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Effect of Thyroid Hormones on the Stage-specific Pigmentation of the Japanese Flounder *Paralichthys olivaceus*

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ABSTRACT—Three experiments were carried out to clarify the effect of thyroid hormones on the pigmentation of larval Japanese flounder *Paralichthys olivaceus*. The first two experiments were conducted to investigate the critical concentration of thyroxine (T_4) which causes abnormal pigmentation, and the third was to determine the sensitive stage of larval development at which albinism is induced by exogenous T_4.
The larvae treated with over 10 nM T_4 showed a significant increase of albinism in Experiments 1 (concentration: 0, 1, 10, 50 nM) and Experiment 2 (concentration: 0, 1, 5, 10 nM). In Experiment 3, seven groups were immersed in 10 nM T_4 at specific stages: between A and B, C and D, E and F, G and I, C and F and A and F. The groups of hormonal treatment at stages E and F (prometamorphosis) produced more than 90% albinism, indicating that differentiation of adult-type melanophores took place at this point. Another 4 groups of larvae were separately treated with levels of 10 nM T_4 at stages A and B, C and D, E and F and G to I. T_4 content of larvae increased corresponding to the developmental stage 180 ng/g-wet weight in A and B, 740 in C and D, and 1350 in E and F, but decreased to 130 in stage G to I ($n=3$, $p<0.01$). Changes in the body content of T_4 were reflected in the groups with higher incidence of albinism. The present experiment has revealed the T_4 sensitive period for the induction of albinism in Japanese flounder. The absence of normal coloration in the juveniles after T_4 treatment clearly indicates the involvement of the thyroid in pigmentation at the early stages of development.

INTRODUCTION

Flounder larvae show a symmetrical body structure until the onset of metamorphosis. During metamorphosis, one of the eyes migrates from one side of the body to the other and other asymmetrical changes occur at the same time. These morphological changes cause a gradual shift in the body axis. A concomitant asymmetric differentiation occurs on the skin pigmentation pattern after the metamorphic climax, rendering a brownish coloration on the ocular side of the body while the blind side becomes white.

Pigmentation of flatfishes, especially Japanese flounder *Paralichthys olivaceus* has been the target of numerous studies (Fukusho *et al.*, 1986; Kanazawa, 1993; Seikai, 1985). Abnormal pigmentation, identified by the lack of black color on the ocular side, has been widely observed in the fingerling production (Seikai, 1985). The appearance of unpigmented juveniles render them more susceptibile to predation when released into the wild (Houde, 1971), besides lowering their market value. It is therefore necessary to understand the mechanism of color anomaly and establish methods for its effective prevention.

Many environmental, genetic and nutritional factors have been suggested as possible causes of albinism in flatfish (Houde, 1971; Seikai *et al.*, 1987a; Tabata, 1991; Dickey, 1993; Gronas *et al.*, 1993; Denson and Smith, 1997; Baker *et al.*, 1998). Kanazawa (1993) suggested that the rodopsin production in the retina of fish is inhibited if the diets deficient in vitamin A, docosahexaenoic acid (DHA) and phospholipids were administered to the fish, resulting in the interruption of melanin synthesis.

Adult type of melanoblasts, derived from neural crest cell, normally appear on both sides of the body during the stages prior to metamorphosis (Seikai, 1992). Flounder metamorphosis is mediated by thyroid hormones (Inui and Miwa, 1985; Miwa and Inui, 1987a; Tagawa *et al.*, 1990; Inui *et al.*, 1995) and the response of the retina to thyroid hormones has been a subject of recent studies. The impairment of the response

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when thyroid hormone blockers are used suggests changes in the composition of the visual pigments and/or a decrease in neural crest cell migration (Nacario, 1983; Rockwood and Maxwell, 1996; Alexander *et al.*, 1998; Hutchison and Iwata, 1998). Histological changes in thyroid stimulating hormone (TSH) cells and in thyroid gland have been observed during the metamorphosis of Japanese flounder (Miwa and Inui, 1987b). A marked increase in thyroid hormone concentration was detected at the metamorphic climax (Tagawa *et al.*, 1990).

The metamorphic climax is believed to be the critical period for pigmentation of flounder larvae and probably influenced by thyroid hormones. So far, no experimental study has reported the relationship between albinism and thyroid hormones in flounder larvae. Hence, this study was designed to examine the regulatory roles of thyroid on pigmentation at the early stages of development.

MATERIALS AND METHODS

Feeds and Feeding

The larvae were fed rotifers *Brachionus plicatalis* from day 3 to 20 and *Artemia* nauplii (Utah strain, Bio Marine) from day 11 until day 40 after their hatching. Rotifer and *Artemia* nauplii were maintained at 20°C and enriched with DOCOSA-EM (containing over 50% docosahexaenoic acid, Harima Chemical Co. Ltd.). The enrichment solution was added 2 ml per 100 liter of the rotifer culture medium (250 ind. rotifer ml^{-1}) and 5 ml per 100 liter of the *Artemia* culture medium (100 ind. *Artemia* ml^{-1}) for 16 hr.

Experimental Animals and Rearing Conditions

For the first two experiments, fertilized eggs of Japanese flounder were obtained from the Miyazu Station of Japan Sea-farming Association (JASFA), and for the third experiment from the broodstock of the Fisheries Research Station of Kyoto University. Developmental stages were classified according to Minami (1982). The characteristics of the larvae and juvenile ranging from 3.25 mm (stage A) to 13.20 mm BL (stage I) were described according to the metamorphic pattern; premetamorphosis (stage A to E), climax (stage F to H), postclimax (stage I), and juvenile.

Eggs were hatched at 16°C in a 500 liter polycarbonate tank. The rearing temperature was gradually raised to 18±0.5°C in a water bath. The rearing area was illuminated, for 12/12 (L/D) photoperiods at 500±300 lux (measured at the surface of the tank) illumination. Fifty to 100% of the rearing water was changed daily.

Experiments 1 and 2

On the first day post-hatching (dph) five and four groups of 300 and 400 larvae were stocked into 30 liter transparent tanks in Experiments 1 and 2, respectively. In Experiment 1, the following concentrations of T_4 (L-thyroxine sodium salt) were dissolved in the rearing water: 1 nM, 10 nM, 50 nM, 100 nM and none (control). In Experiment 2, the concentrations used were 1 nM, 5 nM, 10 nM, and none (control). T_4 was dissolved in dimethyl sulfoxide (DMSO). The hormone concentrations were kept constant until 30 dph. At the end of the experiment (40 dph), all fish were harvested and checked for abnormal pigmentation on the ocular side.

Experiment 3

One day after hatching, 7 groups in duplicates of 600 larvae were stocked into 30 liter transparent tanks. Larvae were treated with 10 nM T_4 during stages A and B, C and D, E and F, G to I, C to F and A to F, and the experimental groups were designated as AB, CD, EF, GI, CF and AF, respectively (see Fig. 1). At the end of the experiment, 6 individuals (about 100 mg-wet w./fish) per tank were sampled for hormone analysis. The other fish were harvested and preserved in 10% formalin, and used for total length (TL) measurement and specific growth rate (SGR) calculation (Casselman, 1987). The pigment index of harvested juveniles was calculated as per the following equation: $\Sigma OCA/\Sigma OTA \times 100$, where ΣOCA is the total normally colored area on the ocular side of all survivors, and ΣOTA is total area of the ocular side of all survivors in a group (Seikai, 1985).

In conjunction with Experiment 3, four groups of larvae were separately treated with the same dose of T_4 in 30 liter tanks during the stages A and B, C and D, E and F and G to I. The larvae of these

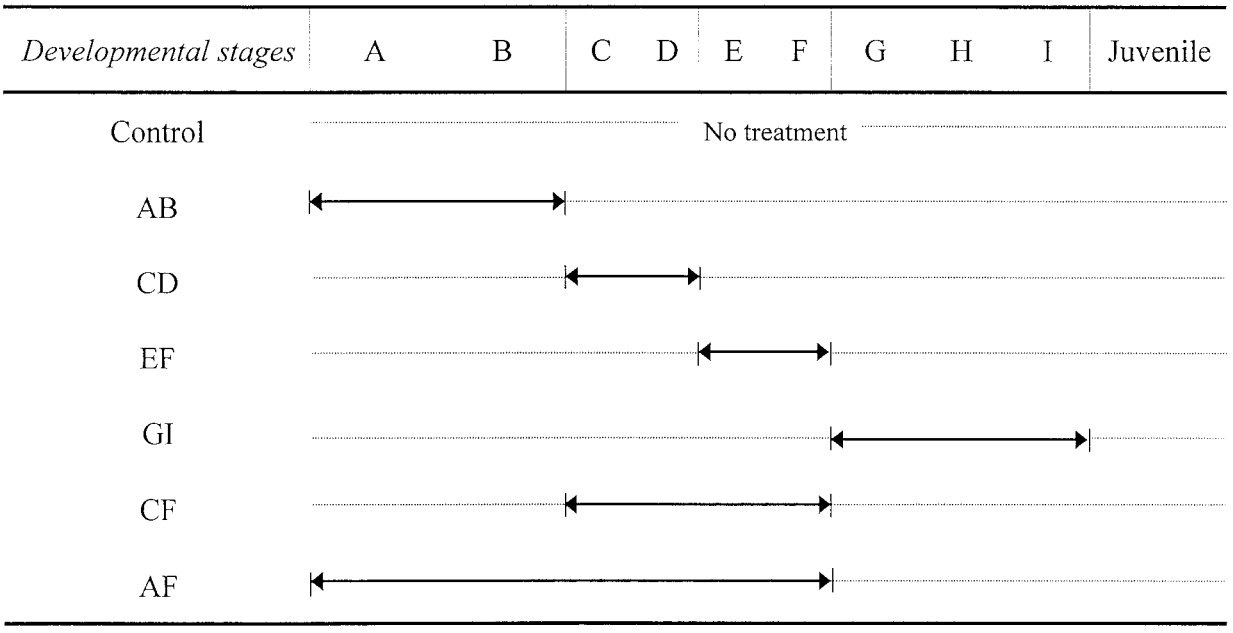


Fig.1. The design of Experiment 3, indicating timing of T_4 treatment, normal pigmentation and judgment of albinism. ····· Culture periods; ◀—▶ immersion periods with 10 nM T_4 .

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treatments were sampled just after the end of T_4 treatment to analyze the T_4 level of the larvae at each developmental stage. All samples, each consisting of about 300 mg of larvae, were stored frozen at –40°C until analysis.

Radioimmunoassay

Hormone extraction and radioimmunoassay were carried out following the procedure described by Tagawa and Hirano (1987). Frozen samples of fish were homogenized for determining the whole body concentration.

Statistics

All data are presented as mean ± standard errors (SE). Analysis of variance (ANOVA) was applied to determine if there was a significant difference ($p<0.05$) among the groups (Steel and Torie, 1980). Turkey's multiple test was applied to compare the means of total length, % survival, % albinism and pigment index between the different groups.

RESULTS

Effect of T_4 concentration on albinism of flounder in Experiments 1 and 2

Figure 2 shows relationship between albinism rate and various T_4 concentration in the Experiments 1 and 2. In Experiment 1, the albinism rates in control (untreated fish) and 1 nM treated fish were 33 and 30%, respectively, and they increased 54% at 10 nM T_4 and 70% at 50 nM T_4. Fish treated with 50 nM T_4 had lower growth rate and higher mortality, while those treated with 100 nM T_4 entirely perished after 19 days (data not shown). In Experiment 2, the control, 1 nM and 5 nM groups showed albinism rate of about 10%, as against a rate of 26% for the 10 nM treated fish.

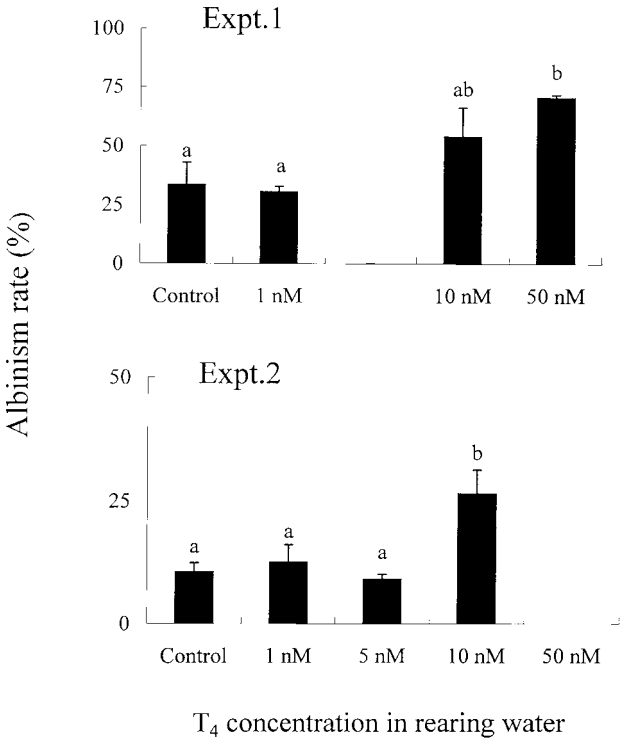


Fig. 2. Relationship between albinism rates at 40 days after hatching and T_4 concentration of rearing water in Experiments 1 (upper) and 2 (bottom). Values are the means for duplicate treatment. Vertical lines show standard errors. Different superscript letters indicate significant differences (ANOVA, $p<0.05$) among experimental groups.

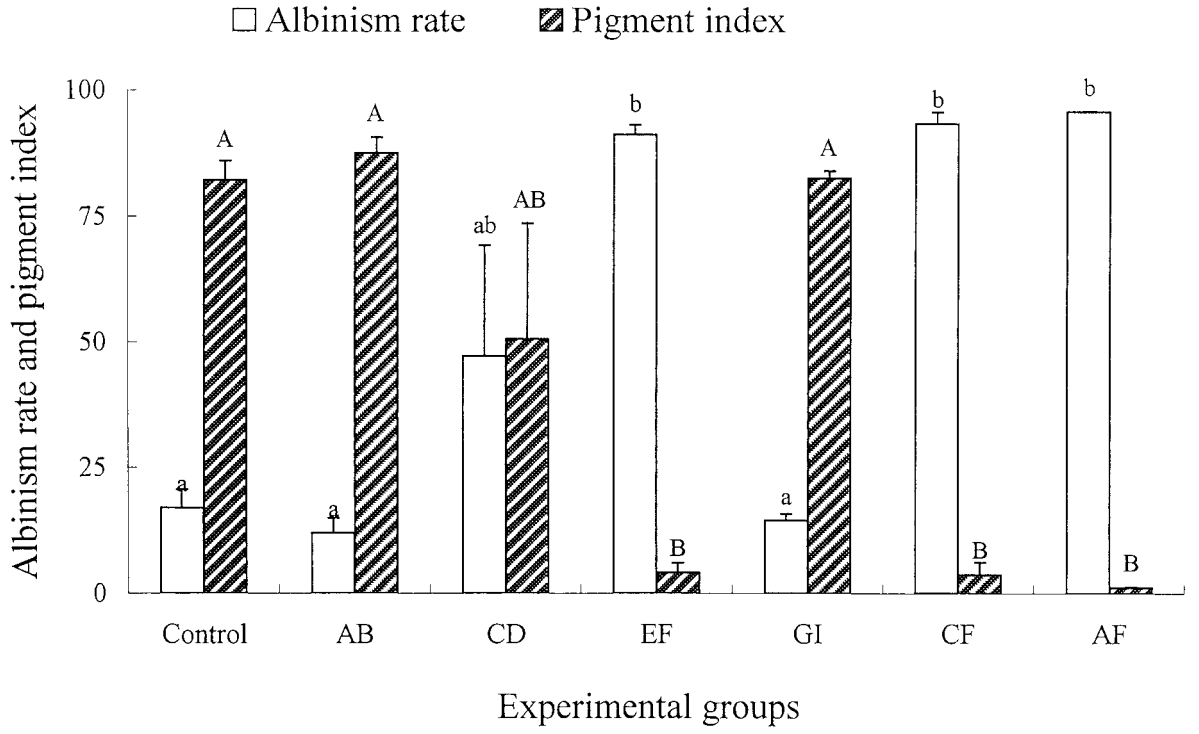


Fig. 3. Albinism rate and pigment index of the final fish sample for 10 nM T_4 treatment. Vertical lines show standard errors and different superscript letters indicate significant differences (ANOVA, $p<0.05$) among experimental groups in albinism rate (a, b) or pigment index (A, B).

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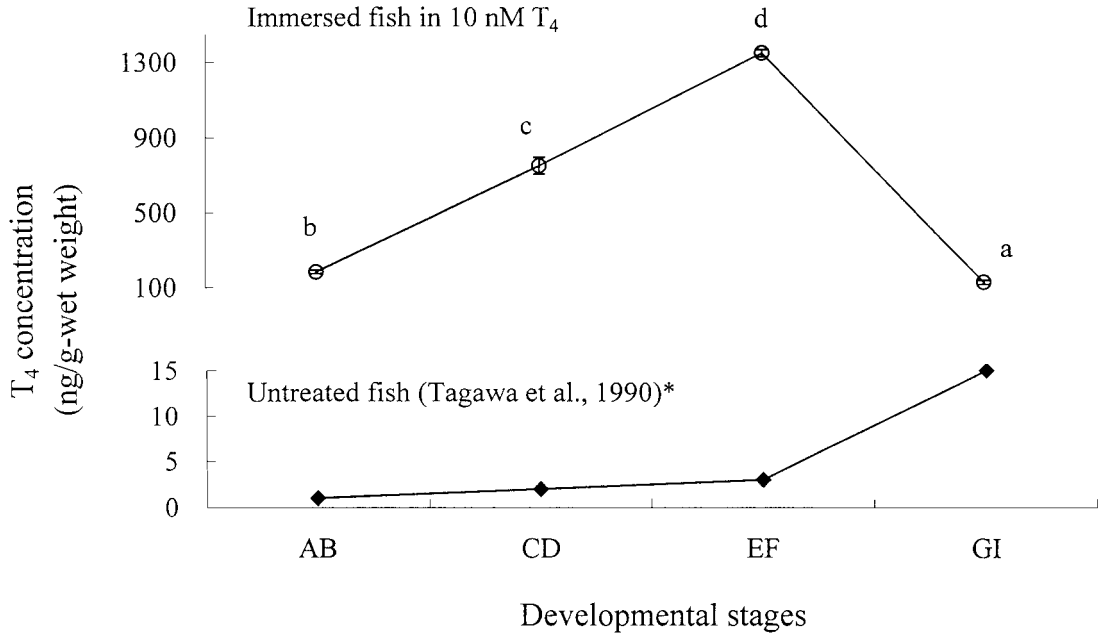


Fig. 4. T_4 concentration in the larvae treated with 10 nM T_4 at different stages of development. Sample was taken just at the end of T_4 treatment. Values are the means of triplicate measurements.
Vertical lines show standard errors and different superscript letters indicate significant differences among experimental groups (ANOVA, $p<0.05$).
* This data is quoted from Tagawa *et al.* (1990).

Survival rate, growth rate and T_4 concentration in flounder in Experiment 3

Survival rate, total length, and specific growth rate (SGR) and T_4 concentration of the different groups at the end of experiment (40 dph) are shown in Table 1. Survival rates in all groups were about 60%, hence not significantly different. Final total length varied between 17.1 mm to 19.9 mm, and SGR varied between 4.3 and 4.6 per day with no significant differences among the groups. The accumulation of exogenous T_4 by the juveniles in all groups at 40 dph were not significantly different: 7.7 ng/g-wet weight in control, 9.3 in AB, 9.4 in CD, 7.4 in EF, 7.8 in GI, 6.4 in CF and 6.4 in AF, respectively.

Effect of 10 nM T_4 on the stage-specific pigmentation in Experiment 3

Albinism rate and pigment index on treatment 10 nM T_4 are shown in Fig. 3. Larvae were treated with T_4 during the period spanning stages E and F (prometamorphosis) showed a significantly higher incidence of albinism ($p<0.05$), 91% in EF group, 94% in CF group and 96% in AF group. The control, AB and GI groups had almost the same albinism rate of about 15%. Varied values (25% and 70%) were observed when larvae were treated with T_4 during C and D stages (CD group). Pigment indices were significantly different among the treatments ($p<0.05$): 82% in control, 88% in AB, 51% in CD, 4% in EF, 83% in GI, 4% in CF and 1% in AF. Abnormal pigment pattern of albinic fish were similar to those of types I to III (these groups were almost all albino on the ocular side) as described by Seikai (1985).

Radioimmunoassay; Influence of Exogenous T_4 on Larval Development in Experiment 3

Figure 4 shows the T_4 contents of the larval body just at the end of T_4 treatment during different stages of development. T_4 content in wet basis increased from 180 ng/g in AB group to 1350 ng/g in EF group, and decreased dramatically to 130 ng/g in GI group. These values are shown in comparison with the normal values reported by Tagawa *et al.* (1990) in the figure. The normal T_4 levels in flounder ranged between 1 and 15 ng/g during development stages A to I.

DISCUSSION

This study showed that treatment with 10 nM T_4 at the developmental stages E to F induced a high incidence of albinism (about 90%), while in the CD stage the rates varied and at the AB stage it was the least. The stage E and F seems to be the peak period for pigment formation while the CD stage contains melanophores in the process of development. In a previous study using Brazilian strain of *Artemia,* Seikai (1985) reported that stage D is critical for determining juvenile coloration. The differing results could be due to the manipulation techniques employed; direct T_4 administration in the rearing water allows the hormones to reach the target cells quickly through the plasma, while live food needs more time to be digested and absorbed to exert its effect on pigmentation. Upon considering that only 3 to 5 days are needed for the larvae at stage D to reach stage E at 18°C, it is could be possible that the materials included in the live food ingested by larvae at stage D reached their final destination (melanoblasts in the skin) by stage E. Kanazawa (1993) pointed out that the larvae

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Table 1. Survival, growth, SGR and T_4 concentration of Japanese flounder larvae after 40 days of hatching

		Experimental groups						
	n	Control	AB	CD	EF	GI	CF	AF
Survival rate (%)	2	51.2±5.7	67.1±1.9	62.1±5.1	60.7±11.5	51.8±5.4	62.2±1.4	62.7±3.2
Total length[1] (mm)	100	19.9±2.1	17.9±1.7	17.3±1.9	17.1± 1.7	18.9±1.6	17.5±1.9	18.5±2.0
SGR[2]	100	4.6±0.06	4.4±0.01	4.3±0.08	4.3± 0.07	4.5±0.01	4.3±0.01	4.4±0.09
T_4 (ng/g)	6	7.7±1.5	9.3±1.7	9.4±0.7	7.4± 1.1	7.8±1.2	6.4±0.7	6.4±0.5

[1] Initial length=3.12 ±0.1 mm.
[2] Specific growth rate (day^{-1})={(Log_e YT-Log_eYt)/(T-t)}×100,
YT is final length (at time T) and Yt is the initial size (at time t).
– Each value represents mean±SE.

at 8–9 mm of total length is in a critical stage for pigmentation, which corresponds to stage E to F. Based on these points, it is reasonable to consider stage E as the critical stage for pigmentation of Japanese flounder.

Thyroid hormones play a major role in controlling the development of the normal pigmentary pattern by secreting melanocyte stimulating hormone (MSH) involved in the synthesis of melanin and the regulation of chromatophore motility (Gorbman, 1969; Inui *et al*., 1995). T_4 administration to frog induced bleaching of the skin color, suggesting an inhibition of MSH release by T_4 from the pars intermedia of the pituitary by Chang (1957). The direct action of thyroid hormone on pigment cells has been described in an *in vitro* study using frogs (Wright and Lerner, 1960).

The T_4 uptake capacity of the larvae is related to development, increasing as metamorphosis progresses and decreasing thereafter. The very high occurrence of abnormal pigmentation (up to 90%) were characteristic in fish treated with T_4 from E to F stage, when the content of the hormone in the body was at the maximum level, suggesting that the mechanism of pigmentation strongly depends on thyroid hormones. However, the high T_4 concentrations during the metamorphic stages caused by exogenous T_4 treatment, decreased to a level comparable to control fish when the fish metamorphosed and became juvenile (Table 1).

Mammalian pigmentation depends upon the transport of the melanogenic substrate (tyrosine) from the cytosol into melanosomes . The initial, rate-limitation step in pigment production is the hyrdroxylation of tyrosine to DOPA (dihydroxyphenylalanine), which is catalyzed by tyrosinase, one of the key enzymes essential for melanogenesis (Ferguson and Kidson, 1997; Kameyama *et al*., 1995). Seikai *et al*. (1987b) reported that tyrosinase activities in the totally albinic both a ocular and blind side juveniles were of background level. The result indicate that in the skins of metamorphosing larvae of Japanese flounder, increase and decrease of tyrosinase activities are precedent or concomitant with the appearance and disappearance of melanophores.

Abnormal pigmentation in flounder is related to changes in the skin structure prior to metamorphosis, and the development of the larval and adult types of melanophores. Probably albinism appears when the normal development of adult type melanophore is interfered (Seikai *et al*., 1987b). Neural crest cells, which gives rise to melanophores (DuShane, 1934), first emerge on the dorsal surface of the developing neural tube as the neural keel differentiates from the epidermis (Hearing, 1998). During metamorphosis nerve fibers and nerves get linked by synapses (Rager and Rager, 1978), and exogenous thyroxine accelerates metamorphosing process, providing little time for synaptic junctions to form and trigger the normal development of the adult-type melanophore.

Thus the present experiment, revealed the sensitive period during which albinism may be inducted in Japanese flounder. The absence of normal coloration in the juveniles after T_4 treatment clearly indicates an involvement of the thyroid in pigmentation at the early stages of development. The factors controlling the development of pigment cells in fish are not well understood, however we suggest that the action of thyroid hormones on albinism may follow one or more of the listed mechanisms:

1) Thyroid hormones might inhibit tyrosinase expression, impeding the production of melanin from the amino acid tyrosine.

2) Thyroid hormones might induce apoptosis of melanophores.

3) The acceleration of metamorphosis in Japanese flounder might impede the nerve fibers from reaching their target synapses responsible for triggering melanphore development.

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