Prolactin Production during Larval and Early Juvenile Periods of Euryhaline Marine Fish, Black Sea Bream

*Acanthopagrus schlegeli*

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Ontogenetic changes in prolactin (PRL) production during larval and early juvenile periods of reared black sea bream were examined using an immunocytochemical technique. The onset of PRL production was observed at the end of the yolk-sac stage. Percent PRL (the percentage of hypophysis volume occupied by PRL-cell masses), cited as a quantitative index of PRL production, increased from about 10% at yolk-sac and preflexion stages to about 17% at early juvenile stage. PRL cells were activated and Percent PRL significantly increased up to 22% in low salinity (5‰) ambient water. These results suggest that PRL has an important osmoregulatory role in marine fish as previously shown in freshwater fish, this being one of the important physiological backgrounds enabling the larvae to inhabit nearshore low-salinity nurseries.

Black sea bream *Acanthopagrus schlegeli* is a common coastal fish in southern Japan. The pelagic larvae are distributed in the coastal waters, whereas the juveniles inhabit nearshore waters and/or estuaries.

Recently, it has been demonstrated that black sea bream migrates from offshore to inshore waters during the transitional phase from larva to juvenile.1-3 From the physiological point of view, functional development of osmoregulatory organs is of great importance in successful immigration into nearshore waters where salinities are more variable and usually lower than those of offshore waters.

It is well established that prolactin producing cells (PRL-cell) of euryhaline freshwater fish are more markedly activated in fresh water than in sea water.4-8 In mullet, a typical euryhaline marine fish, PRL-cell activation is also observed when they migrate to brackish or fresh waters.9-11 However, during the early ontogeny in marine fish, little information is available on prolactin (PRL) production and its activation by surrounding salinities.

In the present study, we examined the ontogenetic change in PRL production during larval and early juvenile periods of reared black sea bream by using an immunocytochemical technique. The changes in PRL production under low salinities were also investigated and discussed in relation to their inshore migration in the sea.

**Materials and Methods**

**Rearing**

Fertilized eggs of black sea bream were obtained from Hiroshima Municipal Fisheries Center and Fukui Prefectural Fish Farming Center on 14th of May and 18th of June 1987, respectively. They were transported to the Fisheries Research Station, Kyoto University, Maizuru. Larvae and juveniles were reared in 500-l polycarbonate tanks with running sea water, flow rates of which were 0.05-1.5 ton per day depending upon the growth. Light and water temperatures were kept under natural conditions. Water temperatures increased from 18.0 to 26.9°C and salinities fluctuated between 32.4 and 34.5‰. Larvae were reared initially with rotifers cultured on *Nannochlolopsis* sp. and later brine shrimp nauplii together with occasional supplies of wild zooplanktons.

The fish were sampled from 1 day to 37 days after hatching, at intervals of 2 to 7 days. Standard length (SL) was measured after anesthetizing in 1% MS222 solution. After the measurement, samples were fixed with Bouin's solution and then preserved in 90% ethanol. Developmental stages

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were determined by the method of Kendall et al.\textsuperscript{12}

Low Salinity Experiment

In order to evaluate the effect of low salinity on PRL cells, an experiment was conducted on 6–8th of July 1988, using early juveniles (SL 8.5–10.1 mm). Fishes used in the experiment were reared in the same conditions as in 1987, except for the salinity and temperature (29.5–33.8\%, 19.2–25.8\°C). Twenty juveniles were transferred with a pipette from the stocking tank to 2 l glass beakers with diluted sea water (5\%) or normal sea water (about 33\%: control) which were incubated in a water bath (22.2–23.9\°C). During the experiment, food supply was stopped and light condition was 12L12D (L: 8:00–20:00). After being exposed for 48h, percent survival was counted and survived fish were fixed in Bouin's solution. Standard lengths were measured after fixation. Next, t-tests were used for statistical analyses.

Histological Procedure

The fish fixed in Bouin's solution were embedded in mixture of normal paraffin and parahisto (Nakaraitesque) (1:1) and serial sagittal sections were made at 5 μm thickness.

Prolactin producing cells were identified immunocytochemically with unlabeled peroxidase-antiperoxidase (PAP) method as described by Sternberger et al.\textsuperscript{13} and modified by Naito et al.\textsuperscript{14} A rabbit antiserum raised against the salmon PRL was provided by Dr. T. Hirano, Ocean Research Institute, University of Tokyo. It was diluted with phosphate-buffered saline (PBS) at rates of 1:4000 or 1:8000 in ontogeny study and at 1:12000–1:16000 in Low Salinity Experiment, according to optimal density for detection of PRL-cell in a preliminary test. Sections were first incubated in a moist chamber for 20 h at 4\°C with anti-salmon PRL antiserum. After rinsing with PBS, they were incubated with goat anti-rabbit IgG (Cappel) diluted at 1:50 for 60 mins and subsequently with rabbit PAP complex (Cappel) diluted at 1:100 for 80 mins under room temperature. Then, they were reacted with DAB solution (24 mg in 120 ml 0.05 M Tris-HCl buffer, pH 7.6, with 0.2 ml 3% H₂O₂). After DAB reaction, Mayer's hematoxylin was used for counter-staining. The numbers of fish of yolk-sac, preflexion, flexion, postflexion, juvenile stages used for PAP method were 3, 6, 4, 5, and 11, respectively.

All serial sections containing hypophysis and PRL cells were traced in paper under a light microscope. The areas were measured by digitizer (KD4300, Logitec). Based on the areas and thickness (5 μm), total volume of hypophysis and total PRL-cell mass were calculated.

PRL-cell diameter in the sections of the fish of Low Salinity Experiment was estimated as following manner: in the middle section of the sequence, cell maximum length (L) and maximum width (W) were measured, then the average of L and W was cited as cell diameter. All visible cells in the section were counted.

Results

Growth of Fish

Newly hatched larvae absorbed their yolk and began to feed at 4 days after hatching (4d). Growth in length and occurrence of each developmental stage of larvae and juveniles are shown in Fig. 1.

Ontogenetic Changes in PRL Production

Initial signs of PRL production in hypophysis were observed in a 2-day-old prelarva, SL 2.8 mm (Fig. 2a). In the larva, the pituitary was histologically discernible as an organ, the median section of which contained less than five PRL-positive cells. The larva had no opened mouth
and pigmented eyes.

PRL production was observed in all specimens of larvae older than 2 days and juveniles (2-35d, SL 2.8-11.8 mm) (Figs. 2b-d). PRL producing cells occurred in rostral pars distalis of hypophysis. The location was clearly separated from that of growth hormone (GH) producing cells which reacted to anti-salmon GH antiserum*.

Both PRL-cells and hypophysis volumes increased exponentially with larval growth as shown in Fig. 3. The percentage of PRL-cell volume to the total hypophysis volume (Percent PRL) was 10–11% at yolk-sac and preflexion stages, subsequently increased gradually to about 13% at flexion and postflexion stages and finally attained to about 17% at early juvenile stage (Fig. 4).

Low Salinity Experiment

Percent survival of fishes which were exposed to diluted seawater (DSW, 5%) and normal seawater (NSW, 33%) for 48 h were 89% and 100%, respectively. The Percent PRL of these experimental fishes were 22.0±1.89% in 5% DSW and 12.2±0.52% in 33% NSW (mean ± SEM). The former was significantly greater than the latter (P<0.01) (Figs. 5–6). Immuno-reactivity is more evident in all sections of fishes.

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acclimated in diluted seawater than that in normal seawater. Immunoreactivity is more evident in all sections of fishes acclimated in diluted seawater than that in normal seawater.

**Fig. 5.** Sagittal sections through the hypophysis of black sea bream juveniles stained with anti-salmon PRL (1/12000) acclimated for 48h to (5a) normal seawater, 33‰, (5b) diluted seawater, 5‰. Immunoreactivity is more evident in all sections of fishes acclimated in diluted seawater than that in normal seawater.

**Fig. 6.** Percent PRL (bar) and PRL-cell diameter (open circle) of early juvenile fish, acclimated for 48 h in normal seawater (NSW: 33‰) and in diluted seawater (DSW: 5‰). Mean ± SEM; solid circles indicate each Percent PRL of individual fish. Both Percent PRL and PRL-cell diameter in DSW are significantly larger than that in NSW (t-test, P<0.01).

The PRL-cell diameter of each group is shown in Fig. 6. Measured cell numbers were 13–46 in each one section of 5 fishes in DSW, 7–17 in NSW. The cells of the fish in 5‰ DSW were significantly larger than those of the fish in 33‰ NSW (P<0.01). The number of PRL-cells observed were also larger in fish kept in 5‰ DSW than those in 33‰ NSW.

### Discussion

Pickford and Phillips demonstrated an osmoregulatory role of PRL in hyposectomized freshwater fish Fundulus heteroclitus. The role of PRL in marine fish, however, has been scarcely elucidated, in particular during the early ontogeny.

With immunocytochemical technique, it was confirmed that black sea bream initiates PRL production before the end of yolk-sac stage. Miwa and Inui demonstrated that thyroid gland stimulating hormone (TSH) in the hypophysis of Japanese flounder Paralichthys olivaceus has not yet been produced in a 36 h-old larva, but is present in a 4d-old larva (rearing temperature=15°C). The initial time of TSH production in flounder larvae seems to be nearly the same as that of PRL production in the present species. This fact may suggest that the marine fish larvae hatched from pelagic eggs begin to produce hypophysis hormones from the end of yolk-sac stage and/or the earliest phase of postlarval period. On the other hand, Schoots et al. demonstrated using PAP method that Oryzias latipes and Cynolebias whitei, both of which are freshwater species and hatched from demersal eggs, began to produce PRL from prehatching stage. Further comprehensive examinations in various species will be needed in order to generalize about the initial time of PRL production in teleost fishes.

In this study, as a quantitative index of PRL secreting activity, Percent PRL (percentage of PRL-cell masses to whole hypophysis) was used. Consequently, the Percent PRL increased gradually with advances in development. According to field observations on the early life stages, this species migrates from offshore coastal waters to nearshore shallow waters during the final phase of postflexion stage. As shown in Fig. 4, Percent PRL attained higher level at the period from flexion to juvenile stages compared with the previous stages although fish were kept in normal seawater (32.4–34.5‰). Thus, the elevation in PRL production estimated from laboratory-reared larvae is well timed to inshore migration observed in wild larvae.

The Percent PRL of the group immersed in 5‰
diluted seawater (DSW) for 48 h was significantly higher than that of the control group. The PRL-cell size and number of PRL cells in 5% DSW also were larger than those of the control. Ruijter et al. demonstrated that in annual cyprinodont fish Cynolebias whitei reared under isoosmotic conditions for five weeks, the hypophysis volume occupied by PRL cells (same indicator as Percent PRL in this study) is only 25% of that of the fish reared in freshwater. Moreover, Nicoll et al. demonstrated that the content of PRL in hypophysis and serum PRL concentration of young tilapia Sarotherodon mossambicus in freshwater-reared group were about ten times of those in seawater-reared group. These reports suggest that PRL production and/or secretion are much more promoted in freshwater than in seawater, and support our conclusion that high Percent PRL implies an increase in PRL secretion under diluted seawater in the marine species of black sea bream. Thus, we can consider that PRL may be involved in osmoregulation under low osmotic environments such as brackish water vein in marine fish larvae and early juveniles.

The results obtained in the present experimental study elucidate one of the physiological factors by which black sea bream larvae can adapt to nearshore low salinity environments associated with inshore migration. In the future, the relationship between decrease of ambient salinity and increase of PRL production needs to be investigated experimentally, and field observations on PRL production directly related to environmental salinities are also expected to be done.

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