INTRODUCTION

A considerable body of information has been compiled on the role of thyroid hormones in the early development of animals. Thyroid hormones have been reported to enhance growth and differentiation in fishes, and to reduce mortality during the early phase of fish life.1–4 The important roles of hormones in pigmentation and habitat transfer during metamorphosis have been demonstrated in salmonids,5 black seabream,6 and red seabream.7,8 The coral trout Plectropomus leopardus experiences two transformations during its early life,9 similar to Hexagrammos agrammus.10 The first transformation occurs at approximately 11–12 mm in standard length (SL), when larvae transform to juveniles by completing most of their fin formation and the ossification of vertebrae. The second transformation is at approximately 20 mm SL when resorption of the dorsal and pelvic fin spines takes place. Settlement, pigmentation, and a surge of thyroxine also occur during this second metamorphosis.9

To confirm the contribution of thyroid hormone to the second metamorphosis of this species, juveniles between the first and second transformations were treated with thyroxine (T4) and thiourea (TU; an inhibitor of thyroid hormone synthesis). Morphological changes were described as indicators of the second metamorphosis.

MATERIALS AND METHODS

Preparation of treatment media

A stock solution of T4 was prepared by dissolving 100 mg of L-thyroxine sodium salt (Nacalai Tesque, Kyoto, Japan) in 20 mL of absolute ethanol with a few drops of 1 N NaOH to increase solubility, and kept at 4°C. Because TU (Nacalai Tesque) dissolves
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day 3, 6, 9, and 13. The experiment was terminated on day 13, corresponding with the complete pigmentation of fish in the control group. In addition, on days 0, 3, and 13, three fish from each tank were anesthetized, fixed in Bouin’s solution, and examined histologically. Three more fish from each tank were immediately frozen at –80°C for T4 analysis.11,12

Because pigmentation is a good measure of metamorphosis in this species, as shown in a related species,13 the pigmentation index (PI) was determined according to the following stages: A, chromatophores are present only on the dorsal surface of the head; B, a small number of chromatophores appear on the anterior dorsal part of the body close to the head; C, many chromatophores are found on the dorsal side and around the top of the abdomen; D, chromatophores cover 50–75% of the body surface; and E, completion of pigmentation (covering most of the body). More than 20 fish were observed and classified before fixation. The numbers of fish that always stayed along the bottom and swam upward only to take prey were counted in the tanks, and the ratio was used as an indicator of settling behavior.

easily in seawater, TU crystals were added directly to 1 cup of seawater and poured into the experimental tanks.

Rearing experiment

Juveniles, aged 35 days [SL 16.71 ± 0.19 mm (mean ± SD); N=27], previously reared in a mass culture by feeding with rotifer, Artemia nauplii, and an artificial diet were used for the experiment. Thirty fish were randomly selected and stocked in a 25-L black, circular, polycarbonate tank with mild aeration. Using a tank for each group, the experimental groups comprised the control group, the T4 (0.1 p.p.m) group and the TU (30 p.p.m) group. One-fifth of the rearing water was changed daily after cleaning the bottom. Water temperature and dissolved oxygen were 28.6 ± 0.1°C and 4.5 ± 0.1 p.p.m. (mean ± SD), respectively. Survival rates for all groups were nearly 100% during the experiment.

At least five fish from each tank were anesthetized by adding several drops of 1% MS222 solution, and were then fixed in 10% formalin on day 0 (day experiment was commenced), and days 3, 6, 9, and 13. The experiment was terminated on day 13, corresponding with the complete pigmentation of fish in the control group. In addition, on days 0, 3 and 13, three fish from each tank were anesthetized, fixed in Bouin’s solution, and examined histologically. Three more fish from each tank were immediately frozen at –80°C for T4 analysis.11,12

Fig. 1 Distribution of pigmentation indices (PI) on days 0, 3, 6, 9, and 13 of the experiment, which are categorized from stages A–E. T4, thyroxine; TU, thiourea.
Statistical analysis

The effect of treatments on total and standard length (TL, SL), dorsal and pelvic fin spine lengths (DSL, PSL), pigmentation index (PI), serration reduction on the dorsal spine (non-serrated spine, NS), and settlement (ST) were tested for significant differences by ANOVA followed by the Tukey HSD multiple means comparison test using the computer program SPSS (1998) (SPSS Inc. Chicago, IL, USA). The percentage data were transformed to arcsin-square root values before testing by ANOVA.14

RESULTS

As shown in Fig. 1, all fish in the T4 group were synchronized and fully pigmented (stage E) on day 3, whereas only 17% in the control group and 0% in the TU group had complete pigmentation. By day 13, all fish in the control group had complete pigmentation, whereas 28% of the fish in the TU group were fully pigmented (Fig. 1).

As shown typically in Fig. 2, fish in the T4 group on day 9 had an opaque body, and their color was a bright reddish-orange with dark spots and whitish horizontal stripes when alive; a similar color to that of the metamorphosed fish. Thiourea-treated fish had a transparent body with a dark color on the anterior dorsal and lateral fins, and fish in the control group were midway between both treatments. On day 13 (data not shown), the body coloration of fish in the control and T4 groups was similar; that is, a reddish-orange color with dark spots. Some fish in the TU group had complete pigmentation with an abnormal black color while others were still widely transparent with some black coloration in some areas. No fish in the TU group showed any reddish or orange coloration throughout the experimental period.

Detailed observations of the melanophores took place on day 3 (Fig. 3). Some melanophores were stellate in shape while others were similar to the black dots seen in the control group (C3). In the TU group (TU3), most melanophores were stellate, whereas in the T4 group (T4 3) most of the melanophores were similar to black dots. On day 13, most of the melanophores in the TU group were large and stellate in shape (TU13).

Histological observations on day 3 revealed an increase in the epidermal thickness of the dorsal skin of the T4 group compared with that of the control and TU groups (Fig. 4). In the T4 group, melanophores were located over and below the dermal layer, whereas they were present only under the dermal layer in the control and the TU group.

The occurrence of non-serrated dorsal and pelvic fin spines (NS) increased significantly ($P<0.01$) in the T4 group compared with the control and TU groups on day 3 (Fig. 5). The difference among groups became smaller by day 13, but was still significantly higher in the T4 group. Settlement behavior (ST) was also significantly stimulated in the T4 group (Fig. 5). All fish in the T4 group completed settlement by day 3, whereas no settled fish were observed in the TU group by day 3. In the control group, only 1% of the fish had settled by day 3; however, by day 13, all fish in the control group had finished settlement, but significantly fewer fish had settled in the TU group.

There were no significant differences in the TL and SL among all groups on day 3 (Fig. 6); but SL was significantly shorter in the T4 group on day 13.
Supplementation of T4 into the rearing water accelerated the resorption of the dorsal and pelvic fin spines. Conversely, TU retarded the resorption of both spines; these spines were calcified judging by alizarin red staining. A similar effect on larval fin resorption has been clearly observed in Epinephelus coioides and the Japanese flounder Paralichthys olivaceus. In rats, Mundy and colleagues have shown a direct increase in osteoclastic bone resorption by T4 and 3,5,3'-triiodo-L-thyronine (T3) in vitro, which could be the reason for the enhancement of larval fin spine resorption by T4 treatment in the coral grouper. A stimulating effect by T3 in bone formation and resorption in rainbow trout Oncorhynchus mykiss has also been reported.

In the TU group, changes in morphological characteristics (fin rays and color) were significantly inhibited, but settling behavior was inhibited to a lesser degree. Similar differential effects of thyroid hormone have been reported in the Japanese flounder; that is, TU did not completely...
inhibit right eye migration but effectively retarded settling behavior and the resorption of elongated fin rays. There are two possible explanations for this effect. First, although TU suppressed the endogenous level of T4, a small amount of T4 might still be sufficient and necessary to stimulate settling behavior at a slow rate. Second, settling behavior does not require T4 at all. This seems to suggest differences in the extent that the thyroid hormone is required among different tissues and for behavior.

The effects of T4 treatment on pigmentation are known in several fishes; for example, silverying during smoltification in salmonid fishes, earlier appearances of melanophores on the black stripe in red seabream Pagrus major, and body color changes in the spottybelly greenling Hexagrammos agrammus. In the present study, the treatment of T4 clearly accelerated pigmentation. Thyroxine-treated fish were of an opaque reddish color, whereas TU-treated fish were transparent and partially covered by a black color. During the metamorphosis of the Japanese flounder, juvenile-type (smaller size) melanophores appear in addition to the larval type (bigger size). In the present study, the ‘appearance’ of melanophores after fixation was also affected by the long-term treatment of the thyroid hormone; that is, dot-like appearances in the T4-treated fish and stellate appearances in the TU-treated fish. The results of the experiment suggest the contribution of the thyroidal system in the function and/or formation of melanophores in the coral trout. Chromatophores other than melanophores were not examined in the study, but detailed observation of erythrophores is necessary to understand the mechanisms of T4-dependent pigmentation during the second metamorphosis of this species.

In addition to the effect on melanophores, hypertrophy of the epidermis was observed in the T4-treated group in the present study. Increased thickness of the epidermis by thyroid hormones has been found previously in platyfish and in tilapia Sarotherodon niloticus. Thickness of the dermis was also increased by T4 treatment in salmonid fish. These findings are in accordance with the results of the present experiment.

The present study has clearly demonstrated that T4 stimulates metamorphosis in coral trout, and that TU retards the process. This suggests that the thyroidal system plays a major role in the regulation of metamorphosis in the coral trout.

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Fig. 5  Percentage of fish with (□) non-serrated dorsal spines (NS) and (◆) those that settled (ST) on days 0, 3 and 13 of the experiment. More than 20 fish were examined from each tank for NS. Vertical bars indicate standard errors of the means; \( n = 3 \) tanks; T4, thyroxine; TU, thiourea. Significantly different from control at *\( P < 0.01 \) and **\( P < 0.05 \).

Fig. 6  (□) Total length and (◆) standard length on days 0, 3 and 13 of the experiment. Vertical bars indicate standard errors of the means; \( n > 20 \) fish; T4, thyroxine; TU, thiourea. *Significantly different from control (\( P < 0.01 \)).
**Fig. 7** Standard length of the dorsal (DSL; □) and pelvic (PSL; □) spines on days 0, 3 and 13 of the experiment. Vertical bars indicate standard errors of the means; \(n=15\) fish; T4, thyroxine; TU, thiourea. *Significantly different from control \((P<0.01)\).

**Fig. 8** Whole body T4 (thyroxine) levels on days 0, 3 and 13 of the experiment. Vertical bars indicate standard errors of the means; \(n=3\) pools of three fish; TU, thiourea. *Significantly different from control \((P<0.01)\).
REFERENCES