# Developmental Changes in Low-Salinity Tolerance and Responses of Prolactin, Cortisol and Thyroid Hormones to Low-Salinity Environment in Larvae and Juveniles of Japanese Flounder, Paralichthys olivaceus

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Source: Zoological Science, 14(6) : 987-992

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.987

ZOOLOGICAL SCIENCE 14: 987–992 (1997)


# Developmental Changes in Low-Salinity Tolerance and Responses of Prolactin, Cortisol and Thyroid Hormones to Low-Salinity Environment in Larvae and Juveniles of Japanese Flounder, *Paralichthys olivaceus*

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**ABSTRACT**—In Japanese flounder (*Paralichthys olivaceus*), metamorphic period involves not only transformation from larva to juvenile but also migration from offshore areas to estuaries. In the present study, the role of endocrine systems in low-salinity adaptation was examined during early development and metamorphosis of the flounder. Survival rate 48 hr after transfer to 1/8 SW was relatively high in yolk-sac larvae, decreased gradually to 0% at premetamorphosis, and increased to 100% at metamorphic climax. The ratio of prolactin (PRL)-immunoreactive part to whole pituitary increased gradually during larval stages and reached a constant level during metamorphosis. When the larvae at premetamorphosis and metamorphic climax and the benthic juveniles were transferred from SW to 1/4 SW, PRL-immunoreactive part increased significantly 48 hr after the transfer at all stages examined. Whole-body concentration of cortisol was measured with a modified extraction method which is much robuster to lipid-rich sample than the ordinary method, but no significant difference was observed after the transfer. Whole-body concentrations of thyroid hormones decreased slightly but significantly at premetamorphosis and metamorphic climax. These results suggest possible involvement of PRL and thyroid hormones in low-salinity adaptation of the flounder during metamorphosis and inshore migration.

## INTRODUCTION

In teleosts, it is well established that prolactin (PRL) is a hormone essential for freshwater (FW) adaptation, and cortisol for seawater (SW) (Evans, 1979; Henderson and Garland, 1981; Hirano, 1986; Hirano *et al.*, 1987). However, most of the studies have been carried out using adult euryhaline fishes, and little is known on the endocrine control of osmoregulation in larvae or juveniles, especially of marine teleosts. Generally, larvae of marine teleosts hatching from small pelagic eggs spend planktonic life in offshore areas. The larvae of some species migrate to inshore habitats during metamorphosis, and this migration is named "inshore migration" (Creutzberg *et al.*, 1978; Tanaka, 1991). Japanese flounder (*Paralichthys olivaceus*) also migrates from offshore areas to estuaries during metamorphosis in nature (Minami, 1982), and occasionally encounters low-salinity water. Although endocrine control of flounder metamorphosis has been extensively studied (see a review by Inui *et al.*, 1994), no information is available on the hormonal control of osmoregulation during their early development and metamorphosis. In the present study, changes in low-salinity tolerance and PRL-cell volume were examined during flounder development, as well as the responsiveness of PRL, cortisol, and thyroid hormones to low-salinity environment.

## MATERIALS AND METHODS

### Fish

Naturally spawned eggs of Japanese flounder (*Paralichthys olivaceus*) were collected from the brood-stock tank in the Fisheries Research Station of Kyoto University. Larvae and juveniles were reared in a polycarbonate tank (500 l) with running SW. Water temperature was maintained at 18°C, and salinities ranged between 30.6 and 32.2 ppt. They were initially fed rotifers (*Brachionus plicatilis*) cultivated with *Nannochrolopsis* sp. and ω-Yeast (Kyowa Hakko Kogyo, Japan), and later brine shrimp (*Artemia* spp.) nauplii enriched with Ester-85 (Nippon Chemical Feed, Japan).

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**Transfer to low-salinity water**

Forty fish at different developmental stages were transferred from the stocking tank (SW: 32 ppt) to 2-l beakers containing FW (0 ppt), 1/8-diluted SW (4 ppt), 1/4-diluted SW (8 ppt), 1/2-diluted SW (16 ppt) or SW (32 ppt) using a pipette, and survival rates were examined 48 hr after the transfer. Water temperature was maintained at 18°C. Photoperiod was 13 L (6:00 - 19:00) and fish were transferred at 17:00. They were not fed during the experiment.

Responsiveness of hormones to low-salinity water was examined at three developmental stages: premetamorphosis (18 days after hatching, 6.5 - 7.0 mm body length, body symmetrical, D stage); metamorphic climax (33 days after hatching, 9.5 - 10.2 mm, right eye at dorsal ridge, H stage); juvenile (49 days after hatching, 16.5 - 18.0 mm, benthic, $I_4$ stage). The developmental stages were described by Minami (1982) and Goto *et al.* (1989). They were transferred from the stocking tank to 2-l beakers containing 1/4 SW or SW (control), and fed *Artemia* nauplii once a day. Samples taken at 0 and 48 hr after the transfer were fixed in Bouin's solution or preserved at –30°C. The samples for cortisol measurement were also taken at 1, 3, 6, 12, and 24 hr after the transfer. Each frozen sample (about 100 mg) consisted of 30, 7 and 3 individuals at premetamorphosis, metamorphic climax and juvenile, respectively.

**Prolactin immunohistochemistry**

Larvae and juveniles at various developmental stages were fixed in Bouin's solution for 2-6 hr at 4°C, dehydrated through graded ethanol, cleared in xylene, and embedded in Parahisto (Nacalai Tesque, Japan). Serial sagittal sections were made at 4 μm thickness and mounted on slides. The sections were deparaffinized, rehydrated, and stained by the peroxidase-antiperoxidase (PAP) method (Sternberger *et al.*, 1970) using commercial reagents (Dako, Denmark). The sections were incubated sequentially with: (1) 3% $H_2O_2$ for 10 min, (2) normal goat serum diluted 1:20 with 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 30 min, (3) rabbit anti-tilapia $PRL_{177}$ serum (Ayson *et al.*, 1993) diluted 1:4000 for 20 hr at 4°C, (4) goat anti-rabbit IgG serum diluted 1:100 for 60 min, (5) rabbit PAP diluted 1:100 for 80 min, (6) 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.005% $H_2O_2$ for 5 min. The sections were then dehydrated, cleared, and mounted.

In all serial sections containing a portion of the pituitary, outlines of both PRL cells and the whole pituitary were traced on paper with a camera lucida. The images were digitized with a flat bed scanner (Sharp, Japan) and areas were measured on an Apple Macintosh computer using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/). Total volumes of PRL cells and of the pituitary were calculated from the areas of the each section and the thickness (4 μm), and the percentage of PRL-cell volume to pituitary volume was calculated as a quantitative criterion of PRL-cell activity (Kimura and Tanaka, 1991; Tanaka *et al.*, 1995).

**Cortisol and thyroid hormone measurements**

In cortisol radioimmunoassay (RIA), we encountered extremely high level of nonspecific binding (NSB) by the use of a standard extraction method for tissue cortisol (de Jesus *et al.*, 1991). This extraction method without the steps to remove lipids seems to be applicable only for lipid-less tissue or tissue free from some specific fatty acids. In the recent sea-farming scene, live feeds for marine fish larvae are enriched with n-3 highly unsaturated fatty acids (see a review by Watanabe and Kiron, 1994). Consequently, tissues of the reared marine fish larvae would contain high quantity of lipids. Since lipids strongly interfere with steroid RIAs (Rash *et al.*, 1979, 1980), we established and validated an extraction method using tetrachloromethane to eliminate lipid contamination.

The frozen samples weighing 100-400 mg were homogenized in five-fold volume of ice-cold PBS using Polytrone homogenizer on a 10S blade (Kinematica, Switzerland). The homogenate (300 μl) was extracted twice with 3 ml of diethyl ether by mixing vigorously for 2 min. After freezing at –80°C, the ether layer was collected by decantation and dried at room temperature. To reconstitute the dry residue, 300 μl of tetrachloromethane was added and mixed for 4 min. PBS containing 0.1% gelatin (300 μl) was then added, and mixed for 2 min. After centrifugation (3000 rpm, 10 min, 4°C), the aqueous layer was divided into four 50-μl aliquots. The two aliquots were used for the cortisol RIA as described by Takahashi *et al.* (1985), and the other two for nonspecific binding (NSB) measurement.

By using the standard extraction method for tissue cortisol, NSB of extracts from metamorphic-climax larvae was 83 ± 9% (NSB / $B_0$, mean ± standard deviation, n = 7). By the present method with tetrachloromethane extraction, NSB was reduced to 1.0 ± 2.7% (n = 20). Figure 1 shows the competitive binding curves for the tetrachloromethane-washed extracts of the larvae in metamorphic-climax. The dilution curve for larval extracts was parallel to the cortisol standard. The curve for the larval extracts added to cortisol was also parallel to the cortisol standard, and the recovery of cortisol was 96.5 ± 15.9% (n = 5).

Thyroxine (T4) and triiodothyronine (T3) were extracted and measured by RIAs as described by Tagawa and Hirano (1989).

**Statistics**

Significant differences in the% PRL-cell volume / pituitary volume and in the levels of thyroid hormones between the group transferred to 1/4 SW and the control group were tested by the Mann-

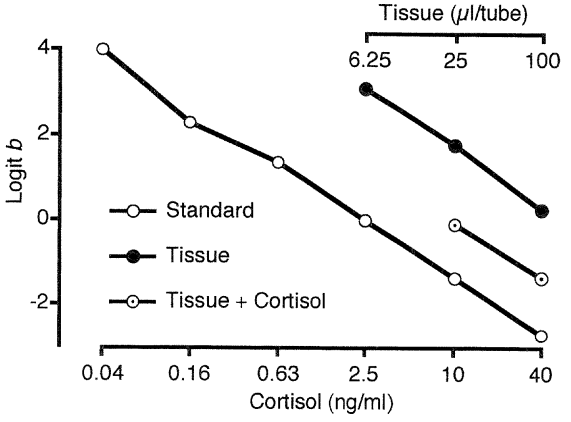


**Fig. 1.** Competitive binding curves for tetrachloromethane-washed extracts of metamorphic-climax larvae of the flounder. Each point represents the average of duplicate determinations. Logit $b = \log_e \{b / (100 - b)\}$. $b$ = Bound / $B_0$ (%).

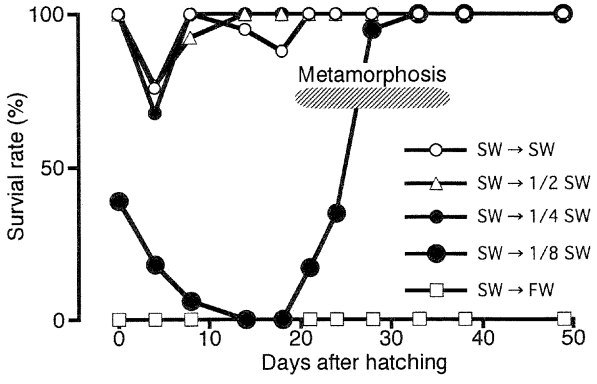


**Fig. 2.** Developmental changes in survival rates 48 hr after the transfer. Each point represents the survival rate of 40 individuals.

Whitney U test. Significant difference in the level of cortisol was examined by two-way ANOVA with repetition (salinity x time), as homogeneity of variance was established.

## RESULTS

Figure 2 shows the developmental changes in survival rates at 48 hr after the transfer. Survival rates of the groups transferred to SW, 1/2 SW and 1/4 SW were more than 90% at most of the stages except for the larvae 4 days after hatching, showing about 70% survival rate even for the larvae maintained in SW. Survival rate of the group transferred to 1/8 SW was relatively high (40%) in yolk-sac larvae, decreased gradually to 0% in premetamorphic larvae (14 days after hatching), started increasing at the beginning of metamorphosis (21 days after hatching), and reached 100% at metamorphic climax. All the larvae and juveniles transferred to FW died within 24 hr at all stages.

PRL-immunoreactive cells were detected in the rostral pars distalis of the pituitary at all stages, except for the larvae just after hatching, in which no positive stain was observed. Figure 3 shows the developmental changes in the% PRL-cell volume / pituitary volume of the larvae and juveniles reared in SW. PRL-cell volume increased gradually during larval stages, and reached a constant level of 10% in the middle of meta-

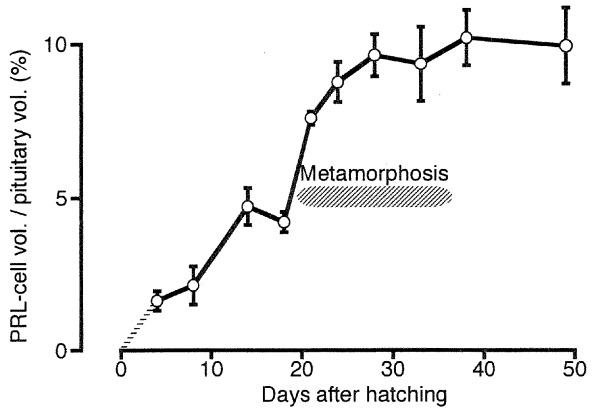


**Fig. 3.** Developmental changes in the percentage of PRL-cell volume to pituitary volume. Vertical bars represent standard errors of the means of 4 individuals.

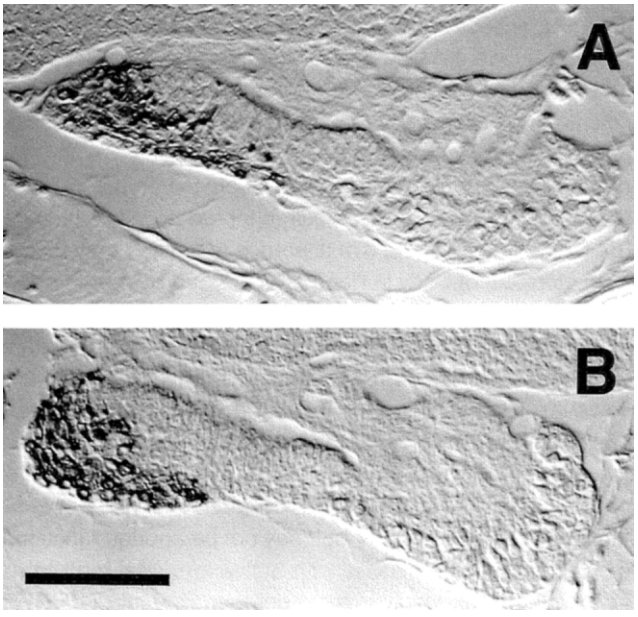


**Fig. 4.** PRL cells in the pituitary of flounder larvae at metamorphic-climax transferred from SW to SW (**A**) and from SW to 1/4 SW (**B**). The bar indicates 50 μm.

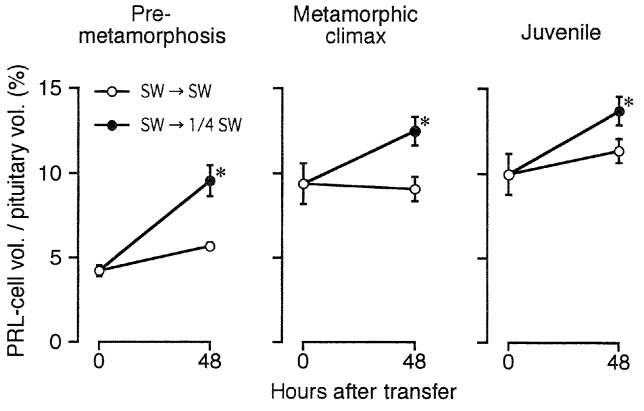


**Fig. 5.** Changes in the percentage of PRL-cell volume to pituitary volume after the transfer. Vertical bars represent standard errors of the means of 4 individuals. *Significantly different ($P < 0.05$) from the control by the Mann-Whitney U test.

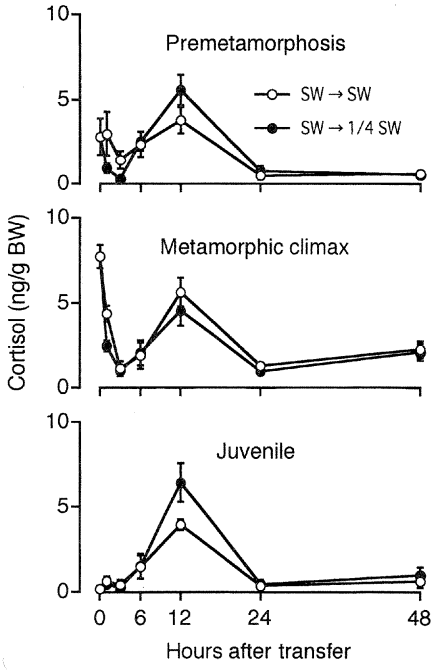


**Fig. 6.** Changes in the whole-body concentration of cortisol after the transfer. Vertical bars represent standard errors of the means of 4 pooled samples.

morphosis (28 days after hatching).

Since 1/4 SW was the lowest salinity in which most of the larvae of all stages survived for 48 hr, effects of low-salinity environment on hormone levels were examined by transferring the larvae at premetamorphosis (18 days after hatching) and metamorphic climax (33 days after hatching) and benthic juvenile (49 days after hatching) to 1/4 SW. Some larvae and juveniles were transferred to SW as controls. No mortality was observed in both groups during the transfer experiments. Figure 4 shows PRL cells in the pituitary of metamorphic-climax larvae after the transfer, indicating that PRL-cell area of larvae transferred to 1/4 SW was larger than that to SW (control). Percent PRL-cell volume / pituitary volume in the groups transferred to 1/4 SW was significantly greater (Mann-Whitney U test, $p < 0.05$) than those in the control groups at all three stages (Fig. 5).

Figure 6 shows the changes in whole-body concentration of cortisol after the transfer. Two-way ANOVA showed a significant effect of time on cortisol concentration, but no significant effect of salinity and a salinity x time interaction at all three stages. At each developmental stages, cortisol concentration reached the highest level 12 hr after the transfer and then decreased. In metamorphic-climax larvae, cortisol concentration was relatively high at 0 hr, decreased 1-3 hr after the transfer, and increased later toward the highest level at 12 hr.

Figure 7 shows the changes in whole-body concentrations of thyroid hormones after the transfer. T4 concentration in the group transferred to 1/4 SW was significantly lower (Mann-Whitney U test, $p < 0.05$) than that in the control group at premetamorphosis and metamorphic climax. T3 concentration was significantly lower (Mann-Whitney U test, $p < 0.05$) in the larvae transferred to 1/4 SW than that in the control group at metamorphic climax.

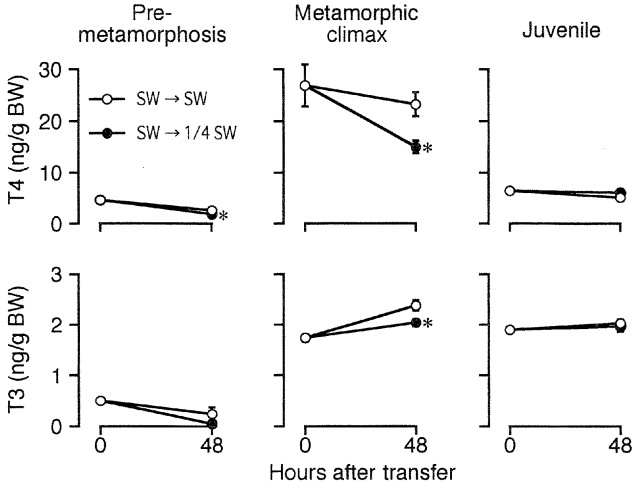


**Fig. 7.** Changes in the whole-body concentrations of T4 and T3 after the transfer. Vertical bars represent standard errors of the means of pooled samples (premetamorphosis, metamorphic climax: n = 4; juvenile: n = 5). *Significantly different ($P < 0.05$) from the control by the Mann-Whitney U test.

## DISCUSSION

A significant number of marine teleosts utilize estuaries as their nursery ground in their early life history, and inevitably encounter brackish water. Japanese flounder, one of the most important species for aquaculture in Japan, spawn in offshore areas of the sea. After spending pelagic life, flounder larvae undergo metamorphosis, migrate to estuaries, and occasionally encounter low-salinity water. In the present study, development of low-salinity tolerance of the flounder was clearly demonstrated in the group transferred to 1/8 SW, even though flounder larvae and juveniles would not be exposed to such a hypotonic environment in nature. In all groups, relatively high mortality was observed at 4 days after hatching when yolk was completely absorbed and feeding started. This stage is generally regarded as "critical period" of early life history in teleosts (Fabre-Demergue and Biétrix, 1897; Minami, 1994). An increase in low-salinity tolerance was observed during metamorphosis (21-28 days after hatching), coinciding with ecologically-observed migration to estuaries (Minami, 1982; Tanaka *et al.*, 1989).

PRL-cell volume of the larvae reared in SW increased gradually during metamorphosis, and PRL-cell volume of the groups transferred to 1/4 SW increased markedly at all stages examined. An increase in the expression of PRL mRNA has been reported in the flounder larvae during metamorphosis (de Jesus *et al.*, 1994), and increases in PRL-cell activity after transfer to low salinity or FW were also observed in the larvae of black sea bream (*Acanthopagrus schlegeli*), amphidromous temperate bass (*Lateolabrax japonicus*), and tilapia (*Oreochromis mossambicus*) (Kimura and Tanaka, 1991; Tanaka *et al.*, 1996; Ayson *et al.*, 1994). The increase in PRL-cell activity may indicate that PRL is involved in the development of low-salinity tolerance during metamorphosis. However, yolk-sac larvae showed better tolerance to 1/8 SW than the larvae 4-14 days after hatching, even though PRL cells were not detected in the pituitary. These results may indicate that PRL is secreted as soon as they are synthesized in the newly-hatched larvae. The other possibility is that the ion and water permeability is extremely low in the yolk-sac larvae, and thus PRL is not required for low-salinity tolerance. The subsequent decrease in tolerance to 1/8 SW may be due to an increase of body surface area caused by mouth opening and gill differentiation of these stages. Gills are the site of active ion secretion in SW and possibly the site of ion uptake in FW (see reviews by Lin and Randall, 1995; Flik *et al.*, 1995). However, the fish living in FW or a hypotonic environment loses ions and absorbs water mainly through gills because gills occupy the most area of body surface with thin respiratory epithelia. The gills of premetamorphic larvae would be effective in SW adaptation, but may not be enough functional to compensate the ion loss with active ion uptake, or to reduce water permeability in hypotonic environment (1/8 SW). The hyperosmoreguratory ability of flounder gills may develop during metamorphosis.

The whole-body concentration of cortisol in the fish trans-

ferred to hypotonic environment was examined by using a newly-developed extraction method. According to Rash *et al.* (1979), lipids interfere with steroid RIAs in two ways. First, lipids form micelles in aqueous solution and entrap steroids, interfering with the binding between steroids and their antisera. Second, lipids bind to the dextran-coated charcoal, blocking the charcoal absorption of free ligands. By reconstituting and washing with tetrachloromethane, lipids would be removed without forming micelles, and NSB was consequently reduced from 83% to 1%. The extraction with tetrachloromethane was shown to be applicable in other species such as juveniles of yellowtail *Seriola quinqueradiata* and red sea bream *Pagrus major* (data not shown). No significant difference in whole-body concentration of cortisol was observed between the group transferred to 1/4 SW and the control group. Although it has recently been reported that cortisol is involved in ion uptake in teleosts in FW (see a review by McCormick, 1995), the role of cortisol in hyperosmoregulation in flounder larvae and juveniles is still unclear. Elevations of cortisol concentration 12 hr after the transfer in both experimental and control groups at all three stages may be due to the transfer stress. Stress-induced increase in whole-body concentration of cortisol has been reported in larval salmonids (Pottinger and Mosuwe, 1994; Barry *et al.*, 1995a,b), although the peak levels were observed at 1 hr poststress. It is also possible that the peak at 12 hr reflects diurnal rhythms.

Concentrations of cortisol and thyroid hormones at 0 hr of the transfer show basal levels of the hormones during development, so that the differences among three stages reflect ontogenetic changes of the hormones. The highest basal levels of cortisol, T4 and T3 at metamorphic climax are consistent with the previous studies (de Jesus *et al.*, 1991; Miwa *et al.*, 1988; Tanangonan *et al.*, 1989; Tagawa *et al.*, 1990).

The whole-body concentrations of thyroid hormones at premetamorphosis and metamorphic climax decreased after low-salinity transfer. Thyroid hormones are well known to induce metamorphosis in flounders (Inui and Miwa, 1985; Miwa and Inui, 1987), whereas PRL antagonizes the thyroid hormone effects (de Jesus *et al.*, 1994). In the present study, PRL-cell volume increased whereas thyroid hormone concentrations decreased after low-salinity transfer. In striped bass (*Morone saxatilis*), the whole-body concentrations of thyroid hormone increased after the transfer from FW to SW at premetamorphosis and metamorphosis (Parker and Specker, 1990). The decreased thyroid hormone concentrations after exposure of the flounder larvae at metamorphic climax may indicate an interaction between thyroid hormones and PRL, although the mode of actions of the hormones is totally unclear. When flounder larvae were exposed to low-salinity water during metamorphosis, metamorphosis is expected to be delayed by increase in PRL and decrease in thyroid hormones. The contribution of endogenous PRL to the control of metamorphosing process would be evaluated experimentally by long-term exposure to low-salinity water.

## ACKNOWLEDGMENTS

We wish to express our gratitude to Professor Tetsuya Hirano, Ocean Research Institute, University of Tokyo, for his encouragement and helpful advice on the establishment of cortisol extraction method. We are grateful to Professor Toyoji Kaneko, Ocean Research Institute, University of Tokyo, for his helpful advice on immunohistochemistry and photomicrography. Thanks are also due to Professor Jennifer L. Specker, University of Rhode Island, for critical reading of the manuscript. This study was supported in part by Grants-in-Aid from the Ministry of Education, and the Fisheries Agency, Japan. J.H. and Y.S. were supported by Research Fellowships of Japan Society for Promotion of Science for Young Scientists.

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(Received July 11, 1997 / Accepted August 16, 1997)