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Pigmentary Responses of *Oryzias latipes* to Darkness

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A freshwater teleost, *Oryzias latipes,* responded to darkness with paling of the skin, melanosome aggregation within melanophores. The response took about 24 hr to become equilibrated, intermediate tones, being different from the background response in light in the time-course of the response. Eye removal, hypophysectomy and pinealectomy did not affect this response to darkness. Spinal sectioned and chemically sympathectomized fishes failed to respond to darkness, remaining dark in darkness. Dibenamine-treated animals did not become pale in darkness. These results suggest that the sympathetic nerves play a primary role in the response to darkness.

Introduction

It is well known that teleost fishes rapidly change their integumentary colors according to environmental tints. When the animals are placed on a white background under illumination, they take on a pale tone and remain so indefinitely. On a dark background, their skin color darkens. These color responses of the skin to environmental tones in light are designated as a white background response and a dark background response, respectively. These rapid changes of hue are due to the motile activities of integumentary colored cells, the chromatophores, pigment translocation within the cell. In these responses, photic stimuli received by the retina are transmitted to the chromatic center of the brain and the information treated there is sent to the chromatophores through nervous and/or hormonal pathways.

On the other hand, in darkness some fishes tend to blanch or take intermediate tones (cf. Waring, 1963). Matthews (1933) reported that the removal of the pituitary in *Fundulus heteroclitus* did not affect its color changes to darkness. The same results were obtained by Khokhar (1970) in the catfish,*Ictalurus melas*. He also examined the chromatic responses to darkness of the blinded catfish. The degree of melanophore aggregation is approximately the same at darkness equilibrium in intact and blinded fishes (Khokhar, 1971). The effects of spinal section on responses of melanophores to darkness were observed by Healey (1951) in the minnow, *Phoxinus laevis*. The operated minnows still showed a slow color change in darkness, although the degree of melanophore aggregation in darkness equilibrium was lower than that of intact fishes. He discussed these results on the basis of double innervation of the melanophores. However, the interpretation of the results was uncertain.

Recently it has been reported that the pineal gland plays an important role in color changes of fishes (Reed, 1968; Kavaliers et al., 1980; Fujii and Oshima, 1986). Kavaliers et al. (1980) demonstrated that, in the killifish, *Fundulus heteroclitus,* which displayed a circadian rhythm of color change, pinealectomy eliminated the rhythm of color change but does not affect background adaptation.

In the course of studies on color changes of *Oryzias latipes,* we found that the fish became pale in darkness. The present experiments, thus, were designed to examine the mechanisms of the paling response to darkness. The results suggested that the sympathetic nerves may play a primary role in the paling of *Oryzias latipes* in darkness.

Materials and Methods

Material used in the present experiments is a freshwater teleost, *Oryzias latipes* (wild type) in body length of 25-28 mm. Fishes collected locally from a stream were kept in a container for at least a week to accustom them to experimental conditions before use.

For experiments on background responses in light, a black or white painted plastic aquarium (35×20×25 cm) was used. About twenty fishes were placed in each aquarium, which was supplied with water in 17 cm depth. The air was continually sent into the water through an air stone connected to an air pump. The aquaria were illuminated with a fluorescent lamp (40W×2) placed 25 cm above them. The intensity of illumination was about 6,500 lux on the surface of the water.

Recording methods

The melanophore index (MI) was used as a method of estimating the degree of dispersion of the melanophores (Fig. 1). At suitable time intervals after background change, fishes were quickly fixed by immersing into hot water for some 5 sec and then a scale was isolated from a given position of the dorso-lateral surface of the trunk in each side. Indexes of all melanophores distributed in the two scales were measured

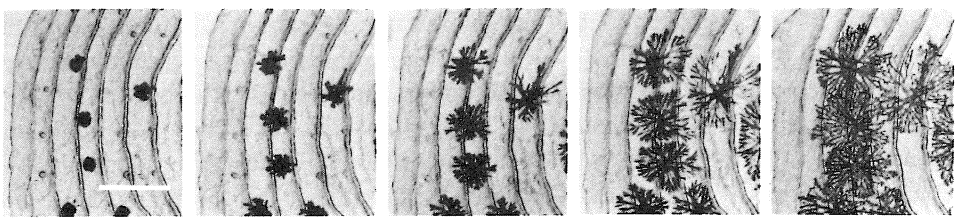

Fig. 1. The melanophore index. The figure, 1 to 5, indicates the melanophore index, stage No, representing different degrees of melanosome dispersal within the melanophores ; the most aggregated state is designated as stage 1 and the most dispersed as stage 5. Bar=100 μm.

microscopically. The mean of the indexes was estimated as the MI of the fish at that time.

Operations

To examine the mechanisms controlling the responses of the fish to darkness, various kinds of operations were made on the fishes. Fishes were anesthetized in a solution of 0.05% chloreton (Kanto Chemicals, Tokyo) and wrapped in wet absorbent cotton to prevent desiccation. All operations were made with a fine forceps and a fine needle. After operations no attempt was made to close the incision. The animals were merely kept in a physiological saline (128 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, 5 mM Tris-HCl buffer, pH 7.2) for 30–60 min and then removed to tap water.

Hypophysectomy: The pituitary gland was removed by a method described by Utida et al. (1971). The gape of the left operculum was enlarged by cutting alone the floor of mouth toward the lower lip. The operculum was then opened widely and gills retracted to expose the roof of the pharynx. The dorsal buccal mucosa was removed, and the pituitary was seen beneath the skull just posterior to the optic chiasma. A cut was carefully made near the gland. A fine glass pipette with an oblique tip was inserted through the slit, the tip was directed to the pituitary stalk, and the gland was removed by oral suction. After experiments, the animals were examined to determine whether or not the hypophysis had been completely removed.

Pinealectomy: The operation was done by a method described by Urasaki (1971). The parietal bone covering the pineal region was incised to form a small pit. The meninges were opened and the pineal vesicle and stalk were removed by oral suction. Sham operations involved exposure of the meninges.

Spinal section: The spinal was sectioned between the 4th and 6th vertebra. The operated fishes attained a maximal darkening in order to remove the neural control completely.

Eye removal: The treatment was done by a small modification of method described by Terami and Watanabe (1977). The surface of the cornea was coated with 0.5 N NaOH solution by means of a tiny piece of cotton thread. After the cornea was soaked up with a small piece of tissue paper, the animal was placed in tap water containing Green-F, an antibiotic. Two or three days after the treatment, the eyeball was removed with a forceps. The eyeball-removed fishes were again kept in tap water containing Green-F for 4 days before use.

Chemical sympathectomy: A method described by Iga and Takabatake (1982) was used. Chemical denervation of melanophores was done by injecting the animals intraperitoneally with 80 μg/g body weight of 6-hydroxydopamine hydrobromide (Sigma Chemical, St Louis) 2 days before use. With this treatment, denervation of melanophores in the skin persisted for at least a week.

The water temperature was between 18 and 23°C during the present experi-

ments.

Results

1. *Background responses under illumination*

Oryzias latipes, like many other teleost fishes, shows color changes of the skin to background tones in light. When the fishes that had been adapted to a black background were transferred to a white background, they became complete pale (MI =1) within 5 min. The time graph of the background response was composed of the initial rapid stage and the following slow one. On the other hand, when the fishes were transferred to a black background, whose fishes had been adapted to a white background, they became almost dark (MI=4) during the initial 15 min, but their complete darkness (MI=5) required a longer time, about 30 min. Thus, in the time courses of the background responses, the white background response was faster than the black background one. These background responses are shown in Fig. 2.

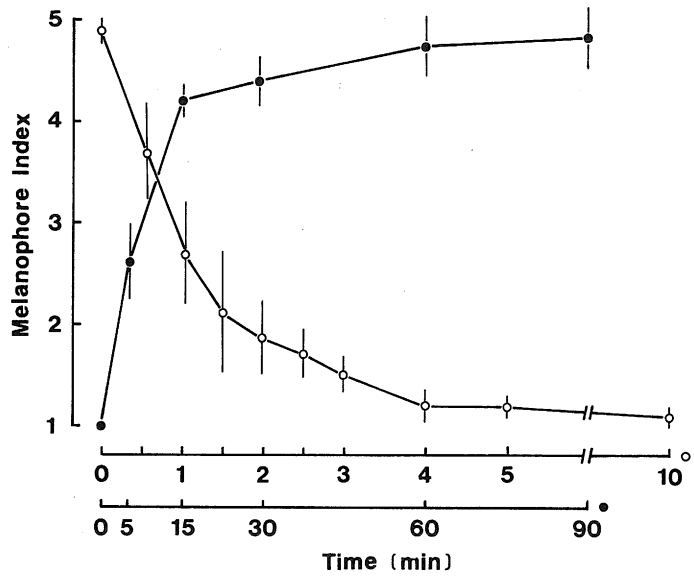

Fig. 2. Background responses of the melanophores of *Oryzias latipes* in light. ○, white-backgnround response. ●, black-background response. Each point is the mean of measurements from 5 fishes. Vertical lines indicate standard deviations. Note the representation of the time axis in each background response.

2. *Pigmentary responses to darkness*

After adapted to a dark background, the fishes were transferred to complete

darkness. They took intermediate tones in darkness. The time graph of the response is shown in Fig. 3. The MI indicated 2.8 and 2.4 at 6 hr and 12 hr respectively after transferring the fishes to darkness. After that, the paling proceeded slowly and equilibrium in darkness was only reached after about 24 hr, a very much longer time than that required for illuminated background equilibrium. We shall designate this *in vivo* response as a darkness response for convenience.

3. *Effects of various operations on the darkness response*

Eye removed fishes and hypophysectomized ones showed the darkness response as the intact fishes did. Pinealectomy also did not affect the response.

On the other hand, both spinal sectioned, and sympathectomized fishes lost their ability to respond to darkness, maintaining their skin color dark in darkness. These results are illustrated in Fig. 3.

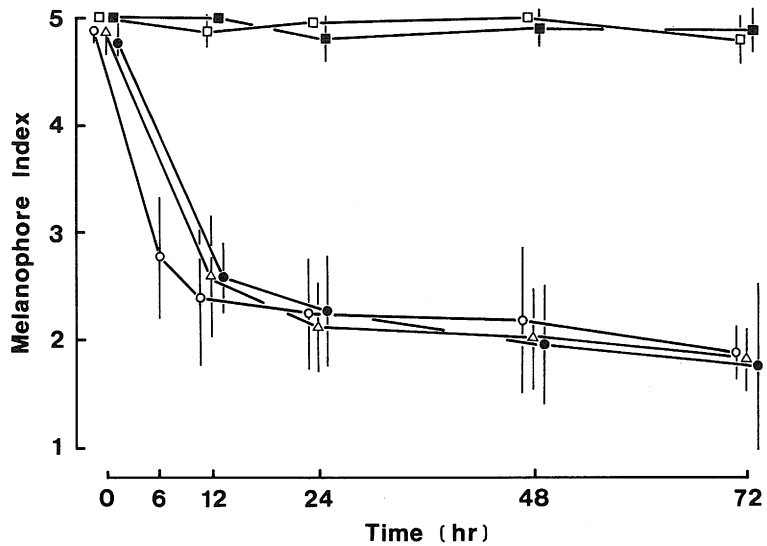

Fig. 3. Responses of the melanophores of intact and operated fishes to darkness. The fishes, which had been previously adapted on a black background under illumination, were transferred to darkness. ○, intact; ●, eye removed; △, hypophysectomized; □, spinal sectioned; ■, sympathectomized. Each point shows the mean of measurements (N=5). Vertical lines indicate standard deviations.

The experiments of spinal section and of denervation suggested that the darkness response was induced by an activity of the sympathetic adrenergic nerves. Thus, effects of adrenergic blocking agents were examined. The fishes were placed in tap water containing 50 μM dibenamine (Kanto Chemicals), an alpha adrenergic antagonist. The treated fishes did not show a pale tone in darkness, keeping their skin

color dark (Fig. 4). Addition to, these fishes did not show the background responses in light.

All experimental results described in this section are summerized in Table 1, where the MI is shown as the value at the time of 12 hr after exposure to darkness.

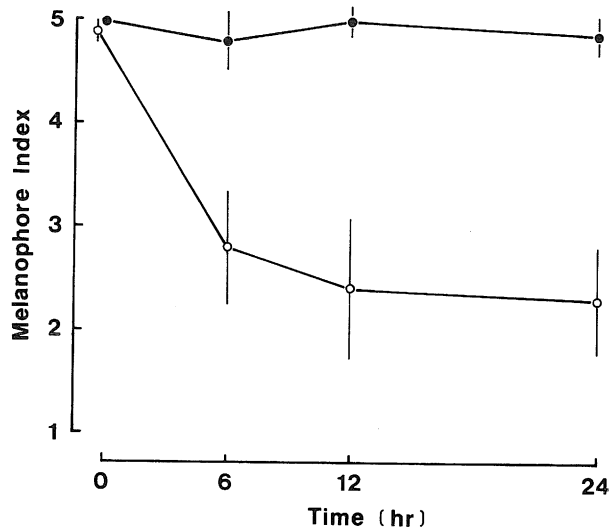

Fig. 4. The effect of dibenamine, an alpha adrenergic antagonist, on the darkness response of melanophores of *Oryzias latipes*. The fishes, which had previously been adapted on a black background in light, were transferred to darkness. ○, intact; ●, treated with 50 μM dibenamine. Each point shows the mean of measurements (N=5). Vertical lines indicate standard deviations.

Table 1. Effects of various operations on the responses of *Oryzias latipes* melanophores to darkness.

Condition of fish	Melanophore index (±SD)
Intact	2.4±0.67
Eye removed	2.6±0.32*
Hypophysectomized	2.5±0.64*
Pinealectomized	2.7±0.65*
Sham-pinealectomized	2.3±0.40*
Spinal sectioned	4.9±0.09+
Sympathectomized	5.0±0.02+
Dibenamine-treated	5.0±0.09+

Melanophore indexes are given with the values when the animals (N=5) were subjected to darkness for 12 hours. *, not significantly different from intact animals ($P>0.4$). +, significantly different from intact animals ($P<0.01$).

Discussion

For reading the MI of the skin melanophores in this experiments, fishes were quickly fixed by hot water. The MI shown by this method appeared to express correctly the state of melanophores just before fixation. Hence, this method can be recommended as a simple but accurate one in small fishes, although the method is not able to read in series the MI of a given fish.

In light, *Oryzias latipes* became pale within 5 min when they were transferred from a black background to a white one, whereas they took about 15 min to become dark when white adapted fishes were placed on a dark background. Hypophysectomy in the present fish did not appear to affect the time course of the background responses (data was not shown). In isolated scale preparations of *Oryzias latipes,* the time course of aggregation response of melanophores was comparable with that of *in vivo* melanophores on a white background. On the other hand, the course of melanosome dispersion took more times in *in vivo* melanophores on the black background response. These rapid background responses can be explained solely by changes in activities of melanosome aggregating nerves, if some endocrine organs play a role on their color changes,because there is no evidence of the existence of melanosome dispersing nerves. In intact fishes, the aggregating nerves may maintain a slight tonic influence even when the fishes were trasferred from a white background to a dark one and produce a darkness equilibrium at fairly low MI value.

Oryzias latipes became pale in darkness and its pigmentary behavior in darkness resembled that in *Phoxinus laevis* and in *Fundulus heteroclitus*. On the other hand, the time-course of the response to darkness was significantly slow in comparison with that of the background response. This suggests that the mechanisms which are responsible for the color changes following transition from illuminated backgrounds to darkness are different from those following a change of illuminated background.

Removal of the eye did not affect the darkness response of *Oryzias latipes*. This result agreed with that obtained in the catfish, *Ameiurus nebulosus* (cf. Waring, 1963) and *Ictalurus melas* (Khokhar, 1971). Thus, the eye is not responsible for the darkness response in fishes.

Quite lately, melanin-concentrating hormone (MCH) was extracted from chum salmon pituitary (Kawauchi et al., 1983). MCH was effective enough to induce melanosome aggregation on melanophores in some teleost fishes including *Oryzias latipes* (Obika and Negishi, 1985; cf. Fujii and Oshima, 1986). Hypophysectomy in *Oryzias latipes* did not affect the response to darkness, as observed in other hypophysectomized fishes (cf. Waring, 1963; Khokhar, 1970). Therefore, the pituitary gland does not appears to play a significant role in regulating the response of melanophores to darkness.

Circadian rhythm in color changes has been reported in some teleost fishes. Reed (1968) observed that the pencil fish, *Nannostomus beckfordi anomalus,*

changed its colorations with day and night. Blinded fishes in normal day-night lighting showed normal color changes indistinguishable from intact fishes. He suggested that the pineal organ played an important role on their circadian pigmentary changes. Kavaliers et al. (1980) have reported that *Fundulus heteroclitus* displays a circadian rhythm of color changes. Pinealectomy in the fish eliminated the circadian rhythm of color change but did not affect physiological color changes and background responses. Urasaki (1971) reported that pinealectomy in *Oryzias latipes* did not affect its color change. In this experiments, pinealectomized fishes became pale in darkness as intact fishes did so. The darkness response in *Oryzias latipes* was significantly slow unlike the change from day-pattern to night-pattern in the pencil fish, whose change took place over 15–30 min. Thus, the pineal organ does not appear to participate directly in the response induced by darkness.

Healey (1951) showed that in the minnow, *Phoxinus laevis,* spinal sectioned fishes can still show a slow color change in darkness, but the MI value at equilibrium in darkness was higher than that obtained in intact fishes. Further, the condition of darkness equilibrium in the case of the spinal minnow is only reached after a much longer time than that required by the intact minnow. He discussed these differences in chromatic behavior of normal and spinal minnows on the basis of double innervation. However, the interpretation of these results remained uncertain.

In *Oryzias latipes,* however, the spinal fishes never responded to darkness with melanosome aggregation, remaining their skin dark in darkness. Chemically sympathectomized fishes also behaved in the same manner in darkness as the spinal ones did. These results strongly suggest that the sympathetic nerves are responsible for the darkness response in *Oryzias latipes.* Dibenamine-treated fishes also did not become pale in darkness, indicating that the aggregating response of melanophores to darkness is mediated through alpha adrenoceptors of the melanophore membrane.

Difference in results obtained in *Phoxinus* and in *Oryzias* may depend on that of mechanisms controlling the chromatophores: The former is regulated primarily neural but requires the participation of the pituitary gland for full dispersion and complete aggregation, while in the latter the chromatic responses are mainly if not exclusively neural.

The conclusion reached is that the darkness response in *Oryzias latipes* is induced solely by a role of the adrenergic aggregating nerves. At present we have no direct evidence that the aggregating nerves fire continually in darkness and it is beyond the scope of the present experiments how the tonic excitation of the aggregating nerves is brought about.

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