast (*) migrating in the space of the dermis. E: epidermis, B: basement membrane, C: subepidermal collagenous lamella, N: nucleus, G: Golgi's apparatus, PM: pre-melanosome, M: mitochondria, scale bar: 2 μm.

ocular side skin in this species also, and hence the developmental changes in the ratio of mucus cell densities (left side/right side) were considered to be useful for detecting a difference between the left side skin and the right side skin. The increasing values in the normal group (Fig. 5) clearly suggested that the components of skin began to change between the left and right sides from the onset of metamorphosis.

Skin structure and pigment cell differentiation in the albinic group

No differences in the fine structure of pigment cells and chromatoblasts were observed between the normal and albinic groups until the onset of metamorphosis. After the middle of mid-metamorphosis (G stage) however, cytolysis and shrinkage of chromatoblasts were observed on both the left and right sides of the albinic group larvae. This process was the same as that observed only on the right side of the normal group larvae (Figs. 3-3, 3-4).

The ratio of mucus cell densities in the albinic group was approximately equal to one throughout metamorphosis (Fig. 5). This result suggests that no differences in skin components were detected between the left and right side skin during metamorphosis.

Discussion

Flatfish show a high degree of bilateral asymmetry in their body form. One of these characters is body coloration. Like other ectothermic vertebrates, flatfish have three types of pigment cells; melanophores, xanthophores, and iridophores. In the case of the Japanese flounder, melanophores play the most important role in body color formation (Seikai et al., 1987). Larvae of this species have large melanophores (larval type melanophores: 70–90 μm when in a dispersed state and 30 μm in width when aggregated) on both sides of the skin. At the climax of metamorphosis, numerous small melanophores (adult type melanophores: 30 μm in width when in a dispersed state) appear rapidly on the left side only. The appearance of these adult type melanophores are responsible for the asymmetrical body color formation at the climax metamorphosis. One of the objects of this study was to reveal the process of pigment cell

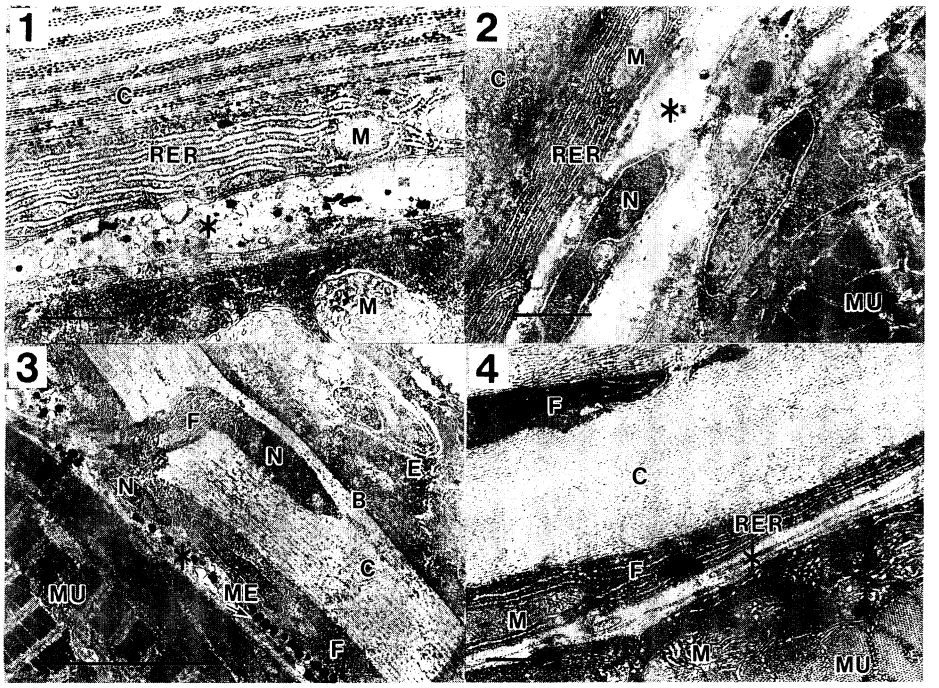

Fig. 3. Ontogenetic asymmetrical melanophore differentiation corresponding to metamorphosis of the normal group. Figs. 3-1 and 2 are electron micrographs of left (3-1) and right (3-2) dorsal skin at mid-metamorphosis (H stage). Note the small, black Dopa assay positive vesicles (about 0.1 um) in the cytoplasm of the chromatoblast (*) in Fig. 3-1. Figs. 3-3 and 4 respectively, are those of left (ocular) and right (blind) skin at the climax metamorphosis (I stage). Note the cytoplasm with destroyed contents (*). (Fig. 3-2), and the shrunken plate-like chromatoblast (*) with little cytoplasm (Fig. 3-4). C: subepidermal collagenous lamella, RER: rough endoplasmic reticulum of fibroblast, M: mitochondria, N: nucleus, MU: muscle, ME: melanosome, F: fibroblast, B: basement membrane, E: epidermis. Scale bar: 1 μm, 2 μm, 10 μm, and 1 μm in Figs. 3-1 to 4, respectively.

differentiation with TEM observation on the fine structures of the pigment cells.

Melanoblasts were detected by Dopa assay in the same densities in the skin on both sides of the body in Japanese flounder at the onset of metamorphosis. However, only the ocular side was pigmented later at the climax metamorphosis (Seikai et al., 1987). This study suggested that chromatoblasts differentiated successfully into melanophores only on the left side, and that those on the right side showed shrinkage and collapse as metamorphosis progressed.

The normal process of pigment cell differentiation through metamorphosis was understood as follows. In the case of normal development, chromatoblasts migrated to the skin on the left and right sides with the same density until the onset of metamorphosis. Some of the chromatoblasts differentiated into larval type pigment cells, and the remainder existed as stem cells (chromatoblasts). The stem cells on the left side differentiated and proliferated into three types of adult type pigment cells. On the right side, this process was inhibited, and cytolysis induced at the climax metamorphosis. The surviving chromatoblasts on the right side supposedly differentiated to iridophores subsequently. It is also very interesting to study the branching off of the two lineages (larval type pigment cells and adult type pigment cells) from a single origin during larval development.

Dopa assay is generally used for the identification of chromatoblasts (Laidlaw, 1932). However, this method has a strong possibility of detecting cells which have not only tyrosinase (key enzyme of melanin synthesis) activities but also other peroxidase activities. Use of monoclonal anti Human Natural Killer Cell (HNK-1) antibody (Anti-LEU-7,

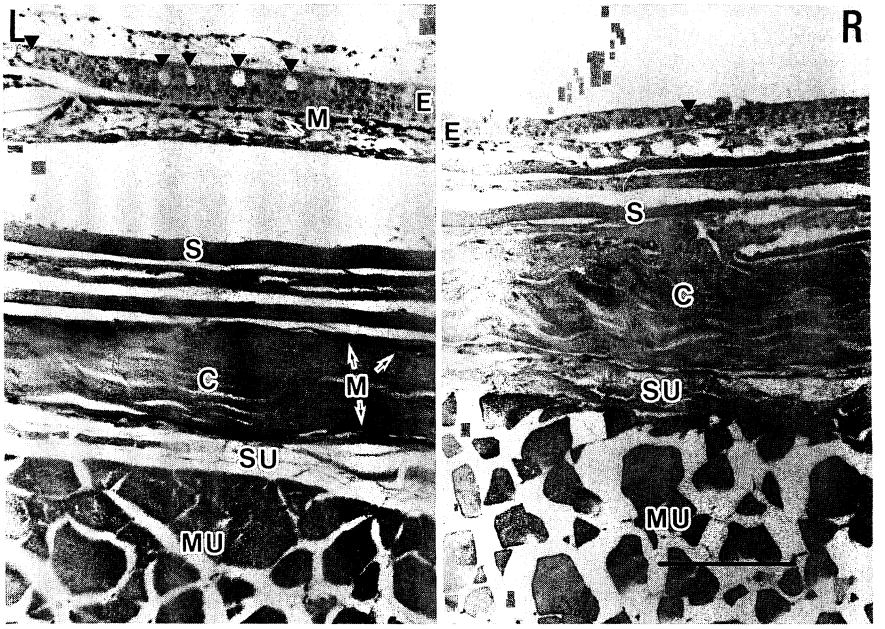

Fig. 4. Cross sections of dorsal skin through a posterior part of the trunk of wild Japanese Flounder *P. olivaceus* (326 mm TL). Note the different mucus cell densities between the left and right sides. L: left (ocular) side, R: right (blind) side, E: epidermis, M: melanophore, S: scale, C: subepidermal collagenous lamella, SU: subcutis, MU: muscle, ▼: mucus cell, scale bar: 200 μm.

Becton Dickinson), crossreacting neural crest antigen, should be better for further studies in this area.

Tyrosin (Lovtrup et al., 1984), calf serum (Jerdan et al., 1985), chick embryo extract (Derby and Newgreen, 1982), and melanophore stimulating hormone (MSH) (Satoh and Ide, 1987) have been reported as promoting factors for the differentiation or melanization of melanoblasts. Phenylalanine (Miyamoto and Fitzpatrick, 1957), 12-O-tetra -decanoylphorbol-13-acetate (TPA) (Oetting et al., 1985), collagen (Loring et al., 1982), fibronectine (Loring et al., 1982), hyaluronate (Tucker and Erickson, 1986), and melanization inhibiting factors (MIF) (Fukuzawa and Ide, 1988) have been reported as inhibiting factors. Only MIF, found in the ventral skin, has sufficient localized specificity to be an effective agent in determining dorso-ventral color pattern. Although asymmetric color pattern in flatfish is a modification of dorso-ventral color pattern, there is a lacking of information regarding such pattern formation in flatfish. Therefore, as a next step, biochemical and cell culture methods should be introduced for further analyses for the control system of color pattern formation in flatfish.

In addition, the present study showed that the ratio of mucus cell densities, a useful indicator of asymmetrical differentiation in flatfish skin, began to increase from the onset of metamorphosis only in the normal group. However, until the middle of mid-metamorphosis, no difference in fine structure of chromatoblasts were observed between the left and right sides.

These results suggest strongly that some components of skin change prior to the differentiation of pigment cells and some environmental factors regulate the differentiation of pigment cells. Evidence for this phenomenon lay in the different process of squamation and the different shapes of scales on the ocular and blind sides are induced by their different body color in normally colored juveniles of the Japanese flounder. Moreover, the same process of squamation as on the blind side and cycloid scales, which are commonly observed on the blind side, are also induced on the pseudoalbinic part of the ocular side in juveniles (Seikai, 1980). On the other hand, in ambicolored juveniles of the Japanese flounder, ctenoid scales were developed also on the blind side, but with a different squamation process from that of

normal fish. It has been suggested that an unknown factor(s) controlls the squamation and formation of ctenoid scales through dermal tissue (Kikuchi and Makino, 1990). These relationships between pigmentation and squamation show high similarity to the relationship between mucus cells and pigment cell differentiation. Therefore, the results suggest that the process of pigment cell differentiation in flatfish skin is dependent upon tissue environmental control. However, further *in vivo* studies are needed to elucidate such mechanisms.

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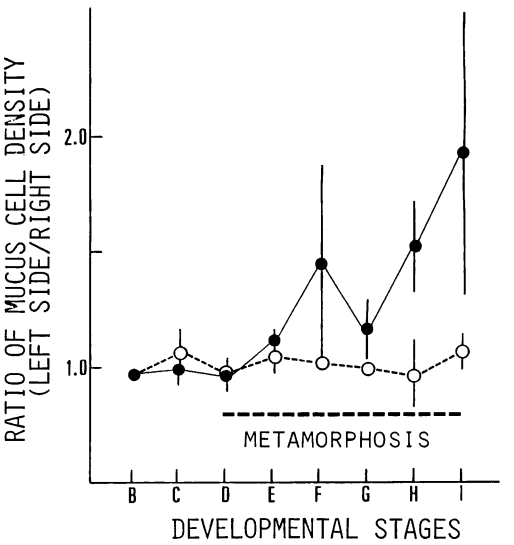

Fig. 5. Ontogenetic change in the ratio of mucus cell densities (left side/right side) from the two different feeding groups (normal and albinic). Stages A–C, D–E, F–H, and I correspond to pre-metamorphosis, onset of metamorphosis, mid-metamorphosis, and climax metamorphosis, respectively. Normal group (closed circles) was fed on wild zooplankton. Albinic group (closed circles) was fed on Brazilian *Artemia* nauplii. Note the gradual increase of this ratio in the case of the normal group from the onset of metamorphosis (E stage).

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ヒラメの変態期における左右体側皮膚の色素細胞の分化過程

青海忠久

変態完了後に正常な体色になる正常群とほぼ完全な白化となる白化群の2群のヒラメ *Paralichthys olivaceus* の仔魚を飼育し，変態の過程における黒色素細胞の分化について，透過型電子顕微鏡による観察を行った．正常群の仔魚では，変態の進行にともなって色素芽細胞は左体側の皮膚では分化が促進され，一方右体側の皮膚ではそれらは萎縮崩壊した．色素芽細胞の判別は，ドーパ反応と細胞の微細構造の観察によって行った．カレイ目魚類成魚の皮膚では，粘液細胞が有眼側において発達し密度が高いことが知られている．そこで，左右体側の粘液細胞密度比を求めたところ，色素芽細胞の形態的相違が認められる以前の変態始動期より増加し始めた．一方，白化群では両体側の皮膚において，色素芽細胞が萎縮崩壊することが観察され，粘液細胞密度比も変態期を通じて変化しなかった．

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