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Response of a Neotenic Goby, Ice Goby (*Leucopsarion petersii*), to Thyroid Hormone and Thiourea Treatments

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ABSTRACT—In order to clarify the mechanisms of neoteny in the ice goby (*Leucopsarion petersii*), we examined effects of thyroid hormone and thiourea (TU) treatments on their neotenic characteristics and the pituitary-thyroid axis. Adult ice goby were exposed to 3, 5, 3'-triiodothyronine (T3, 0.1 ppm), TU (inhibitor of thyroid hormone synthesis, 30 ppm), or the combination of the two for 2 weeks. Observations of whole body T3 levels, thyroid follicles and TSH immunoreactive cells in the pituitary suggests the presence of a functioning thyroidal system. However, all of the neotenic features did not disappear in T3-treated fish, suggesting the absence of T3 responsiveness in peripheral tissues. These results indicate the similarity between neoteny of the ice goby and obligatory-type neoteny of urodeles.

Key words: ice goby (*Leucopsarion petersii*), neoteny, thyroid hormone, thiourea, TSH

INTRODUCTION

Metamorphosis, widely observed in amphibian and fish species, is a drastic change in the morphology and physiology between larval and juvenile stages, being controlled mainly by thyroid hormones (Bentley, 1998). Some urodele species sexually mature without undergoing metamorphosis, and therefore mature adults have a larval appearance. This phenomenon has been well known as neoteny, and has attracted many scientists working on amphibian metamorphosis (Huxley, 1920; Lynn, 1961; Dent, 1968; Gould, 1977; Frieden, 1981; Armstrong and Malacinski, 1989; Wakahara, 1996). *Ambystoma mexicanum* is one of the most famous neotenic salamanders, and information has been accumulated on the physiological mechanisms of neoteny in this species. Since exogenous thyroid hormone can induce metamorphosis of *A. mexicanum*, the shortage of thyroid hormone secretion is considered to account for neotenic development of this species (Taurog, 1974; Rosenkilde and Ussing, 1996). However, no physiological study has been carried out concerning neoteny in fish.

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Most gobies change their life style from nektonic to benthic during transformation from larva to adult associated with metamorphosis. Air bladders are present only during the larval period, while pigmentation, scale formation and extension of the pelvic fin occur during metamorphosis (Dotsu and Uchida, 1979). Consequently, the typical appearance of gobius fish is completed during the juvenile period. However, some gobies remain nektonic throughout their life without drastic changes in appearance, and therefore are regarded as neotenic species (Dotsu, 1979). The ice goby, *Leucopsarion petersii*, is one of the neotenic gobius species. Sexually mature individuals of this species do not have any of the characteristics of adult gobies (Dotsu and Uchida, 1979). Although thyroid gland activity in relation to reproduction was previously reported (Tamura and Honma, 1970), there has been no physiological research relating thyroid gland activity to neotenic features.

This study was undertaken to clarify the mechanisms of neoteny in fish using the ice goby, especially focusing on the thyroid system. First, to explore a possible involvement of thyroid hormone in neoteny in the ice goby, we examined effects of exogenous thyroid hormone on neotenic characteristics. Second, activities of the thyroid gland and thyroid stimulating hormone (TSH) producing cells were examined

in fish treated with thyroid hormone and thiourea.

MATERIALS AND METHODS

Mature male and female ice goby (shirouo), *Leucopsarion petersii*, were caught by set net in the Isazu River, Kyoto Prefecture during their spawning migration in March 1999 and transported to the Fisheries Research Station of Kyoto University. The total length of the fish was about 5 cm. They were reared in fresh water under natural temperature (8–12°C) and natural photoperiod for three days without food, as they do not normally feed during this time. Thereafter, undamaged fish were randomly divided into 4 groups of 50 individuals each. The fish were placed in plastic tanks (10 liter) containing the following media: fresh water (control); 0.1 ppm 3, 5, 3'-triiodothyronine (T3); 30 ppm thiourea (TU); and 0.1 ppm T3 and 30 ppm TU (T3+TU). Half of the rearing water was replaced daily. The treatment was continued for two weeks.

Fish were sampled before and after the experiment. Ten fish from each group were fixed in 10% neutral formalin for 24 h and preserved in 70% ethanol for morphological examination for neotenic features. Four of the fixed fish were further cleared and stained with alcian blue and alizarin red (Dingerkus and Uhler, 1977) to observe osteological development. The neotenic features of this species (transparent body color, lack of scale, large air bladder, undeveloped pelvic fin and so on), as listed by Dotsu and Uchida (1979), were examined under a stereomicroscope (Nikon SWZ800). For observations on the thyroid gland, another 10 fish from each group were fixed in Bouin's solution for 24 h and then preserved in 70% ethanol. These samples were embedded in histoparaffin, sectioned at 5 μm thickness, and stained with hematoxylin-eosin, according to a standard procedure. From randomly selected 10 thyroid follicles per individual (n=3), the epithelial cell height was measured and the number of the vacuoles in the colloid was counted, to serve as indices of thyroid activities. Another 10 fish were fixed in 4% paraformaldehyde for 24 h to detect TSH cells in the pituitary. Paraffin sections were immunohistochemically stained following the procedures described by Kaneko *et al.* (1993). Anti-salmon TSH β (1:800) (Moriyama *et al.*, 1997) preadsorbed with salmon GTH/TSH α-subunit (625 ng/ml) was used as a primary antiserum, and visualized with commercial reagents (strept ABC complex / HRP, DAKO A/S, Denmark). Ten fish from each group were frozen at –80°C, and T3 concentrations were measured according to Tagawa and Hirano (1989).

The data on T3 concentrations and thyroidal activity were tested for significant differences by Scheffe test after one-way analysis of variance using Stat View J4.02 (Abacus concepts, Inc.).

RESULTS

Modification of T3 concentration by T3 and TU treatments

Whole-body T3 concentration was measured before and after treatment (Fig. 1). A significant amount of T3 (average 3.5 ng/g body weight) was present in fish at the beginning of the experiment, and the concentration did not change in the control group (6.5 ng/g). T3-treated fish showed significantly higher T3 concentrations (19.4 ng/g)

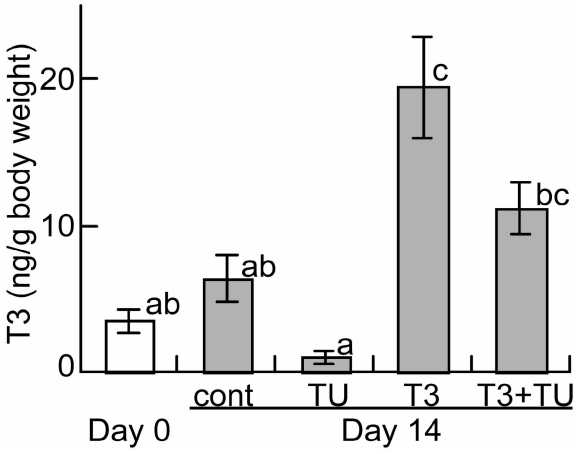

Fig. 1. Whole-body T3 concentrations of adult ice goby before and after treatments for 2 weeks. cont, fresh water; TU, 30 ppm thiourea; T3, 0.1 ppm T3; T3+TU, 0.1 ppm T3 and 30ppm TU. Vertical bars represent standard errors of the means (n=7). Means accompanied by the same letter do not differ significantly (Scheffe test, P>0.05).

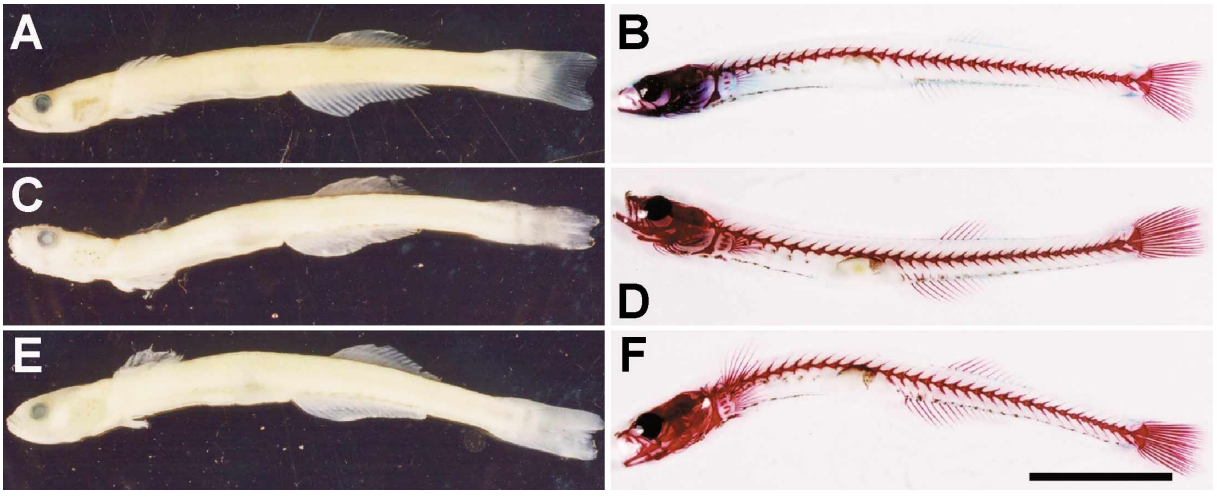

Fig. 2. Ineffectiveness of T3 treatment on neotenic features of adult ice goby. (A, B) Fish before the treatment, (C, D) 2 weeks in freshwater as control, and (E, F) 2 weeks in 0.1 ppm T3. Photographs were taken after fixation in 10% neutral formalin (left column) and further double-stained with alcian blue and alizarin red (right column). Bar indicates 10 mm.

than the control fish. Although not statistically significant, the T3 concentration in TU-treated fish was about 1/6 of that in control fish. There was no significant difference between T3+TU-treated fish and the control.

Effect of T3 on neotenic features

As shown in Fig. 2, there was no change in the appearance of the control fish during the 2-week experimental period. Moreover, T3 treatment failed to induce pigmentation, scale formation, and enlargement of pelvic fin. Air bladder did not reduce in size in T3-treated fish compared to control fish. No additional ossification was observed in any cartilage in T3-treated fish. The appearance of TU-treated fish and the T3+TU-treated fish did not differ from that of the control fish (data not shown).

Effect of T3 and TU on thyroid activity

The thyroid glands of hormone-treated fish were subjected to histological examination. Well-developed thyroid follicles were observed in the lower pharyngeal regions in control fish before and after the experiment (Fig. 3). These follicles consisted of cuboidal epithelial cells, containing colloid and vacuoles. In TU-treated fish, hypertrophy of follicular epithelial cells and shrinkage of the colloid were evident (Fig. 3B). As shown in Fig. 4A, the height of epithelial cells increased significantly in TU-treated fish (average 9.6 μm) compared to controls (5.8 μm). The cell height of T3-treated fish (5.6 μm) was similar to the control (Figs. 3 and 4A), and that in T3+TU-treated fish (3.9 μm) was slightly but significantly lower (Figs. 3 and 4A). The number of vacuoles in colloid in the control (0.08 /μm) and T3-treated fish (0.09 / μm) were similar, but that in T3+TU-treated fish (0.04 /μm) was significantly smaller than that in control fish (Fig. 4B). Vacuoles were not counted in TU-treated fish, since the colloid in the thyroid follicles was very small (Figs. 3B and 4B).

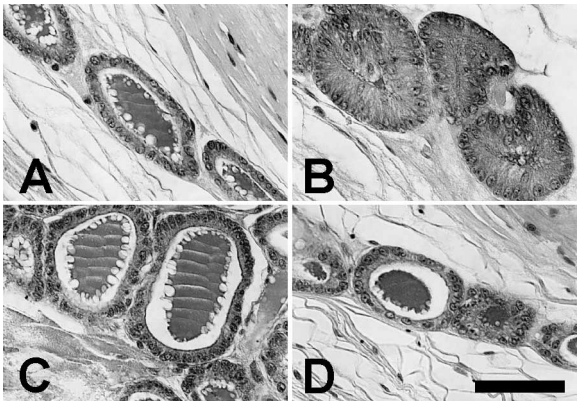

Fig. 3. Photomicrographs of the thyroid glands of adult ice goby 2 weeks after the hormonal treatment. (A) in freshwater as control, (B) in 30 ppm TU, (C) in 0.1 ppm T3, and (D) in 0.1 ppm T3 and 30 ppm TU. Note the height of the thyroid follicular epithelium and presence of vacuoles in the colloid. The thyroid follicles of TU group had thickened epithelial cells and uncountable vacuoles in very small colloid. Bar represents 50 μm.

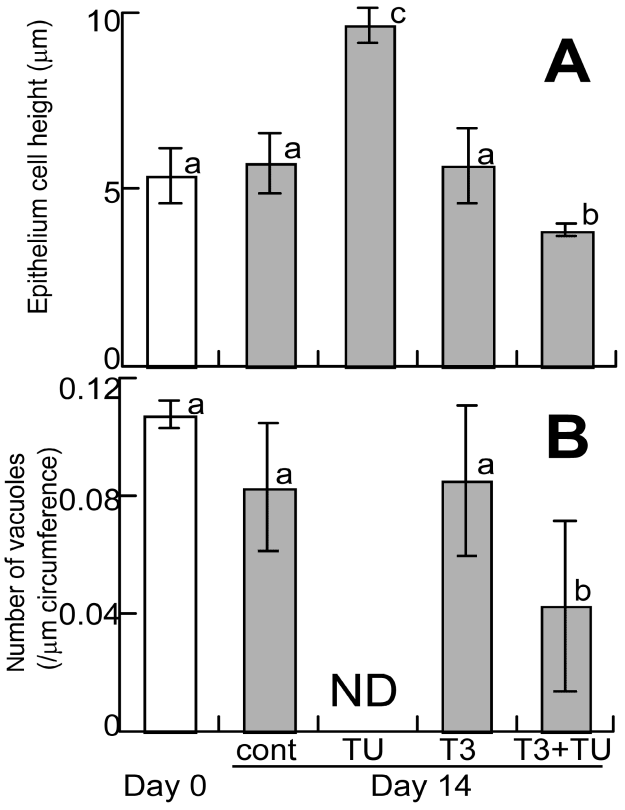

Fig. 4. (A) epithelial cell height and (B) number of vacuoles in colloid of thyroid follicle in adult ice goby. Abbreviations are shown in Fig. 1. Ten follicles from 1 fish, and 3 fish in each group were measured. Vertical bars represent standard errors of the means (n=30). Means accompanied by the different letter differ significantly each other (Sheffe test, P<0.05). ND; not determined.

TSH cells

Since the antiserum was raised against a coho salmon TSH β fraction containing a small amount of sGTH/TSH α subunit, the antiserum was preadsorbed with sGTH/TSH α prior to the immunostaining. When unadsorbed antiserum was applied to the pituitary sections of the ice goby, a large proportion of cells in the proximal pars distalis (PPD) was stained, presumably including both TSH and GTH cells (Fig. 5A). However, the TSH β antiserum preadsorbed with sGTH/TSH α reacted only with a small cluster of cells (putative TSH cells) located at the anterior region of the PPD (Fig. 5B).

Comparable numbers of TSH immunoreactive cells were observed in the control (Fig. 5C), T3-treated (Fig. 5E) and T3+TU-treated (Fig. 5F) fish, whereas the number of immunoreactive cells in TU-treated fish (Fig. 5D) was much smaller than in the control (Fig. 5C).

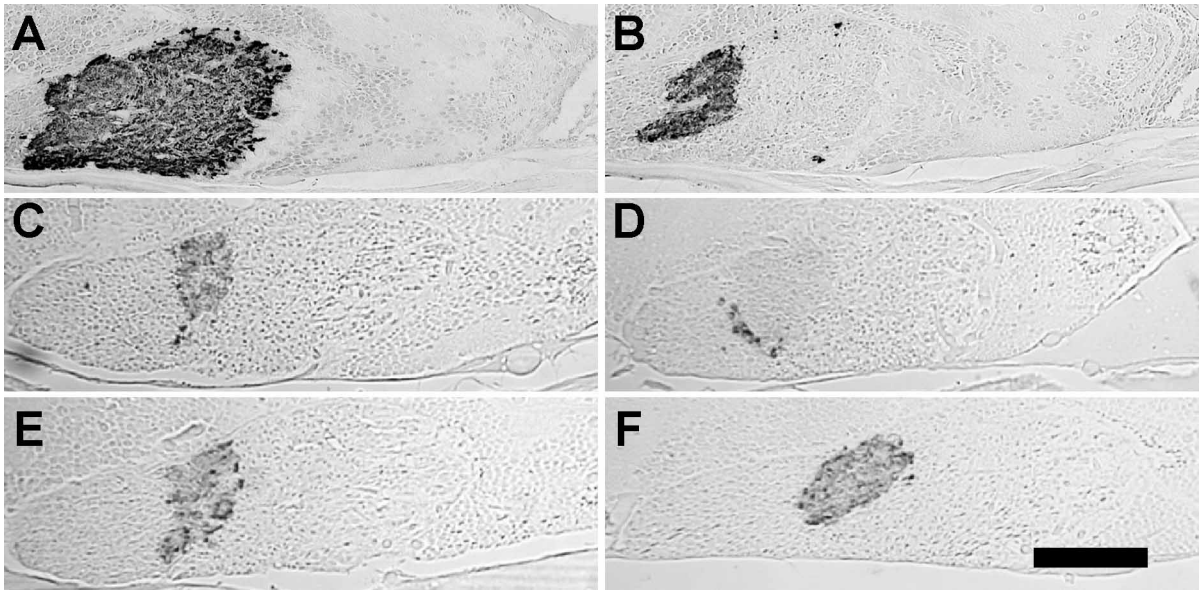

Fig. 5. (A) Serial sagittal sections of the pituitary gland of adult ice goby stained with non-preadsorbed anti-salmon TSH β and (B) salmon GTH/TSH α preadsorbed anti-salmon TSH β. TSH immunoreactive cells 2 weeks after the treatment with (C) freshwater as control, (D) 30 ppm TU, (E) 0.1 ppm T3, and (F) 0.1 ppm T3 and 30 ppm TU, stained with anti-salmon TSH β preadsorbed with salmon GTH/TSH α. Bar represents 100 μm.

DISCUSSION

Thyroid glands play an essential role in metamorphosis of amphibians and teleosts (Bentley, 1998) and incomplete metamorphosis in neotenic urodele species has been attributed to depressed function of the thyroid system (Rosenkilde and Ussing, 1996). There are two types in neotenic urodeles, the obligatory neotenic species and the facultative neotenic species. *Necturus maculosus* is an obligatory type, in which exogenous T3 does not induce metamorphosis (Wakahara, 1996). Recently, cDNAs for thyroid hormone receptors (TRs) of *N. maculosus* have been cloned, and the lack of TRβ mRNA expression has been suggested as the reason for a lack of a T3 response (Safi *et al*., 1997). On the other hand, *Ambystoma mexicanum* is a facultative neotenic species, in which exogenous T3 (Huxley, 1920) and TSH (Taurog, 1974) can artificially induce metamorphosis. Therefore, the deficiency of T4 and/or T3 production seems to develop neoteny in this species.

The ice goby is regarded as a neotenic fish, since adults possess several features characteristic of larvae in ordinary gobies (see Introduction). This study was conducted to examine the inducibility of metamorphosis of this fish by thyroid hormone treatment. When T3 was added to the rearing water of adult ice goby, a significant increase in the whole body T3 level was observed (Fig. 1). Moreover, T3 treatment in addition to TU treatment canceled the hypertrophy of thyroid follicles observed in TU-treated groups (Figs. 3 and 4), suggesting that exogenous T3 was physiologically recognized by the fish. However, no morphological changes were observed, maintaining all the neotenic features listed by Dotsu and Uchida (1979) (Fig. 2). A similar T3 treatment (100 ng/ml in rearing water) did effectively induce metamorphosis in less than two weeks in Japanese flounder (Inui and Miwa, 1985), and the same dose of T4 in greenling (Matsumoto and Tanaka, 1996) and in red sea bream (Hirata *et al*., 1989). In addition, the whole body T3 concentration was 4–6 ng/g even in the control fish (Fig. 1). This level is higher than the T3 level found during the flounder metamorphosis study (Tagawa *et al*., 1990). Consequently, it is highly possible that the neotenic features of adult ice goby do not respond to thyroid hormone.

The activity of thyroid glands seems to be controlled by the plasma T3 level. When thyroid hormone production is inhibited by TU (Fig. 1), it is expected that the TSH cells in the pituitary secrete more TSH by detecting the decrease of T3, and consequently the thyroid gland becomes hyperactivated. Because TU-treated fish showed hypertrophy of the thyroid gland (Figs. 3 and 4) and the addition of T3 to TU treatment cancelled the hypertrophy (Figs. 3 and 4), the TSH-thyroid axis is considered to function normally in the ice goby. On the contrary, increases in T3 concentration did not inactivate the thyroid gland (Figs. 3 and 4), suggesting the absence of negative feedback system in this species. However, we can not exclude the possibility that the increase in whole body T3 concentration was not sufficient to induce the inactivation of the thyroid gland, as well as the metamorphosis in the adult ice goby.

In order to detect TSH cells in the ice goby, we used the antiserum to salmon TSH β subunit, which proved to react only with TSH cells after preadsorption with salmon GTH/TSH α subunit (Moriyama *et al*., 1997). Since the non-

preadsorbed aliquot of this antiserum cross-reacts with GTH α in salmon (Moriyama *et al*., 1997), the immunoreactive cells in the PPD of the pituitary in Fig. 5A may correspond to TSH and GTH cells of the ice goby. After preadsorption of the antiserum with salmon GTH/TSH α subunit, the number of immunoreactive cells decreased (Fig. 5B), probably because TSH cells were specifically detected with GTH cells unstained. Thus, the cells that reacted with the preadsorbed antiserum were considered to be TSH cells of the ice goby. This is also supported by their specific location in the anterior region of the PPD, in accordance with TSH cells observed in the pituitary of a tidepool-living goby, *Chasmichthys dolichognathus* (Kaneko *et al*., 1984). Exogenous T3 did not affect the number of TSH immunoreactive cells (Fig. 5C, E). This is consistent with the constant activity of the thyroid gland in T3-treated fish (Figs. 3 and 4). On the other hand, TSH immunoreactive cells were markedly reduced in TU-treated fish (Fig. 5C, D). This could be explained by active degranulation of TSH cells in response to decreased T3 levels caused by TU treatment. Immunocytochemical detection of hormone-secretory cells is generally based on visualization of hormone-containing secretory granules in the cytoplasm. Extensive localization of secretory granules in the cytoplasm results in labeling over the cell with the nucleus unstained. Thus, the reduced immunoreactivity to TSH is accounted for by activation of TSH cells, in which TSH secretion predominates over its storage in the form of secretory granules. A similar observation was reported in Japanese flounder treated with TU (Miwa and Inui, 1987).

Although no conspicuous changes in morphology were observed during the development of this species (Dotsu, 1979; Dotsu and Uchida, 1979), there existed a comparable period corresponding to the larva-to-juvenile transformation (metamorphosis) of other teleost species (Harada *et al*., 2003), judging from the upward flexion of the notochord end (Kendall *et al*., 1984). Since the tissue responsiveness to thyroid hormone may be different among the developmental stages, the possibility can not be ruled out that the ice goby has thyroid hormone responsiveness during early life stages.

The results of this study indicate that the thyroid axis of the ice goby is functioning in mature adult stages, producing a significant amount of thyroid hormone with the activity being regulated, especially by the lowered T3 concentration. When taking into account the absence of thyroid hormone responsiveness of the neotenic features in adult stages, the neoteny of this fish may resemble that of obligatory-type urodeles. In order to clarify this point, however, it is necessary to study the development and function of the thyroid gland as well as T3 responsiveness during the larval period.

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