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Changes in Expression of Genes Encoding Gonadotropin Subunits and Growth Hormone/Prolactin/Somatolactin Family Hormones During Final Maturation and Freshwater Adaptation in Prespawning Chum Salmon

Takeshi Onuma,[1] Takashi Kitahashi,[1] Shinya Taniyama,[1] Daisuke Saito,[1] Hironori Ando,[1] and Akihisa Urano[1, 2]

[1]Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Hokkaido, Japan; and [2]Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Hokkaido, Japan

The pituitary levels of mRNAs encoding gonadotropin (GTH) subunits (GTH α2 and IIβ), prolactin (PRL), and somatolactin (SL) increased in chum salmon during the last stages of spawning migration. In the present study, changes in pituitary levels of mRNAs encoding GTH α2, Iβ, and IIβ; growth hormone (GH); PRL; and SL were examined in homing chum salmon of Sanriku stock to clarify whether the changes are associated with final maturation or freshwater (FW) adaptation. In 1993, fish were caught at four areas: off the coast of Sanriku (off-coast), the mouth of Otsuchi Bay (ocean), inside of Otsuchi Bay (bay), and the Otsuchi River (river). In addition, effects of hypoosmotic stimulation by transition from seawater (SW) to FW were examined in 1994 and 1995. The amounts of mRNAs were determined by dot-blot analyses or real-time polymerase chain reactions. The levels of GTH α2 and IIβ mRNAs in the ocean, bay, and river fish were two to five times those in the off-coast fish, and the levels of SL mRNAs in the bay fish were two to four times those in the off-coast fish. The levels of GH and PRL mRNAs in the ocean and bay fish were significantly lower than those in the off-coast fish, and those in the river fish were three to five times those in the ocean and bay fish. In the SW-to-FW transition experiment in 1994, the levels of GTH α2, Iβ, and IIβ mRNAs transiently increased, whereas changes were insignificant in 1995. The levels of GH, PRL, and SL mRNAs increased in both SW and FW environments, and no apparent effects of SW-to-FW transition were observed. The present study suggests that in prespawning chum salmon, expression of genes encoding GTH α2, IIβ, and SL elevates with final maturation regardless of osmotic environment. Hypoosmotic stimulation by transition from the SW-to-FW environment is not critical to modulate expression of genes for PRL. PRL gene expression can be elevated in SW fish that were sexually almost matured.

Key Words: Gonadotropins; growth hormone; prolactin; somatolactin; gene expression; chum salmon.

Introduction

In our previous studies, expression of genes encoding gonadotropin (GTH) subunits (GTH α2 and IIβ), prolactin (PRL), and somatolactin (SL) was elevated in the pituitaries of chum salmon during the last stages of spawning migration in the Ishikari River, Hokkaido, Japan *(1,2)*. Expression of PRL gene seemed to be stimulated by the entrance into the freshwater (FW) environment from coastal seawater (SW), whereas that of GTH α2, IIβ, and SL genes increased during upstream migration from the coast to the hatchery. An arising question is, Are the changes in expression of genes encoding GTH subunits and GH/PRL/SL family hormones during the last stages of spawning migration in the Ishikari River general biologic phenomena, because salmon stocks in different rivers may have different genetic and environmental backgrounds?

During upstream migration, two main factors are considered to be involved in the regulation of gene expression of pituitary hormones. One is a hypoosmotic stimulation by transition from the SW to FW environment, and the other is a progress of final maturation. The question is, Are the levels of hormonal mRNAs elevated with final maturation or FW adaptation? Effects of hypoosmotic stimulation on expression of pituitary hormone genes have not been clarified in prespawning salmonids.

In the present study, we determined changes in pituitary levels of mRNAs for GTH α2, Iβ, and IIβ; growth hormone (GH); PRL; and SL from 1993 to 1995 in prespawning chum salmon of Sanriku stock, whose environmental backgrounds

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Author to whom all correspondence and reprint requests should be addressed: Takeshi Onuma, Division of Biological Sciences, Hokkaido University Graduate School of Science, Sapporo, Hokkaido 060-0810. Japan. E-mail: takeo@sci.hokudai.ac.jp

Table 1
GSIs (gonad wt/body wt × 100) and Spermiation or Ovulation Ratio (Ratio of Fish Spermiated or Ovulated) of Prespawning Chum Salmon Used in Experiment 1[a]

	Males			Females		
Year 1993	GSI (%)	*n*	Spermiation ratio (%)	GSI (%)	*n*	Ovulation ratio (%)
Off-coast	4.8 ± 0.3	3	0.0	15.3 ± 1.4	5	0
Ocean	5.0 ± 0.4	7	0.0	17.8 ± 0.9	8	0
Bay	4.7 ± 0.2	7	28.5	22.7 ± 1.1*	9	100
River	3.7 ± 0.2*	10	100.0	21.8 ± 0.7*	10	100

[a]Fish were caught at four areas probably along their migratory pathway, and they were referred to as off-coast fish, ocean fish, bay fish, and river fish. Data are the mean ± SEM.

*$p < 0.05$ compared to the off-coast fish by one-way ANOVA followed by Tukey test for multiple comparison.

including river length are different from Ishikari stock in the previous studies *(1,2)*. We further assessed progress of final maturation by gonadosomatic index (GSI), spermiation and ovulation ratio; and plasma levels of testosterone, 11-ketotestosterone (11KT), estradiol-17β (E_2) and 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), and determined plasma levels of Na^+ and cortisol to estimate whether fish adapted to osmotic environments. In the first experiment in 1993, fish were caught at four areas along their migratory pathway: off the coast of Sanriku (off-coast), the mouth of Otsuchi Bay (ocean), inside of Otsuchi Bay (bay), and the Otsuchi River (river) to elucidate naturally occurring temporal changes during the last stages of spawning migration. In the second experiment in 1994 and 1995, the effects of hypoosmotic stimulation on the mRNA levels were examined by transition from SW to FW. The fish used in the present study were the same as those used in the previous researches *(3,4)*, so that the GSI, plasma levels of Na^+, and DHP were based on these reports.

Results

Field Experiment in 1993

Gonadal Maturity

In the males, the GSI in the river fish was significantly lower than that in the off-coast fish (Table 1). About 30% of the bay fish and all of the river fish were spermiated. In the females, the GSI in the bay and river fish were significantly higher than those in the off-coast fish. All of the bay and river fish were ovulated. Thus, chum salmon of Sanriku stock used in the present study completed sexual maturation near or in the natal river.

Pituitary Levels of GTH α2, Iβ, and IIβ mRNAs

The levels of mRNAs encoding GTH subunits increased during the spawning migration from the off-coast to the river (Fig. 1). In the females, the levels of GTH α2 and IIβ mRNAs in the ocean, bay, and river fish were two- to five-fold those in the off-coast fish. The level of GTH Iβ mRNA in the river fish (7.1 ± 1.0 fmol) was significantly higher than that in the off-coast fish (3.4 ± 0.7 fmol). In the males, the levels of GTH α2 and IIβ mRNAs showed similar tendency, whereas noticeable changes were not observed in the level of GTH Iβ mRNA.

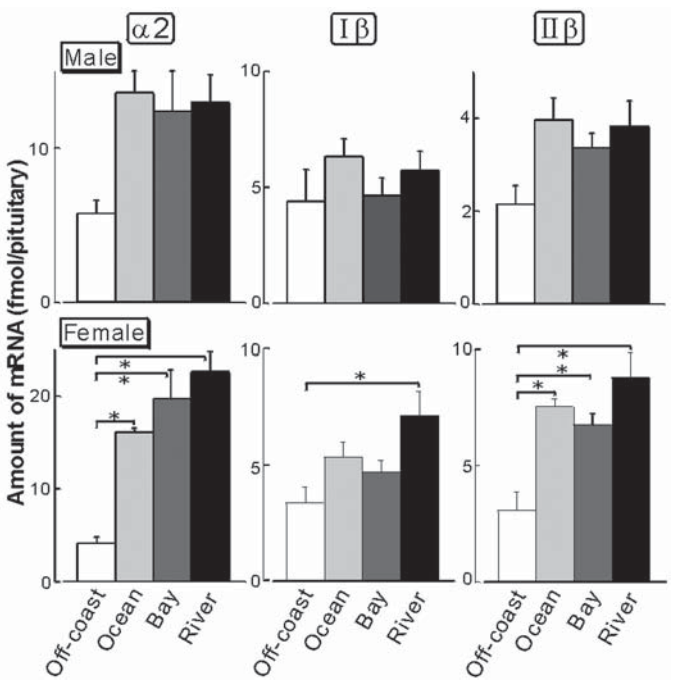

Fig. 1. Pituitary levels of GTH α2, Iβ, and IIβ mRNAs in prespawning chum salmon during last phases of spawning migration. Data are the mean ± SEM (see Table 1 for the number of fish in each group). *$p < 0.05$ compared to the off-coast fish (one-way analysis of variance [ANOVA] followed by Tukey test for multiple comparison).

Pituitary Levels of GH, PRL, and SL mRNAs

There were two different patterns of changes in the levels of mRNAs for GH/PRL/SL family hormones: a noticeable

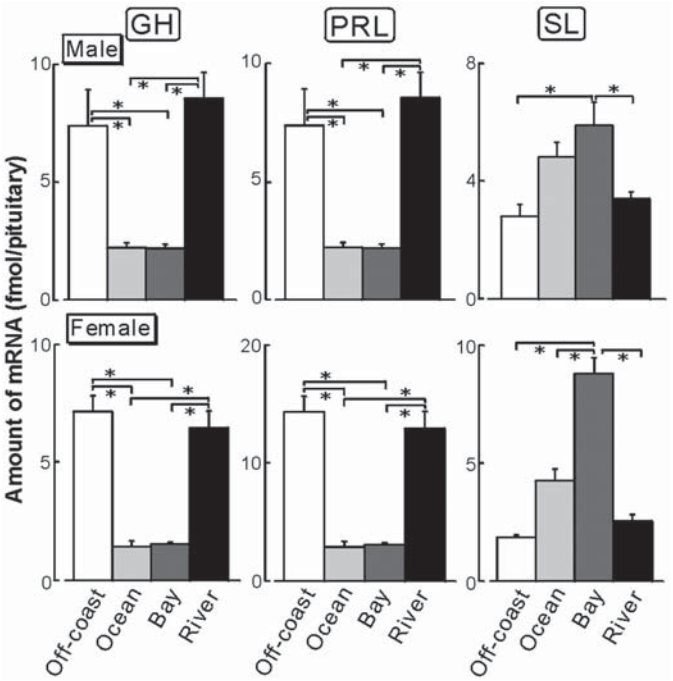

Fig. 2. Pituitary levels of GH, PRL, and SL mRNAs in prespawning chum salmon during last phases of spawning migration. Data are the mean ± SEM (see Table 1 for the number of fish in each group). *$p < 0.05$ compared to the off-coast fish (one-way ANOVA followed by Tukey test for multiple comparison).

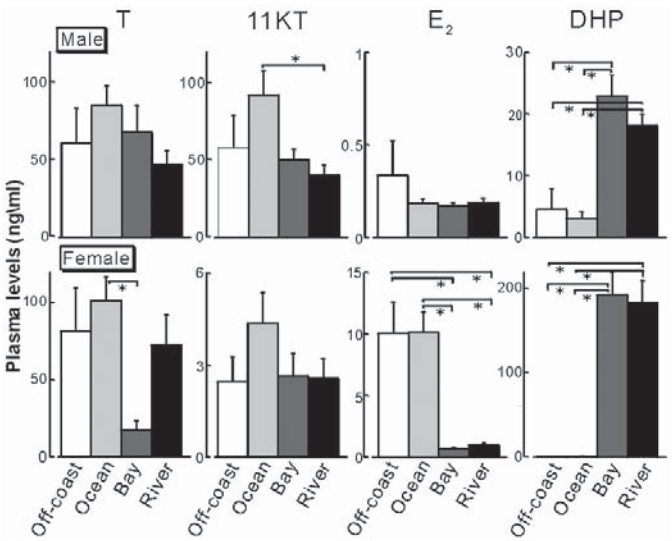

Fig. 3. Plasma levels of testosterone, 11KT, E_2, and DHP in prespawning chum salmon during last phases of spawning migration. Data are the mean ± SEM (see Table 1 for the number of fish in each group). *$p < 0.05$ compared to the off-coast fish (one-way ANOVA followed by Tukey test for multiple comparison).

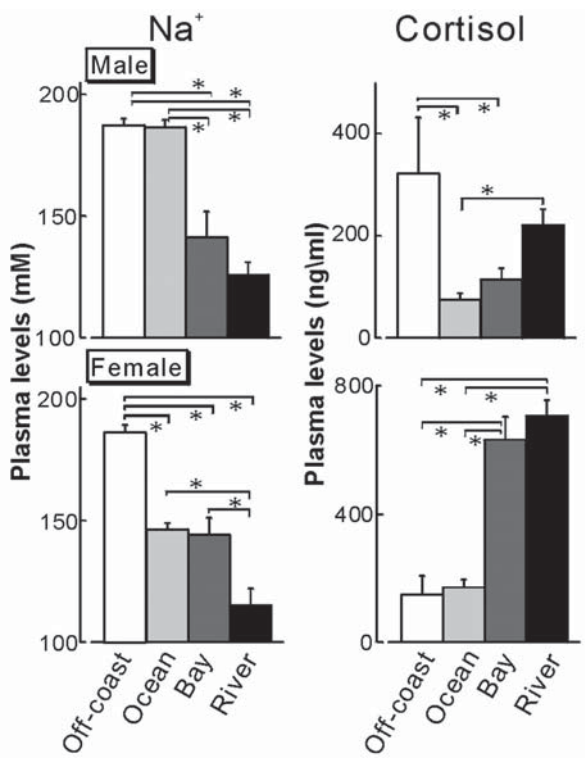

Fig. 4. Plasma levels of Na^+ and cortisol in prespawning chum salmon during last phases of spawning migration. Date are the mean ± SEM (see Table 1 for the number of fish in each group). *$p < 0.05$ compared to the off-coast fish (one-way ANOVA followed by Tukey test for multiple comparison).

decrease followed by an increase in the levels of GH and PRL mRNAs, and a gradual increase in the levels of SL mRNA (Fig. 2). The levels of GH and PRL mRNAs in the ocean and bay fish were one third to one fourth times those in the off-coast fish, and the levels in the river fish were significantly higher than those in the ocean and bay fish of both sexes. The levels of SL mRNA in the bay fish in both sexes were about two and five times those in the off-coast fish. The levels of SL mRNA in the river fish were significantly lower than those in the bay fish.

Plasma Levels of Testosterone, 11KT, E_2, and DHP

The levels of testosterone and 11KT showed the maximum values in the ocean fish in both males and females (Fig. 3). In the males, the levels of DHP in the bay and river fish were more than 10 times those in the off-coast and ocean fish. In the females, the levels of E_2 in the bay and river fish were about 0.05-fold those in the off-coast and ocean fish, whereas the levels of DHP in the bay and river fish were more than 100 times those in the off-coast and ocean fish.

Plasma Levels of Na^+

The plasma levels of Na^+ in the off-coast fish were about 190 m*M* in both males and females (Fig. 4). In the males, the levels of Na^+ in the bay fish were significantly lower than those in the off-coast and ocean fish, and in the females, the levels in both the bay and ocean fish were significantly lower than those in the off-coast fish. The levels of Na^+ in the ocean and/or bay fish were rather similar to the levels of the river fish. The plasma levels of Na^+ thus decreased before the fish entered the natal river.

Table 2
The GSIs and Spermiation or Ovulation Ratio
of Prespawning Chum Salmon Used in SW-to-FW Transition Experiments[a]

	Males			Females		
Environment/ year	GSI (%)	*n*	Spermiation ratio (%)	GSI (%)	*n*	Ovulation ratio (%)
SW/1994						
d 0	4.4 ± 0.3	6	16.0	19.1 ± 0.6	6	0.0
d 1	4.1 ± 0.6	6	33.3	21.4 ± 0.5	6	33.3
d 2	4.1 ± 0.5	6	0.0	19.5 ± 0.6	7	33.3
d 4	3.2 ± 0.6	2[c]	0.0	24.2 ± 0.7[b]	4[d]	100.0
FW/1994						
d 1	4.2 ± 0.5	6	33.3	20.6 ± 0.7	6	16.7
d 2	4.0 ± 0.2	6	50.0	21.5 ± 0.4	6	33.3
d 4	4.5 ± 0.2	6	33.3	22.1 ± 0.7	6	83.3
d 7	4.4 ± 0.2	6	33.3	20.7 ± 1.0	7	71.4
SW 0 d/1995						
d 0	4.0 ± 0.3	6	0.0	20.1 ± 0.8	8	0.0
d 1	4.6 ± 0.6	5	20.0	19.5 ± 0.7	9	22.2
d 3	5.0 ± 0.5	7	28.6	21.7 ± 0.7	10	25.0
SW 1 d/1995						
d 0	4.2 ± 0.2	8	0.0	19.7 ± 0.8	9	11.1
d 1	4.3 ± 0.3	6	0.0	20.4 ± 0.7	8	0.0
d 3	3.9 ± 0.3	7	57.1	22.3 ± 0.9	8	75.0
SW 3 d/1995						
d 0	4.1 ± 0.3	5	0.0	18.7 ± 1.9	8[e]	25.0
d 1	4.4 ± 0.6	7	42.9	20.8 ± 1.1	6	66.7
d 3	4.0 ± 0.5	6	16.7	23.1 ± 0.6	7	85.7

[a] In 1994, the ocean fish were transferred to an SW or FW environment. In 1995, the ocean fish were transferred to an FW environment after 0–3 d of retaining in SW.
[b] $p < 0.05$ compared to the FW group (Student's *t*-test).
[c] Individuals dead and not sampled was 66.6%.
[d] Individuals dead and not sampled was 33.3%.
[e] Individuals dead and not sampled was 20.0%.

Plasma Levels of Cortisol

In the males, the levels of cortisol in the off-coast fish were higher than those in the ocean and bay fish, whereas the levels in the river fish were higher than those in the ocean fish (Fig. 4). In the females, the levels of cortisol in the bay and river fish were significantly higher than those in the off-coast and ocean fish.

SW-to-FW Transition Experiments in 1994 and 1995

Gonadal Maturity

The GSI of initial controls (SW d 0 fish in 1994, and d 0 fish of SW 0-d group in 1995) were consistent with those of the bay and river fish in 1993 rather than those of the ocean and off-coast fish (Table 2). Neither SW retaining nor FW replacement changed the GSI in both sexes. The spermiation and ovulation ratio increased in both SW-retained fish (SW fish) and FW-replaced fish (FW fish), indicating that final maturation occurred during the 1-wk experimental period regardless of the osmotic environments.

Pituitary Levels of GTH α2, Iβ, and IIβ mRNAs

Changes in the levels of mRNAs for GTH subunits were different between 1994 and 1995 (Fig. 5). In 1994, the levels of GTH α2, Iβ, and IIβ mRNAs significantly increased during the experimental period in both SW and FW fish. In 1995, changes in the levels of GTH Iβ, and IIβ mRNAs were not noticeable, whereas the level of GTH α2 mRNA decreased in the females.

Pituitary Levels of GH, PRL, and SL mRNAs

Appreciable increases in the pituitary levels of GH, PRL, and SL mRNAs were seen in both 1994 and 1995 (Fig. 6). In 1994, the pituitary levels of GH, PRL, and SL mRNAs

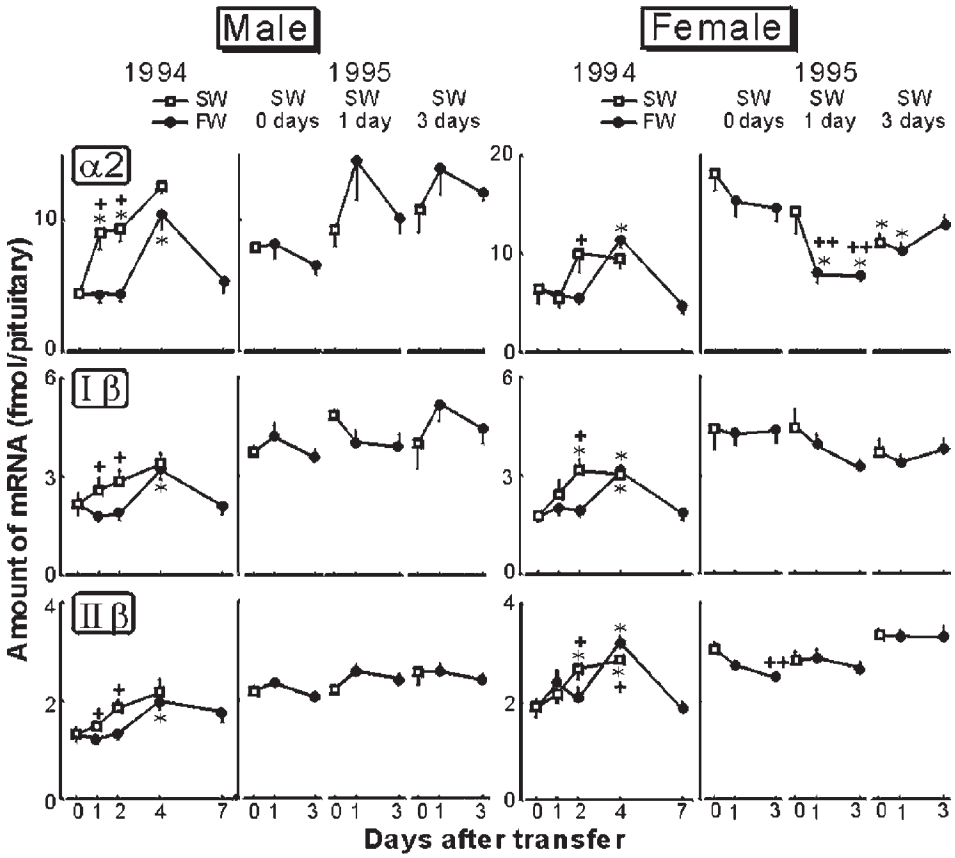

Fig. 5. Effects of SW-to-FW transition or SW retaining on the pituitary levels of GTH α2, Iβ, and IIβ mRNAs in prespawning chum salmon. In 1994, the ocean fish were transferred to an SW or FW environment. In 1995, the ocean fish were transferred to an FW environment after 0–3 d of retaining in SW. Data are the mean ± SEM (see Table 2 for the number of fish in each group). *$p < 0.05$ compared to the initial control (SW d 0 in 1994, d 0 of SW 0-d group in 1995); $^{++}p < 0.05$ compared to the SW control (d 0 of each group) (one-way ANOVA followed by Tukey test for multiple comparison); $^{+}p < 0.05$ compared to the FW group (Student's *t*-test).

increased during 4 d in both SW and FW fish. In 1995, the pituitary levels of GH, PRL, and SL mRNAs increased during the experimental period in the males, whereas no significant changes were seen in the females. In the males, the levels in d 1 fish of SW 3-d group (GH, 30.1 ± 7.0 fmol; PRL, 32.9 ± 9.9 fmol; and SL, 145.5 ± 46.6 fmol) were significantly higher than those in the initial controls. In the females, however, the levels of GH, PRL, and SL mRNAs in the initial controls (19.7 ± 2.5, 20.2 ± 5.0, and 36.2 ± 6.7 fmol, respectively) were near the peak values in the males, and retained higher levels during the experimental period. No apparent relationship to the SW-to-FW transition was observed for the levels of mRNAs for GH, PRL, and SL.

Plasma Levels of Testosterone, 11KT, E_2, and DHP

In the males, the levels of testosterone transiently increased one day after SW to FW transition in both 1994 and 1995 (Fig. 7). Afterward, the levels declined in both SW and FW fish. Changes in the level of 11KT were similar to those of testosterone. In the females, the levels of testosterone decreased during the experimental periods in both SW and FW fish.

Like the changes in the levels of GTH α2, Iβ, and IIβ mRNAs, the patterns of changes in the levels of E_2 and DHP were different between 1994 and 1995. In 1994, the levels of DHP in the males increased to about 20 ng/mL in both SW and FW fish. In the females, the levels of E_2 in the initial control were <2.0 ng/mL and retained similar levels during the experimental period, whereas the levels of DHP increased to 109.0 ± 33.3 ng/mL in SW fish and 200.3 ± 56.4 ng/mL in FW fish. In 1995, changes in the levels of DHP were not noticeable in both males and females except those in d 3 fish of SW 1-d group in the males. In the females, the levels of E_2 in the initial controls were >5.0 ng/mL and decreased during the experimental period.

Plasma Levels of Na^+

The levels of Na^+ decreased within 1 d after the SW-to-FW transition in both males and females (Fig. 8), indicating that fish rapidly adapted to the FW environment within 1 d after the SW-to-FW transition in both 1994 and 1995. Changes in the levels of Na^+ in the SW fish, however, were varied between the 2 yr. In 1994, the levels of Na^+ in the SW fish were high and did not change significantly. Further,

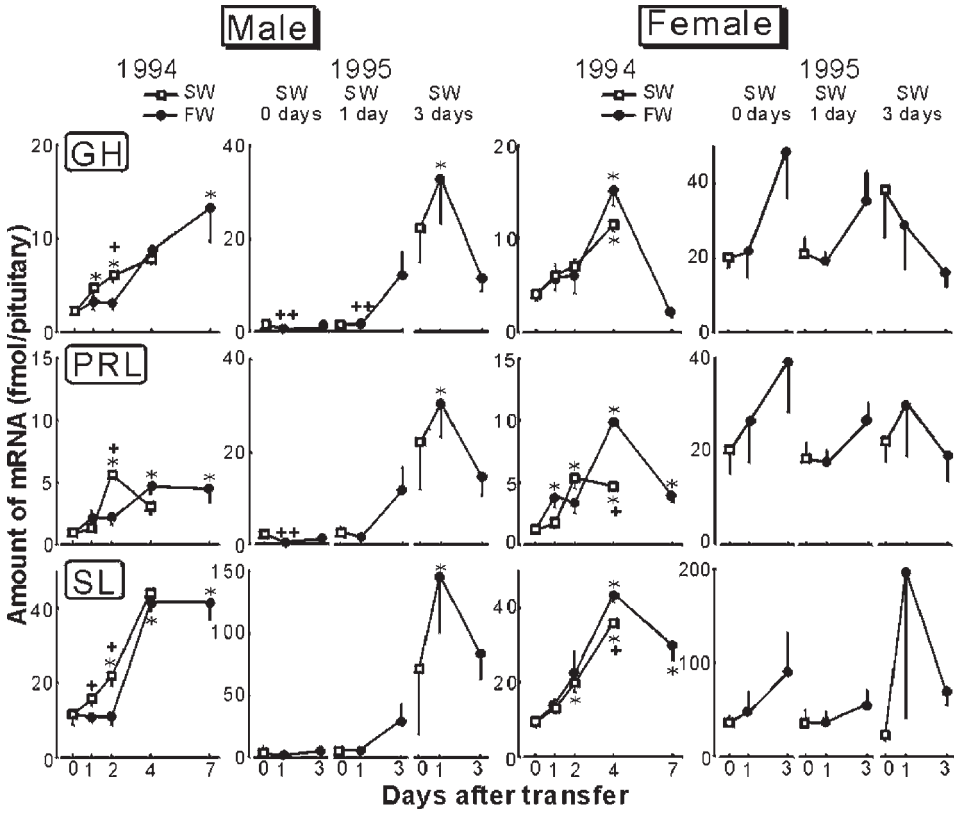

Fig. 6. Effects of SW-to-FW transition or SW retaining on pituitary levels of GH, PRL, and SL mRNAs in prespawning chum salmon. In 1994, the ocean fish were transferred to an SW or FW environment. In 1995, the ocean fish were transferred to an FW environment after 0–3 d of retaining in SW. Data are the mean ± SEM (see Table 2 for the number of fish in each group). $^{*}p < 0.05$ compared to the initial control (SW d 0 in 1994, d 0 of SW 0-d group in 1995); $^{++}p < 0.05$ compared to the SW control (d 0 of each group) (one-way ANOVA followed by Tukey test for multiple comparison); $^{+}p < 0.05$ compared to the FW group (Student's *t*-test).

the SW-retained fish showed higher mortality, 66.6% on d 4 (Table 2). In 1995, plasma Na^{+} levels in SW-retained fish decreased during the experimental period, as was seen in the ocean and bay fish in 1993.

Plasma Levels of Cortisol

The levels of cortisol in the males transiently decreased after the SW-to-FW transition, whereas the change was not significant in the females (Fig. 8).

Discussion

The present study showed that the pituitary levels of GTH α2 and IIβ mRNAs in the ocean, bay, and river fish were two to five times those in the off-coast fish, and the levels of SL mRNAs in the bay fish were two to four times those in the off-coast fish. The levels of GH and PRL mRNAs in the ocean and bay fish were significantly lower than those in the off-coast fish, and the levels in the river fish were three to five times those in the ocean and bay fish. In SW-to-FW transition experiments, the pituitary levels of GTH α2, Iβ, and IIβ mRNAs and plasma levels of DHP transiently increased in 1994, whereas the changes were not noticeable in 1995. The levels of GH, PRL, and SL mRNAs increased in both SW and FW environments, and no apparent effects of the SW-to-FW transition were observed in the levels of PRL mRNA.

We adopted different procedures to determine the pituitary levels of GH, PRL, and SL mRNAs in the samples obtained in 1994 and 1995. Nonetheless, the pattern of changes might not be affected by the assay procedure itself, since the levels of mRNAs were determined by a single assay within each year. We further considered that when the sources of standards were the same, the determined values for the same samples were within a certain level, since the values in 1994 and 1995 were in similar ranges and intra- and interassay coefficients of variation (CVs) showed reliability of the present assays. Thus, the estimated values are reliable enough to discuss the changes in the levels of mRNAs in association with physiologic events, such as final maturation and osmoregulation.

The elevation in pituitary levels of GTH α2, IIβ, and SL mRNAs during homing migration in prespawning chum salmon in 1993 coincided well with the changes reported in chum salmon of Ishikari stock *(1,2)*. We consider that elevations of GTH α2, IIβ, and SL gene expression are general biologic phenomena during homing migration of prespawn-

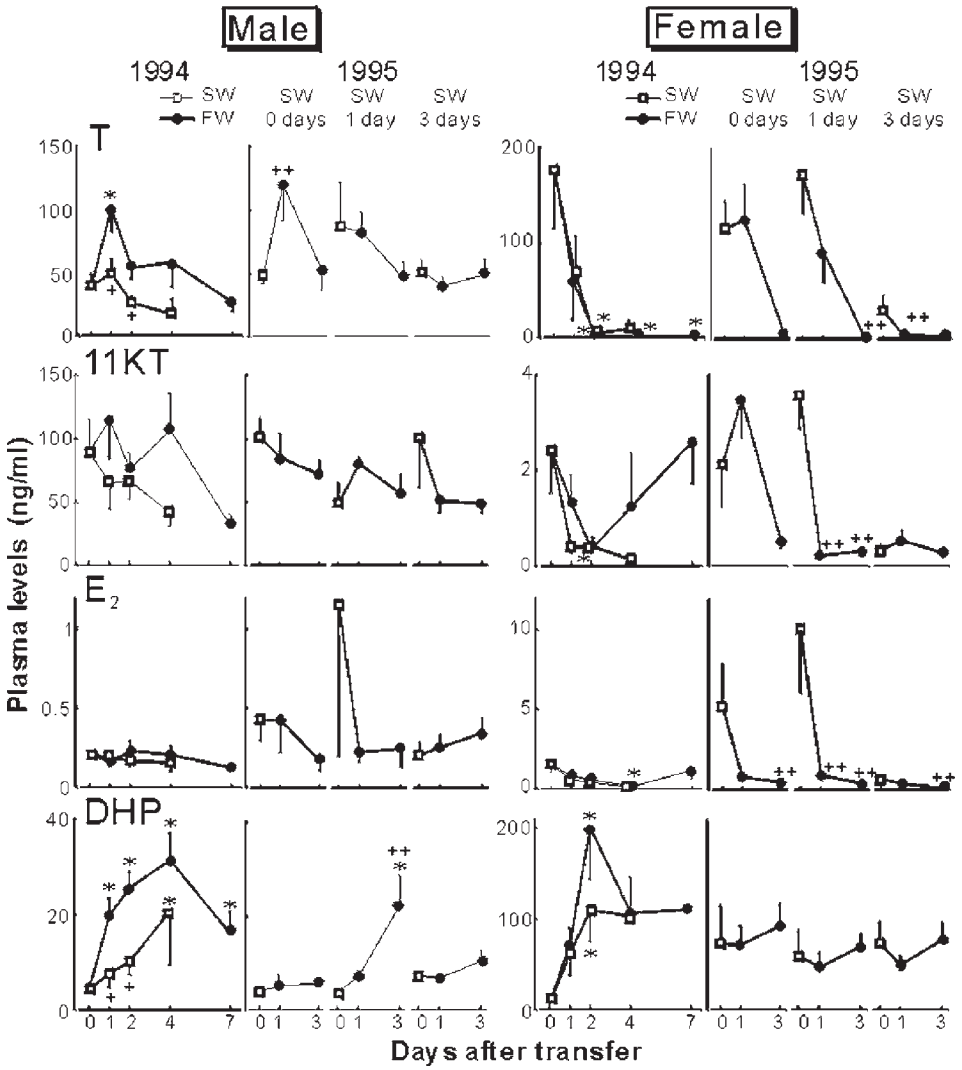

Fig. 7. Effects of SW-to-FW transition or SW retaining on plasma levels of testosterone, 11KT, E_2, and DHP in prespawning chum salmon. In 1994, the ocean fish were transferred to an SW or FW environment. In 1995, the ocean fish were transferred to an FW environment after 0–3 d of retaining in SW. Data are the mean ± SEM (see Table 2 for the number of fish in each group). $*p < 0.05$ compared to the initial control (SW d 0 in 1994, d 0 of SW 0-d group in 1995); $^{++}p < 0.05$ compared to the SW control (d 0 of each group) (one-way ANOVA followed by Tukey test for multiple comparison); $^{+}p < 0.05$ compared to the FW group (Student's *t*-test).

ing chum salmon. However, the changes were different between Sanriku stock and Ishikari stock, since the levels of GTH α2, IIβ, and SL mRNAs increased at the hatchery after the fish entered the natal river in Ishikari stock. In Ishikari stock, homing chum salmon complete gonadal maturation after the fish enter the natal river *(5)*, whereas, in Sanriku stock, homing fish complete gonadal maturation before entering the natal river *(4)*. It is thus probable that the elevations in GTH α2, IIβ, and SL gene expression observed in maturing fish are initiated by temporal neuroendocrine information related to final maturation regardless of environmental salinity.

Our concern for the present results is whether the higher levels of GH and PRL mRNAs in the off-coast fish represent general phenomena during homing migration to the natal river, because consistent changes in the levels of GH and PRL mRNAs were not observed in the Ishikari stock *(2)*. As mentioned earlier, the changes in the levels of GTH α2, IIβ, and SL mRNAs were similar to those observed in the Ishikari stock. We thus consider that the changes in the levels of GH and PRL mRNAs during homing migration through the off-coast to the bay represent physiologic changes in the levels of hormonal mRNAs, although we do not have any lines of evidence to explain biologic meanings of the transient decreases in the levels of GH and PRL mRNAs.

In the SW-to-FW transition experiments, no apparent relationships to osmoregulation were observed in the levels of PRL mRNA. In prespawning chum salmon, the turnover rate of PRL increased 6 d after the females were transferred to the FW environment, although both males and females

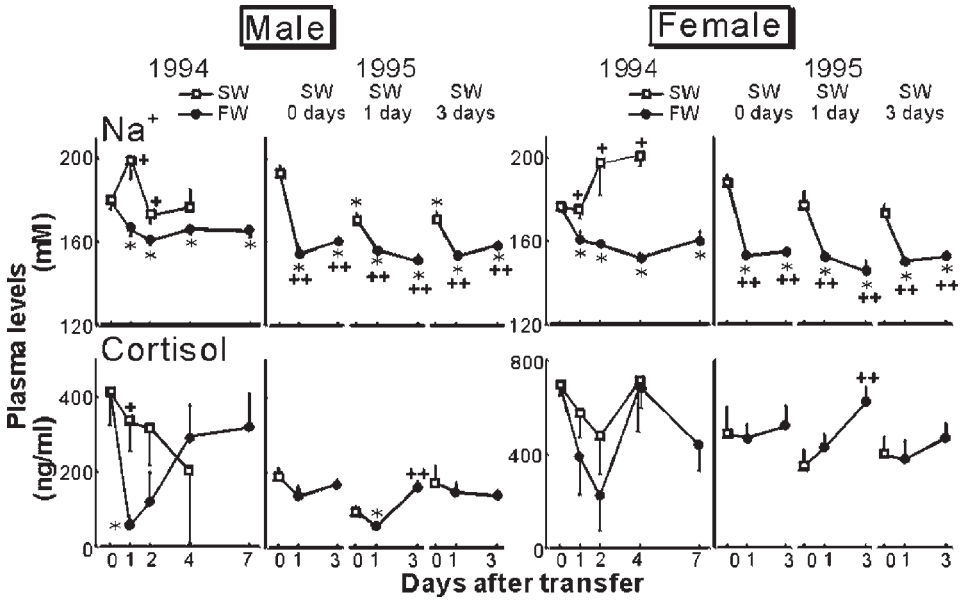

Fig. 8. Effects of SW-to-FW transition or SW retaining on plasma levels of Na^+ and cortisol in prespawning chum salmon. In 1994, the ocean fish were transferred to an SW or FW environment. In 1995, the ocean fish were transferred to an FW environment after 0–3 d of retaining in SW. Data are the mean ± SEM (see Table 2 for the number of fish in each group). $^*p < 0.05$ compared to the initial control (SW d 0 in 1994, d 0 of SW 0-d group in 1995); $^{++}p < 0.05$ compared to the SW control (d 0 of each group) (one-way ANOVA followed by Tukey test for multiple comparison); $^+p < 0.05$ compared to the FW group (Student's *t*-test).

adapted to the FW environment within several hours *(6)*. Further, no difference was found in the rate of GH and PRL release from the pituitaries between those incubated in isotonic medium (325 mos*M*) and those in hypotonic medium (250 mos*M*) in matured chum salmon *(7)*. Although PRL is considered to be important for FW adaptation in teleosts *(8,9)*, the present results indicate that hypoosmotic stimulation by transition from SW to FW is not critical to enhancing expression of PRL gene in prespawning chum salmon.

The pituitary levels of PRL mRNA in the river fish were significantly higher than those in the ocean and bay fish. Such changes were consistent with those observed in homing chum salmon of Ishikari stock *(2)*. It is thus noteworthy that no apparent effects of the SW-to-FW transition were observed on the levels of PRL mRNA. A plausible explanation for this discrepancy is that, regardless of SW or FW environment, elevation in PRL gene expression is initiated by temporal information related to the fish's arrival at the natal river. Actually, in masu salmon of the Mori hatchery strain, which usually return to their natal river from March to May, PRL gene expression in FW-reared fish was elevated from early through middle spring *(18)*.

The pituitary levels of PRL mRNA increased in both SW and FW fish. Since evidence for the involvement of PRL in the regulation of sexual maturation, including increases in the plasma levels of PRL during sexual maturation, have been accumulated in salmonids *(6,8,9)*, we consider that the elevation of PRL gene expression may be related to final maturation. In prespawning chum salmon that were almost matured, expression of PRL gene would be stimulated even in an SW environment by some mechanisms not yet clarified.

In summary, expression of genes encoding GTH α2, IIβ, and SL was elevated during the last stages of spawning migration. Expression of genes encoding PRL also increased during homing migration from the bay to the river, whereas no apparent effects of the SW-to-FW transition were observed on the levels of PRL mRNAs. In prespawning chum salmon, expression of genes encoding GTH α2, IIβ, and SL were elevated with final maturation regardless of osmotic environment. Hypoosmotic stimulation by transition from the SW-to-FW environment is not critical to modulating expression of genes for PRL. PRL gene expression can be elevated in SW fish that are sexually almost matured.

Materials and Methods

Experiment 1: Changes in Expression of Genes for GTH Subunits and GH/PRL/SL Family Hormones During Spawning Migration

We used prespawning chum salmon of Sanriku stock. This population has genetically characteristic haplotype distribution *(10)* and migratory behavior *(4)* when compared to chum salmon of Ishikari stock that were used in our previous studies *(1,2)*. Homing chum salmon of Sanriku stock usually complete gonadal maturation just before entering the river, because their spawning ground is about 1 km upstream from the river's mouth *(4)*. By contrast, homing chum salmon of Ishikari stock require 3 wk to reach the hatchery

from the river mouth *(11)* and complete gonadal maturation at the hatchery *(5)*, because the distance from the mouth of the Ishikari River to the hatchery is about 70 km.

Animals

Fish were captured at four areas along their migratory pathway in the Sanriku coast of the Pacific Ocean, as described in the Introduction. The off-coast fish were caught on the Sanriku coast north to Otsuchi Bay by salmon long lines during the cruise of the research vessel *Tansei-Maru* of Ocean Research Institute, University of Tokyo in early to mid-November 1993. The long lines were set in the early morning before dawn and were recovered within 1 h after the set, and only actively moving fish were sampled. Afterward, maturing and matured chum salmon were caught in late November and early December 1993, by a salmon settled-net placed 1 km outside Otsuchi Bay (ocean), a net settled in the bay close to the mouth of the Otsuchi River (bay), and a trap settled in the Otsuchi River 500 m upstream to the river mouth (river) *(3)*. Most of the off-coast fish still showed silver color, whereas the ocean fish developed a faint nuptial color of mature fish. The bay and river fish had well-developed nuptial color.

Preparation of Tissue

Fish were anesthetized with buffered 0.02% tricaine methane salfonate (MS-222; Sigma, St. Louis, MO) and measured for body weight and folk length. Body weights were 3.5 ± 0.1 kg ($n = 27$) in the males and 3.9 ± 0.1 kg ($n = 32$) in the females, while folk lengths were 67.8 ± 1.2 cm in the males and 69.2 ± 0.8 cm in the females. Blood samples were collected from the caudal vasculature, kept on ice, and centrifuged to separate plasma at 3000 rpm at 4ºC for 15 min. The plasma was stored frozen at –20ºC until assayed. Plasma levels of Na^+ were later determined by an electrolyte analyzer (AVL9130; AVL-Scientific, Graz, Austria). After the collection of blood samples, spermiation or ovulation was checked by gentle abdominal massage. Fish were killed by decapitation, and gonads were dissected out and weighed to calculate GSI (gonad wt/body wt × 100). Immediately after decapitation, pituitaries were removed, frozen in liquid nitrogen, and stored at –80ºC. Total RNA was extracted from single pituitaries by the guanidium thiocyanate/hot phenol method *(12)*.

Quantitative Dot-Blot Analysis

Preparation of Single-Stranded Standard DNA and cDNA Probes. The levels of mRNAs for GTH α2, Iβ, and IIβ; GH; PRL; and SL in the pituitaries were quantitatively determined by dot-blot analyses as previously described *(1,2)*. In brief, single-stranded sense DNAs of the same sequences of mRNAs for salmon GTH subunits and GH/PRL/SL family hormones were prepared by polymerase chain reactions (PCRs), and used as the standards. Labeled cDNA probe that was specific to mRNA for GTH subunits, PRL, or SL was prepared by a primer extension method using a Megaprime DNA labeling system (Amersham, Tokyo) and [α^{32}-P]dCTP (Amersham) with a specific oligonucleotide primer. The probes for GTH subunits, PRL, and SL were about 200, 350, and 500 bases long, respectively, and specific to corresponding standard DNAs. Since cDNA probes for GH mRNAs were seriously crossreacted with PRL and SL mRNAs in preliminary experiments, we used oligonucleotide sequences that were at the positions of 400–419, 441–470, and 499–528. They were labeled with a 3'-end labeling kit (Amersham) and [α^{32}-P]dATP according to the manufacturer's instructions. The serially diluted standard DNA and 1/50 or 1/150 vol of the total RNA from individuals were blotted onto a membrane using a MilliBlot-D (Millipore, Nihon Millipore, Tokyo) and hybridized with the labeled probe.

Dot-Blot Analysis. For the assay of mRNAs for GTH subunits, PRL, and SL, hybridization was performed at 65ºC overnight in a solution containing 5X Denhardt's solution, 5X SSPE, 0.5% sodium dodecylsulfate (SDS), 100 μg/mL of denatured calf thymus DNA, and a labeled probe (2×10^6 cpm/mL). For the assay of mRNA for GH, hybridization was performed at 42ºC overnight. The membranes were washed with 2X SSPE/0.1% SDS at room temperature for 15 min, twice with 2X SSPE/0.1% SDS at 50 or 65ºC for 30 min, twice with 0.1X SSPE/0.1% SDS at 50ºC for 30 min, and exposed to a Fuji imaging plate for 4 h. Hybridization signals were analyzed by a Bioimaging analyzer (Fuji Photo Film, Tokyo). The levels of mRNAs were estimated from the standard curves. All samples within the same year were analyzed in a single assay to reduce interassay variation. The sensitivity of the assay for GTH α2 and IIβ, GTH Iβ, GH, PRL, and SL was 1.0, 3.0, 20.0, 2.0, and 1.3 amol, respectively. The ranges of intra- and interassay CVs were 6.2–17.0%, and 2.5–14.0%, respectively.

Enzyme Immunoassay

Plasma levels of testosterone, 11KT, E_2, DHP, and cortisol were determined by enzyme immunoassays as previously described *(4,13)*. The sensitivity of the assay for testesterone and 11KT, E_2 and DHP, and cortisol was 30.0, 10, and 300.0 pg/mL, respectively. Ranges of intra- and interassay CVs were 9.6–14.0%, and 7.6–20.0%, respectively.

Experiment 2: Effects of Hypoosmotic Stimulation on Expression of Genes for GTH Subunits and GH/PRL/SL Family Hormones

In the second experiment, environmental SW of salmon in aquaria was replaced with FW to examine the effect of hypoosmotic stimulation on the expression of genes encoding GTH subunits and GH/PRL/SL family hormones. We tried to clarify whether the changes in the levels of hormonal mRNAs are associated with final maturation or FW adaptation.

Animals

Fish caught by the settled net, referred to as ocean fish, were used in the SW-to-FW transition experiments in 1994

and 1995. Fish were captured near the mouth of Otsuchi Bay and transferred by oxygenated-SW aquaria (90 cm × 140 cm × 95 cm) to the Otsuchi Marine Research Center, separated into males and females, and transferred to aerated-running SW aquaria (290 cm × 150 cm × 100 cm, 12ºC, 0.1 t/min) in early December. They had faint to strong nuptial color of maturing salmon in both 1994 and 1995.

SW-to-FW Transition Experiment in 1994

In 1994, the fish were maintained for 1 d in a calm condition to alleviate stresses from capture and transportation. Some of them were then randomly selected, sampled, and served as the initial controls. Most of the fish were divided into two groups: those retained in SW and those whose environmental running SW was replaced with running FW (11ºC, 0.1 t/min). The FW and SW fish were sampled 1, 2, and 4 d after the experimental treatments. The FW fish were additionally sampled on d 7. The fish retained in SW, however, could not survive for more than 1 wk, so that 7-d SW fish were not prepared. Body weights were 3.2 ± 0.1 kg (n = 48) in the males and 3.2 ± 0.0 (n = 44) in the females, while folk lengths were 66.8 ± 0.8 cm in the males and 66.5 ± 0.5 cm in the females.

SW-to-FW Transition Experiment in 1995

In 1995, we examined the effects of SW retention for 0–3 d on expression of GTH subunit, GH, PRL, and SL genes to see the effects of malfunctions by hyperosmotic environment in the maturing salmon *(4,14)*. The fish transported and transferred to the SW aquaria were divided into three groups: those in environmental running SW that was immediately replaced with running FW (SW 0-d group), those retained in an SW environment for 1 d (SW 1-d group), and those retained in an SW environment for 3 d (SW 3-d group). The fish in SW sampled on d 0 in the SW 0-d group served as the initial control. In each group, SW controls (d 0) were sampled just before transfer to the FW environment. Afterward, fish in the FW environment were sampled 1 and 3 d after the experimental treatments. Body weights were 3.7 ± 0.1 kg (n = 60) in the males and 3.6 ± 0.1 (n = 73) in the females, while folk lengths were 69.4 ± 0.8 cm in the males and 68.2 ± 0.6 cm in the females. In both 1994 and 1995, the levels of Cl^- in the water of the aquaria, which were monitored using a chloridometer (Buchler), showed that SW was completely replaced by FW within 1 h.

Preparation of Tissue

Fish were anesthetized with buffered 0.02% MS-222 (Sigma) and sampled as described in experiment 1. Plasma levels of testosterone, 11KT, E_2, DHP, cortisol, and Na^+ were determined in a single assay as in experiment 1. Total RNA extraction was performed also as described in experiment 1. The amounts of total RNA extracted from single pituitaries were 112.0 ± 1.1 µg in the males and 120.0 ± 6.2 µg in the females in 1994, while they were 181.1 ± 6.0 µg in the males and 179.3 ± 6.0 µg in the females in 1995. The levels of mRNAs for GTH subunits were quantitatively determined by the dot-blot analyses as described in experiment 1. The levels of mRNAs for GH, PRL, and SL were determined by the dot-blot analyses in 1994, but were determined by real-time reverse transcriptase (RT)-PCR methods in 1995, because we recently developed highly sensitive quantitative real-time RT-PCR.

Real-Time PCR

Preparation of Samples. Total RNA of the pituitary (200 ng) was used for synthesis of the first-strand cDNA. Reverse transcription was carried out in a final volume of 10 µL containing 500 µ*M* of dNTP, 5 µ*M* oligo $d(T)_{12–18}$ primer (Gibco BRL, Tokyo), 1X RT buffer (50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3), 5.5 m*M* $MgCl_2$, 4 U of RNase inhibitor (Toyobo, Tokyo), and 12.5 U of Multiscribe Reverse Transcriptase (PE Applied Biosystems, Foster City, CA) at 25ºC for 10 min, 48ºC for 30 min, and 95ºC for 5 min.

Preparation of Standards. Plasmid DNAs containing cDNA inserts for GH (a gift from Kyowa Hakko) *(15)* and PRL (a gift from Kyowa Hakko) *(16)* were digested by restriction enzyme *Bam*HI, and SL (a gift from Dr. M. Ono, Rikkyou University) *(17)* by *Eco*RI. Then corresponding cDNA inserts were extracted by a Qiagen gel extraction kit (Qiagen, Hilden, Germany). GH and PRL inserts were ligated into the *Bam*HI site of pBluescript SK, and SL insert into the *Eco*RI site. These GH/PRL/SL plasmid reconstructs were digested by *Eco*RI, *Xba*I, and *Not*I, respectively, at one site just after the sequence of poly (A). Using MAXIscript™ (Ambion, Austin, TX), RNA synthesis was performed according to the manufacturer's instructions. The synthesized RNA was quantified and used as the standard RNA. Serially diluted standard RNA (6×10^3 to 6×10^8 copies/µL) were reverse-transcribed in 10 µL of reaction mixture to prepare the first-strand cDNA, diluted to 1:5, and used as the standard.

Preparation of Primers and Probes. The nucleotide sequences of GH, PRL, and SL mRNAs of chum salmon and rainbow trout *(15–17)* were considered to design primers for PCRs. Briefly, the sequences of primers and probes were selected in the coding region of GH, PRL, and SL mRNA. For the assay of GH mRNA, the sequences of primers were 5'-GTTCAGTCCTGAAGCTGCTC-3' (forward) and 3'-CTCTTCGAGTCGCTGGAGTT-5' (reverse), and that of probe was 5'-(Vic)-TGAATCCTGGGAGTACC CTAGCCAGAC-(Tamra)-3'. For the assay of PRL mRNA, the sequences of primers were 5'-TGTGTCACACCTCCT CACTC-3' (forward) and 3'-CTAGTCCCTTGACGTCCT GA-5' (reverse), and that of probe was 5'-(Fam)-AGAATG AGCTGATC TCCCTGGCTCGCTC-(Tamra)-3'. For the assay of SL mRNA, the sequences of primers were 5'-CGA GCAGGGCAGCATCATA-3' (forward) and 5'-GTTCCT CTCTGGGA GCGCATAG-3' (reverse), and that of probe was 5'-(Fam)CCAA CACGCCGAGCTCATCTACCGT (Tamra)-3' (TaqMan probe, PE Applied Biosystems). In preliminary experiments, the primer sets for GH, PRL, and SL

amplified specific bands of 136, 199, and 158 bp, respectively (data not shown).

Real-Time PCR Assay. Real-time quantitative PCR was carried out using an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The PCR reaction mixture (10 µL) contained 1X TaqMan buffer A (PE Applied Biosystems), 3.5 m*M* $MgCl_2$, deoxynucleotide triphosphates (0.2 m*M* dATP, 0.2 m*M* dCTP, 0.2 m*M* dGTP, and 0.2 m*M* dUTP), 100 n*M* each of forward and reverse primers, 1.3 µmol of fluorogenic probe, and 0.25 U of AmpliTaq Gold polymerase. Reaction was carried out at 95ºC for 10 min, and 45 cycles at 95ºC for 30 s and at 60ºC for 1 min. In this assay, 1 µL of standard cDNA (6×10^2, 6×10^3, 6×10^4, 6×10^5, 6×10^6, 6×10^7, 6×10^8, 6×10^9 copies/µL) were applied in triplicate, and 1 µL of RT products from total RNA was subjected in duplicate. In each assay, a standard sample (about 200 pg of first-strand cDNA from pituitary total RNA of chum salmon) was subjected in triplicate to estimate interassay CV between runs. The sensitivity of the assay for GH, PRL, and SL was 9.96×10^{-4} amol. The ranges of intra- and interassay CVs were 15.0–19.0% and 4.9–19.0%, respectively.

Statistical Analyses

One-way ANOVA followed by Tukey test for multiple comparison was applied when comparing locations or control and treated fish. Student's *t*-test was applied when comparing SW fish and FW fish in 1994.

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References

1. Kitahashi, T., Ando, H., Ban, M., Ueda, H., and Urano, A. (1998). *Zool. Sci.* **15,** 753–760.
2. Taniyama, S., Kitahashi, T., Ando, H., Ban, M., Ueda, H., and Urano, A. (1999). *J. Mol. Endocrinol.* **23,** 189–198.
3. Hiraoka, S., Hyodo, S., Kubokawa, K., Ando, H., and Urano, A. (1996). *Zool. Sci.* **13,** 737–745.
4. Saito, D., Ota, Y., Hiraoka, S., Hyodo, S., Ando, H., and Urano, A. (2001). *Zool. Sci.* **18,** 389–396.
5. Onuma, T., Higashi, Y., Ota, Y., Ando, H., and Urano, A. (2000). *Prog. Jpn. Soc. Comp. Endocrinol.* **15,** 16.
6. Ogasawara, T., Sakamoto, T., and Hirano, T. (1996). *Zool. Sci.* **13,** 443–447.
7. Suzuki, R., Kishida, M., Ogasawara, T., Hasegawa, S., and Hirano, T. (1987). *Gen. Comp. Endocrinol.* **68,** 76–81.
8. Hirano, T. (1986). *Prog. Clin. Biol. Res.* **205,** 53–74.
9. Ogasawara, T., Kubota, J., Sakamoto, T., and Hirano, T. (1988). In: *Prolactin gene family and its receptors.* Hoshino, K. (ed.). Elsevier: Amsterdam.
10. Sato, S., Ando, J., Ando, H., Urawa, S., Urano, A., and Abe, H. (2001). *Zool. Sci.* **18,** 99–106.
11. Kitahashi, T., Ando, H., Urano, A., et al. (2000). *Zool. Sci.* **17,** 1247–1253.
12. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). *Biochemistry* **24,** 5294–5299.
13. Asahina, K., Kambegawa, A., and Higashi, T. (1995). *Fisheries Sci.* **61,** 491–494.
14. Hirano, T., Ogasawara, T., Hasegawa, S., Iwata, M., and Nagahama, Y. (1990). *Gen. Comp. Endocrinol.* **78,** 254–262.
15. Sekine, S., Mizukami, T., Nishi, T., et al. (1985). *Proc. Natl. Acad. Sci. USA* **82,** 4306–4310.
16. Kuwana, Y., Kuga, T., Sekine, S., Sato, M., Kawauchi, H., and Itoh, S. (1988). *Agric. Biol. Chem.* **52,** 1033–1039.
17. Ono, M., Takayama, Y., Rand-Weaver, M., et al. (1990). *Proc. Natl. Acad. Sci. USA* **87,** 4330–4334.
18. Bhandari, R. K., Taniyama, S., Kitahashi, T., et al. (2003). *Gen. Comp. Endocrinol.* **130,** 55–63.