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Iridophores Involved in Generation of Skin Color in the Zebrafish, *Brachydanio rerio*

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Abstract. We examined the distribution of iridophores in the skin of the zebrafish and the effects of a neurotransmitter and hormones on the cells. In the skin of deep blue stripe areas, motile iridophores are distributed over the layer of melanophores, while in the dorsal skin, such cells underlie melanophores and xanthophores. These iridophores assume blue coloration in physiological saline probably through multilayered thin-film interference phenomenon of non-ideal type, and norepinephrine (NE) induces color change to yellow within 1–2 min. The action of NE is mediated through α-adrenoceptors on the iridophore membrane. MCH (melanin concentrating hormone) and MSH (melanophore stimulating hormone) have no effect on the cells. Iridophores manifesting whitish color exist densely in the skin of white stripe regions, and these iridophores are immotile. Immotile iridophores of another type are compactly arranged beneath the melanophore layer in the skin corresponding to deep blue stripes. The role of the iridophores in the generation of skin color and its changes is discussed.

1. Introduction

Skin colors of fish are generated as a result of the absorption of light rays of certain wavelengths by pigmentary substances, and by the scattering and the reflection of light by intracellular structures with refractive indices different from the cytoplasmic matrix. The absorption of light by pigment is achieved by light-absorbing pigment cells ("chromatophores"), which include melanophores, erythrophores, xanthophores and cyanophores, whereas the latter phenomena, in the absence of colored materials, are dependent on the presence of light-reflecting chromatophores, leucophores and iridophores (FUJII and OSHIMA, 1986; FUJII, 2000). Generally, fish chromatophores are present in the dermis of the skin tissue.

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Melanophores (melanin-containing, dark chromatophores), erythrophores (red chromatophores containing carotenoids and pteridines), xanthophores (yellow chromatophores which contain carotenoids and pteridines), cyanophores (blue chromatophores; the chemical nature of blue pigment materials remains to be determined; cf. GODA and FUJII, 1995) and leucophores (light-scattering chromatophores which look whitish when illuminated by incident light) are dendritic, extending their processes parallel to the plane of the skin. In such chromatophores, pigment granules, within which pigments are packed, or light-reflecting microstructures migrate centripetally (aggregation) or centrifugally (dispersion) in response to neurotransmitters, hormones or other signals received by the cells.

By contrast, most iridophores (light-reflecting chromatophores) assume round, oval or multilateral shape without dendritic processes (FUJII and OSHIMA, 1986). The iridophores are concentrated in whitish and silvery belly surface, and also responsible for the metallic glitter on the side skin of many fishes. The reflectivity of iridophores is due to the presence of light-reflecting platelets of guanine within the cells (cf. FUJII, 1993). Electron-microscopic observations revealed that one or a few stacks of very large platelets are present in such iridophores, and that the distance between the adjacent platelets is uniform (KAWAGUTI, 1965). DENTON (1970) and LAND (1972) showed that the cellular reflectivity is due to the multiple thin-film interference phenomenon of the ideal type. There, the optical thickness (i.e., the refractive index multiplied by the actual thickness) of the light-reflecting platelets and that of the cytoplasmic sheet intervening the adjacent platelets are almost the same, being about one-quarter of a wavelength of a spectral peak of light reflected from the iridophores. In this system, the spectral peak becomes very wide and the reflectivity so high with the increase in the number of platelets. Usually, those iridophores are physiologically inactive: the distance between the adjacent platelets in a stack does not vary.

Motile iridophores in which the distance between the adjacent platelets changes in response to stimuli have been found in some teleostean species: the blue damselfish, (KASUKAWA *et al.*, 1987), the blue-green damselfish (FUJII *et al.*, 1989), the neon tetra (NAGAISHI and OSHIMA, 1989, 1992; NAGAISHI *et al.*, 1990), etc. In the skin of the fishes, small, round or ellipsoidal iridophores are densely arranged and the spectral peak of light reflected from the iridophores shifts towards longer wavelengths by adrenergic stimuli. Each cell contains two (neon tetra) or a number of (damselfishes) piles of light-reflecting platelets (guanine). Based mainly on the fine structural observations on these iridophores, we reported that the light-reflecting platelets are too thin (about 5 nm thick) to produce the ideal-type interference, and further suggested that the characteristic hue displayed by the fishes should be due to the interference of the non-ideal nature (OSHIMA *et al.*, 1985). In the "non-ideal" system, where the optical thickness of the light-reflecting platelets and that of the cytoplasmic sheet are not identical (LAND, 1972), the thinner the platelets, the lower is the reflectance, and the color becomes purer with the decrease in the thickness of the platelets. Further, with the increase in the number of thin platelets, the peak heights increase and widths decrease. Actually, a number of thin platelets (about 5 nm thick) pile up regularly in the motile iridophores of the fishes mentioned above to achieve a considerably higher reflectance and to produce a purer color.

In dendritic iridophores of some species of the fresh water goby, including the dark sleeper, *Odontobutis obscura obscura*, tiny platelets can move centripetally and centrifugally,

resembling other common dendritic chromatophores such as melanophores (IGA and MATSUNO, 1986). In striking contrast to the rapid responses of motile iridophores of damselfishes and neon tetra, which lack dendritic processes, the responses of the dendritic iridophores are extraordinarily slow. Usually, it takes more than 30 min to complete either the plate-aggregating or -dispersing response. Under the dark field epi-illumination microscope, FUJII *et al*. (1991) observed that, when the reflecting platelets were aggregated in the perikarya of the iridophores, the cells appeared bluish in color, and that the same cells appeared yellowish, with some greenish shading, when the platelets were dispersed in the cytoplasm. The bluish tone exhibited by the platelet aggregates is considered to be due to the presence of organized piles of platelets, which are gradually formed during the process of aggregation of the platelets.

Thus, we can now easily suppose that both motile and immotile iridophores must play an important role in the manifestation of skin color and its changes in many fish species. Especially, it seems significant for us to understand how iridophores, in addition to pigment-containing chromatophores, participate in the color pattern formation. However, such investigation remains to be done. Recently, the zebrafish having alternate stripes of deep blue and white are often used as materials to study the mechanisms of the pattern formation at the molecular level (PARICHY and JOHNSON, 2001). Therefore, we have examined the distribution of chromatophores in the skin of the zebrafish, paying attention to iridophores, and also have investigated the motility of the iridophores. Further, we discuss the role of the iridophores in the generation of body color and its changes.

2. Materials and Methods

Individuals of both sexes of the zebrafish (Fig. 1), *Brachydanio rerio*, 35–58 mm in length, were purchased from a local commercial source and reared in an aquarium for several days before experiments.

Decapitation of the fish was immediately followed by immersion of the body in a physiological saline solution of the following composition (mM): NaCl 125.3, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-glucose 5.6, and Tris-HCl buffer 5.0 (pH 7.2).

The body of each fish was sliced in half along the back-bone, and the viscera and all bones were removed. Scales and the layer of muscle were carefully removed from each

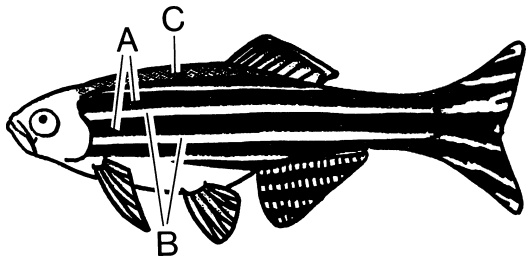


Fig. 1. Illustration of the zebrafish. A: dark blue stripe region, B: white stripe region, C: dorsal part.

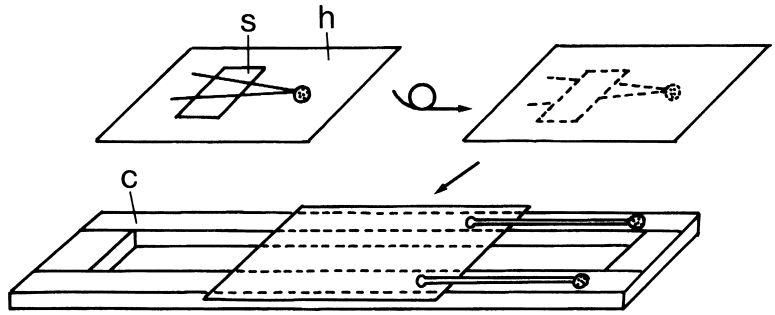


Fig. 2. Illustrations of the holder (h) and the perfusion chamber (c). The skin preparation (s) was fixed on the holder so that the skin surface (epidermis side) came to contact with the coverslip. The holder was then turned over and placed on the chamber.

sliced side with finely pointed forceps, and the integument was left intact. Such preparations of the skin were used as the experimental materials.

The skin preparation was attached, epidermis side down, to a holder with two glass fibers, one side of which had been attached to a coverslip with epoxy adhesive. The holder was then turned over and placed on the perfusion chamber with a narrow trough filled with physiological saline (Fig. 2; OSHIMA and FUJII, 1984). The chamber was placed on the stage of a dark-field epi-illumination microscope (Optiphoto, CF-BD plan lenses; Nikon). The responses of the iridophores to various chemicals were microphotographed.

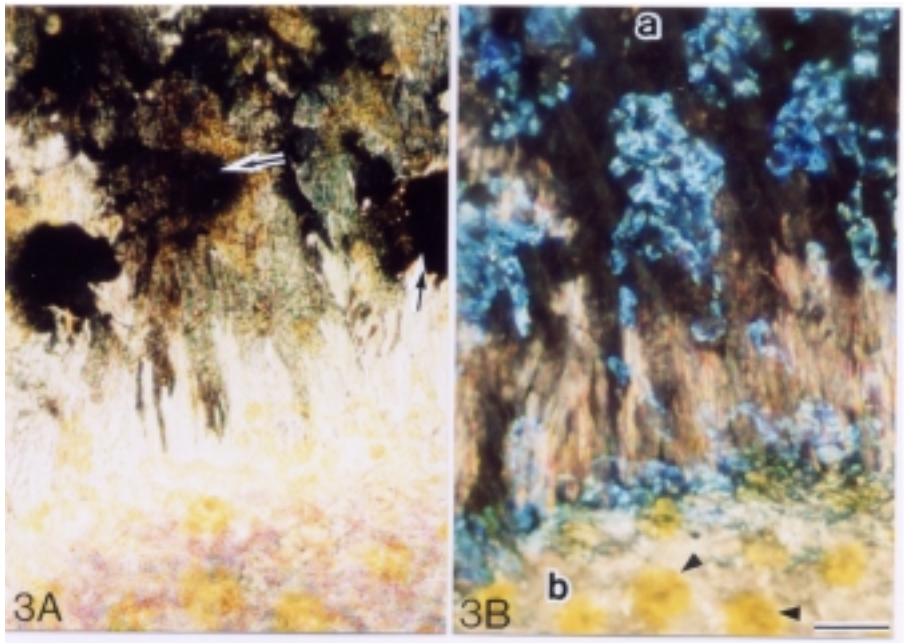
The drugs used were norepinephrine hydrochloride (Sankyo), isoproterenol hydrochloride (Nikken Chemicals), phentolamine mesylate (Ciba-Geigy), propranolol hydrochloride (ICI), α-melanophore stimulating hormone (α-MSH, Sigma Chemical) and melanin-concentrating hormone (MCH) synthesized at the Peptide Institute (Minoh, Osaka).

All experiments were performed at room temperature (18–25°C).

Fig. 3. Photomicrographs of the skin corresponding to dark blue stripe (a) and white stripe (b) regions of the zebrafish. A and B were taken on the same field of the skin with a transmission optics (A) or with a dark-field epi-illumination optics (B). In this preparation, melanophores in blue stripe region (arrows in A) and xanthophores in white stripe region (arrow heads in B) are dispersed. Bar: 50 μm.

Fig. 4. Photomicrographs of the skin corresponding to dark blue stripe region. Melanin granules were aggregated by 100 nM MCH. A: taken with a transmission optics, B: taken with a dark-field epi-illumination optics. Note that the iridophores (orange colored, slender cells which look like chromosomes; arrow heads in A) do not exist in the black areas in photograph B (arrows in B). Bar: 50 μm.

Fig. 5. Photomicrographs showing type 2 iridophores (arrow heads in A) in the dark blue stripe region. In this preparation, melanin granules were aggregated by 100 nM MCH. A: taken with a transmission optics, B: taken with a dark-field epi-illumination optics. Bar: 50 μm.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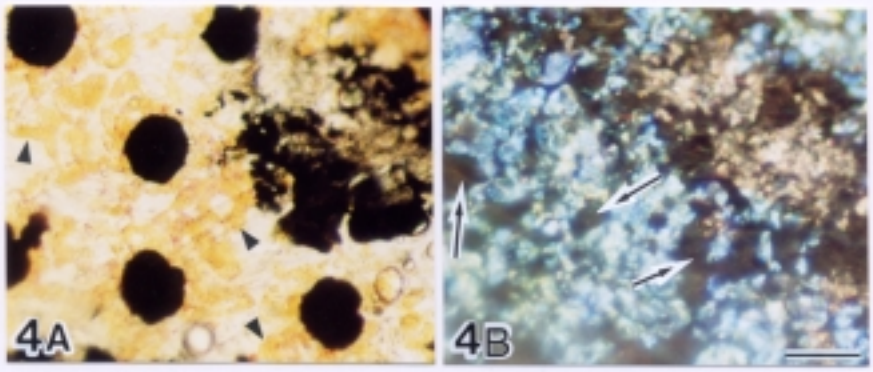
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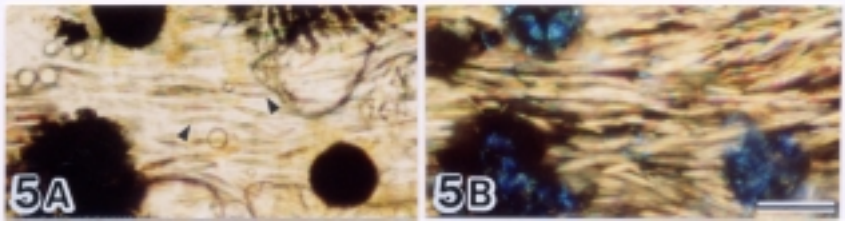
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3. Results and Discussion

3.1. Distribution of iridophores in the integument of zebrafish

In the skin corresponding to vertical deep blue stripes (A in Fig. 1), iridophores displaying blue color (type 1) are present mostly in groups, being underlain by melanophores (Fig. 3). To observe the iridophores under a transmission microscope, melanin granules in melanophores were aggregated by MCH (100 nM). As shown in Fig. 4A, very small iridophores (orange colored, slender cells) are assembled and distributed randomly. The photomicrograph taken on the same field with a dark-field epi-illumination optics (Fig. 4B) indicates that blue coloration of the stripes is dependent on the iridophores, and that the blue iridophores surely overlie melanophores. Light may be reflected though a multilayered thin-film interference of the non-ideal type. However, actual stripe hue is fairly dark compared with highly brilliant coloration of the blue damselfish or the longitudinal stripe of the neon tetra, perhaps because the iridophores of type 1 are not closely arranged over the dark sheet of melanophores in the stripe surface. The layer of spindle-shaped iridophores (type 2; Fig. 5) assuming white or silver color under epi-illumination is also observed beneath the melanophore layer (Fig. 6): When melanin granules were aggregated by MCH (100 nM), whitish iridophores became visible among melanin aggregates (Fig. 6B), which suggests that such iridophores underlie melanophores.

Ellipsoidal iridophores are densely arranged in the white stripe regions (B in Fig. 1) and seem to lie on top of another (Fig. 7A). Since the morphology of the iridophores is different from that of type 2 cells, these iridophores are defined as type 3 iridophores. The iridophores (type 3) assume white color inclining to yellow when epi-illumination is applied (Figs. 7B and 8). Xanthophores are also present over the iridophores, but no melanophores are observed. In Figs. 3B and 7B, xanthophores are fairly dispersed, while, in Fig. 8, they are aggregated.

In the dorsal skin (C in Fig. 1), xanthophores are distributed with many melanophores smaller than those in the dark blue stripe areas (Fig. 9; cf. Fig. 6A). Probably, the

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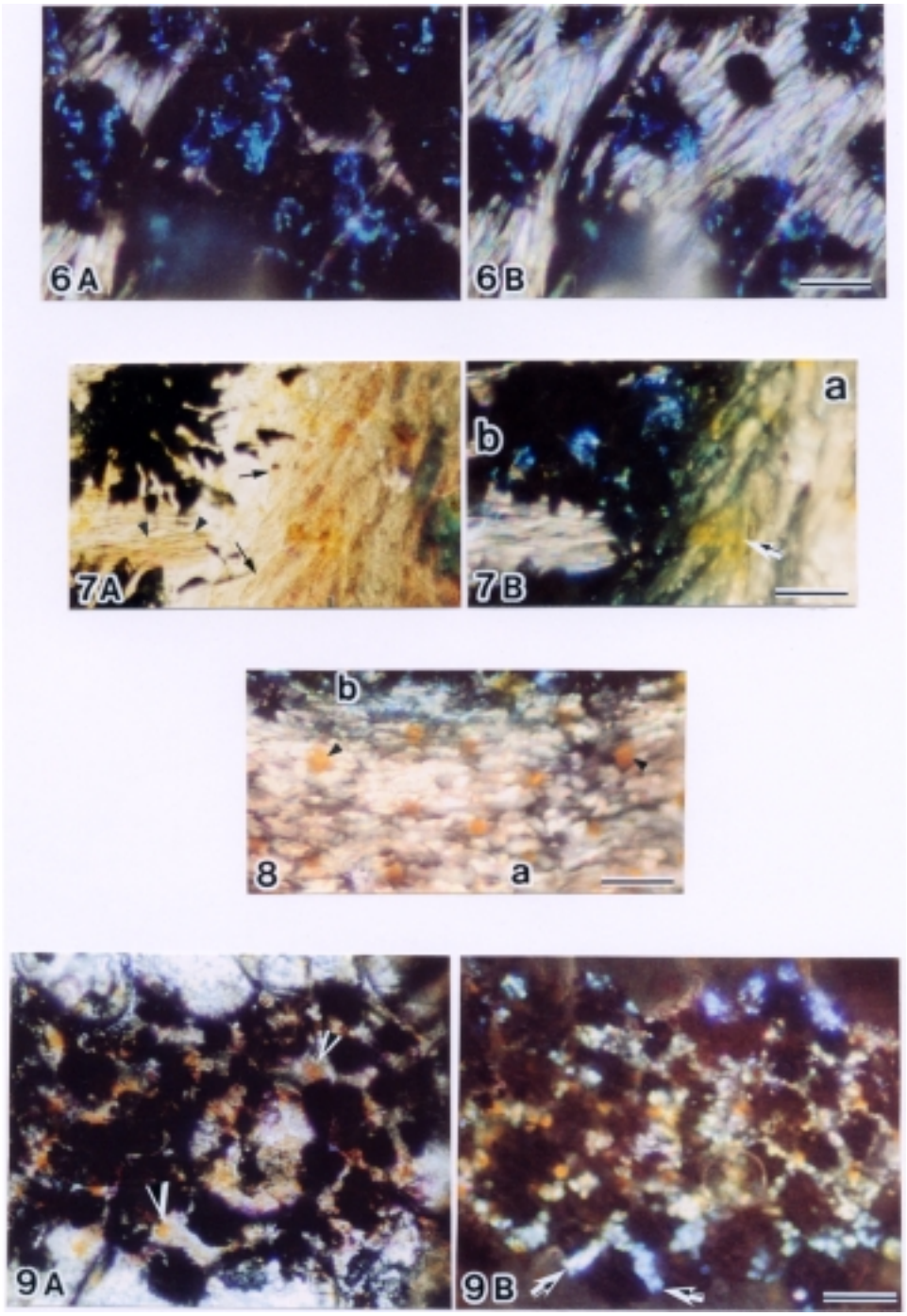
Fig. 6. Photomicrographs (taken with a dark-field epi-illumination optics) showing the dark blue stripe region. Although pigment granules within melanophores are dispersed in physiological saline (A), MCH (100 nM) induces melanin aggregation (B), leading to the exposure of white iridophores. Bar: 50 μm.

Fig. 7. Photomicrographs of white stripe (a) and dark blue stripe (b) regions. A and B were taken on the same field of the skin with a transmission optics (A) or with a dark-field epi-illumination optics (B). Type 2 (arrow heads in A) and type 3 (arrows in A) iridophores are seen. The outline of xanthophore (arrow in B) is not clear since pigment granules within the cell are dispersed. Bar: 50 μm.

Fig. 8. Photomicrographs (taken with a dark-field epi-illumination optics) showing the white stripe (a) and dark blue stripe (b) regions. In this skin preparation, xanthophores (arrow heads) are aggregated and lie over type 3 iridophores. Bar: 50 μm.

Fig. 9. Photomicrographs of the dorsal skin. A and B were taken on the same field of the skin with a transmission optics (A) or with a dark-field epi-illumination optics (B). Although melanin granules are dispersed in this skin preparation, melanophores are smaller than those in the blue stripe region (see Fig. 6A). Xanthophores (arrow heads in A) are also observed. Note that blue light (arrows in B) is reflected from the spaces between the pigment-containing chromatophores. Bar: 50 μm.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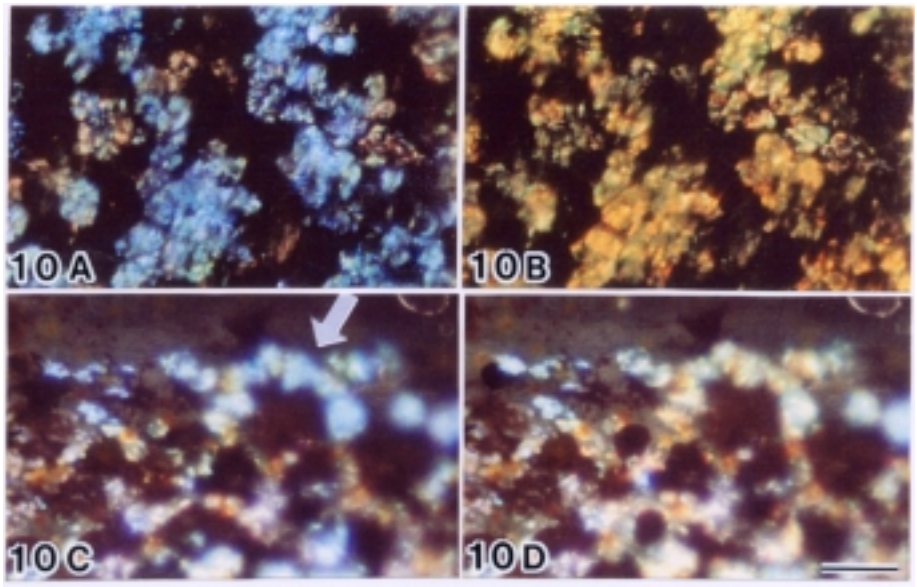
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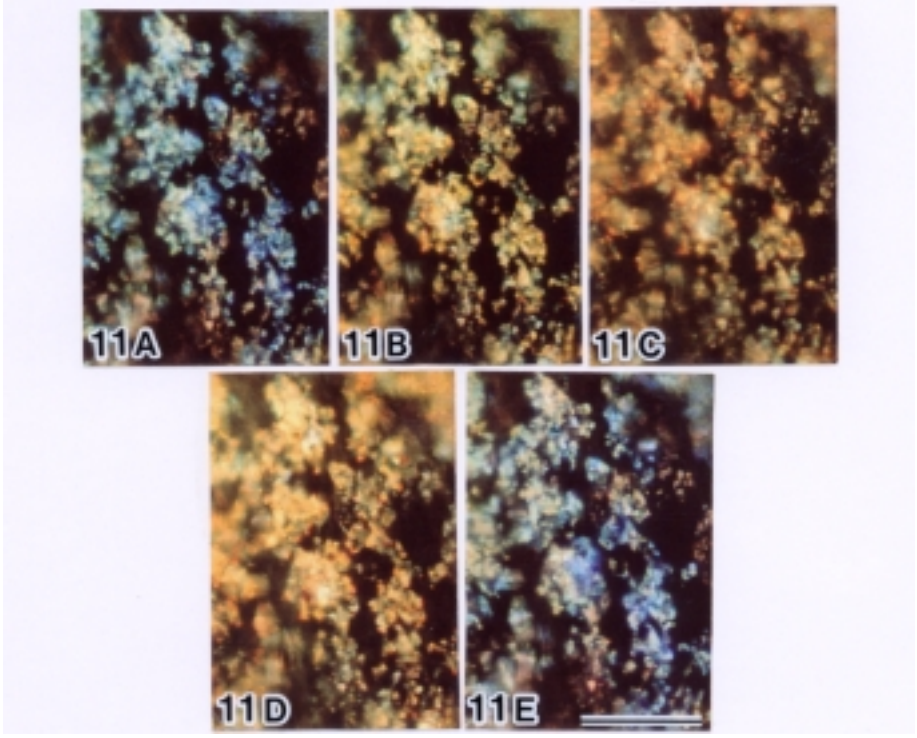
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xanthophores function to add a yellowish tone to black, giving rise to the brownish hue of the dorsal skin. Iridophores are not observed over melanophores and xanthophores under dark-field epi-illumination and blue light is reflected from the spaces among pigment-containing chromatophores (Fig. 9B). Therefore, the iridophores assuming light-blue coloration appear to lie under melanophores and xanthophores. We distinguish these iridophores from type 1 cells which overlie melanophores, and define these as type 1′. The reason why the dorsal skin does not exhibit brilliant blue color, unlike the lateral stripe surface, may be that the iridophores of type 1′ exist beneath melanophores and xanthophores.

3.2. Responses of iridophores to adrenergic agents and hormones

In fish, the motility of chromatophores is controlled neurally and/or hormonally (FUJII and OSHIMA, 1986; FUJII, 2000). In the case of neural regulation, norepinephrine (NE), a neurotransmitter of the sympathetic fibers, is believed to be responsible for the transmission of the signal that leads to pigment aggregation in light-absorbing chromatophores such as melanophores, xanthophores, erythrophores and cyanophores. In this study, therefore, the iridophores of type 1, 1′, 2, and 3 were treated with NE at 10 μM for 3 min. Although the type 2 and 3 cells did not respond to NE, blue color displayed by type 1 and 1′ cells in physiological saline solution changed to brilliant yellowish color when NE was applied (Fig. 10). As typically shown in Fig. 11, the color change was caused within 1–2 min, and the response was reversible. That is, such iridophores recovered from the effect of NE within 2 min. Since NE is known to act on β_1-adrenoceptors in addition to both types of α-adrenoceptors (α_1- and α_2-adrenoceptors) (AHLQUIST, 1984), we then examined the effects of 10 μM isoproterenol, β agonistic synthetic amine, which acts on β_1- and β_2-adrenoceptors, on all types of iridophores. The sympathomimetic amine did not affect zebrafish iridophores at all. From these results, it is suggested that the action of NE on type 1 and 1′ iridophores is mediated through α-adrenoceptors on the cell membrane of the iridophores.

Next, the influences of adrenergic blocking agents on NE action on type 1 and 1′ iridophores were studied. In this series of experiments, skin preparations were pretreated with each blocker for 5 min and then NE was applied for 5 min in the continued presence of the blocker. Phentolamine, adrenergic blocking agent of α type, at 10 μM was found to inhibit the NE action (data not shown), whereas propranolol, adrenergic blocking agent of β type, at 10 μM had no effect. These results also lend support to the supposition that α-adrenoceptors participate in sympathetic-iridophore (type 1 and 1′) transmission.

MCH (100 nM) and α-MSH (100 nM) did not cause the responses of zebrafish iridophores (type 1, 1′, 2, and 3), although MCH induced pigment aggregation (cf. Figs. 4,

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Fig. 10. Photomicrographs (taken with a dark-field epi-illumination optics) showing the responses of motile iridophores to norepinephrine (NE; 10 μM). A and C: type 1 and 1′ (arrow) iridophores, respectively, in physiological saline, B and D: type 1 and 1′ cells, respectively, treated with NE for 3 min. Bar: 50 μm.

Fig. 11. Responses of motile iridophores (type 1) to 10 μM norepinephrine (NE). Photomicrographs were taken with a dark-field epi-illumination optics. A: type 1 iridophores in physiological saline, B: 1 min after application of NE, C: 2 min after application of NE, D: 1 min after the beginning of rinse with physiological saline, E: 2 min after the beginning of saline rinse. Bar: 100 μm.

5 and 6B) and α-MSH accelerated pigment dispersion in melanophores and xanthophores (data not shown). That is, zebrafish motile iridophores (type 1 and 1′) are regulated solely by nerves, which is the case in the blue damselfish (KASUKAWA *et al.*, 1986) and the neon tetra (NAGAISHI and OSHIMA, 1989), too. Exclusively controlled neuronally, the motile iridophores of the zebrafish show the very rapid responses: exogenously applied NE gives rise to a shift in the spectral peak toward longer wavelengths within 1–2 min. As in the cases of the motile iridophores of the damselfish and the neon tetra, such rapid change may be due to a simultaneous increase in the distance between the adjacent light-reflecting platelets in each stack of platelets. Since it is known that color change induced by aggregation or dispersion of the platelets within iridophores requires more than 30 min (see "Introduction"), the possibility that the same mechanism functions in type 1 and 1′ iridophores should be ruled out.

Type 2 and 3 iridophores assuming whitish color are spindle-shaped or ellipsoidal cells without dendritic processes and immotile. At the present time, the mechanism of light-reflection in these cells is not clear, but preliminary electron-microscopic observations (Hirata *et al.*, personal communication) imply that they might reflect light via a multilayered thin-film interference phenomenon of the ideal type. Further histological and fine-structural observations should be made on the integument for better understanding of the mechanism of the light reflection by zebrafish iridophores.

When zebrafish is transferred to a white background from a black one, NE will be released from the endings of sympathetic nerve fibers. In dorsal skin, both melanophores and xanthophores aggregate their pigment granules into the perikaryon and yellowish hue manifested by the underlying motile iridophores (type 1′) in response to NE may become a dominant color of the skin. In the dark blue stripe regions, NE induces the change in color reflected from the motile iridophores (type 1) from blue to yellow. In addition, pigment aggregation occurs in melanophores underlying the motile iridophores, which brings about the exposure of the whitish layer of immotile iridophores, leading to the change in stripe color from dark blue to whitish yellow. These changes are reversible on a dark background. Thus, the motile and immotile iridophores are believed to take an active part in the generation of skin color and its changes in the zebrafish.

4. Summary

Zebrafish iridophores are classified into four groups. In the iridophores present in the dark blue stripe surface (type 1; blue color) and those in the dorsal skin (type 1′; light-blue color), norepinephrine induces the change in color to yellow. Immotile iridophores are also observed in the dark blue stripe surface (type 2) and in the white stripe region (type 3). These iridophores take an important part in producing body color and its changes.

REFERENCES

AHLQUIST, R. P. (1984) A study of the adrenotropic receptors, *Am. J. Physiol.*, **154**, 586–600.

DENTON, E. J. (1970) On the organization of reflecting surfaces in some marine animals, *Philos. Trans. R. Soc. London B*, **258**, 285–313.

FUJII, R. (1993) Cytophysiology of fish chromatophores, *Int. Rev. Cytol*, **143**, 191–255.

FUJII, R. (2000) The regulation of motile activity in fish chromatophores, *Pigment Cell Res.*, **13**, 300–319.

FUJII, R. and OSHIMA, N. (1986) Control of chromatophore movements in teleost fishes, *Zool. Sci*., **3**, 13–47.

FUJII, R., KASUKAWA, H., MIYAJI, K. and OSHIMA, N. (1989) Mechanism of skin coloration and its changes in blue-green damselfish, *Chromis viridis*, *Zool. Sci*., **6**, 477–486.

FUJII, R., HAYASHI, H., TOYOHARA, J. and NISHI, H. (1991) Analysis of the reflection of light from motile iridophores of the dark sleeper, *Odontobutis obscura obscura*, *Zool. Sci*., **8**, 461–470.

GODA, M. and FUJII, R. (1995) Blue chromatophores in two species of callionymid fish, *Zool. Sci*., **12**, 811–813.

IGA, T. and MATSUNO, A. (1986) Motile iridophores of a freshwater goby, *Odontobutis obscura*, *Cell Tissue Res*., **244**, 165–171.

KASUKAWA, H., OSHIMA, N. and FUJII, R. (1986) Control of chromatophore movements in dermal chromatic units of blue damselfish—II. The motile iridophore, *Comp. Biochem. Physiol*., **83C**, 1–7.

KASUKAWA, H., OSHIMA, N. and FUJII, R. (1987) Mechanism of light reflection in blue damselfish iridophore, *Zool. Sci*., **4**, 243–257.

KAWAGUTI, S. (1965) Electron microscopy on iridophores in the scale of the blue wrasse, *Proc. Japan Acad*., **41**, 610–613.

LAND, M. F. (1972) The physics and biology of animal reflectors, *Prog. Biophys. Mol. Biol*., **24**, 75–106.

NAGAISHI, H. and OSHIMA, N. (1989) Neural control of the motile activity of light-sensitive iridophores in the neon tetra, *Pigment Cell Res*., **2**, 485–492.

NAGAISHI, H. and OSHIMA, N. (1992) Ultrastructure of the motile iridophores of the neon tetra, *Zool. Sci*., **9**, 65–75.

NAGAISHI, H., OSHIMA, N. and FUJII, R. (1990) Light-reflecting properties of the iridophores of the neon tera, *Paracheirodon innesi*, *Comp. Biochem. Physiol*., **95A**, 337–341.

OSHIMA, N. and FUJII, R. (1984) A precision photoelectric method for recording chromatophore responses *in vitro*, *Zool. Sci*., **1**, 545–552.

OSHIMA, N., SATO, M., KUMAZAWA, T., OKEDA, N., KASUKAWA, H. and FUJII, R. (1985) Motile iridophores play the leading role in damselfish coloration, in *Pigment Cell 1985: Biological, Molecular and Clinical Aspects of Pigmentation* (eds. J. T. Bagnara, S. N. Klaus, E. Paul and M. Schart), pp. 241–246, Univ. Tokyo Press, Tokyo.

PARICHY, D. M. and JOHNSON, S. L. (2001) Zebrafish hybrids suggest genetic mechanisms for pigment pattern diversification in *Danio*, *Dev. Genes Evol*., **211**, 319–328.