Stimulatory Effects of Insulin-Like Growth Factor 1 on Expression of Gonadotropin Subunit Genes and Release of Follicle-Stimulating Hormone and Luteinizing Hormone in Masu Salmon Pituitary Cells Early in Gametogenesis

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Insulin-like growth factor-I (IGF-I) has been shown to be involved in pubertal activation of gonadotropin (GTH) secretion. The aim of this study was to determine if IGF-I directly stimulates synthesis and release of GTH at an early stage of gametogenesis. The effects of IGF-I on expression of genes encoding glycoprotein α (GPα), follicle-stimulating hormone (FSH) β, and luteinizing hormone (LH) β subunits and release of FSH and LH were examined using primary pituitary cells of masu salmon at three reproductive stages: early gametogenesis, maturing stage, and spawning. IGF-I alone or IGF-I + salmon GnRH (sGnRH) were added to the primary pituitary cell cultures. Amounts of GPα, FSHβ, and LHβ mRNAs were determined by real-time PCR. Plasma and medium levels of FSH and LH were determined by RIA. In males, IGF-I increased the amounts of all three subunit mRNAs early in gametogenesis in a dose-dependent manner, but not in the later stages. In females, IGF-I stimulated release of FSH and LH early in gametogenesis, whereas no stimulatory effects on the subunit mRNA levels were observed at any stage. IGF-I + sGnRH stimulated release of FSH and LH at all stages in both sexes, but had different effects on the subunit mRNA levels depending on subunit and stage. The present results suggest that IGF-I itself directly stimulates synthesis and release of GTH early in gametogenesis in masu salmon, possibly acting as a metabolic signal that triggers the onset of puberty.

Key words: insulin-like growth factor 1, gonadotropin-releasing hormone, gonadotropin, pituitary, puberty, salmon

INTRODUCTION

Insulin-like growth factor I (IGF-I) is a 70-amino-acid polypeptide that has metabolic and mitogenic activities, and acts as a mediator of somatotropic action of growth hormone (GH). Although a major source of circulating IGF-I is the liver, IGF-I and IGF-I receptors are expressed in the

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pituitary (Bach and Bondy, 1992; Aguado *et al*., 1993), and IGF-I exerts a regulatory influence upon hormone release from the pituitary, as reported for GH (Yamashita and Melmed, 1986; Kajimura *et al*., 2002).

In addition to its role in the somatotropic axis, IGF-I has been shown to be involved in reproductive function at multiple levels of the hypothalamic-pituitary-gonadal axis. In mammals, the elevation of circulating IGF-I levels that occurs at puberty is considered to be connected to pubertal activation of gonadotropin (GTH) production (Daftary and Gore, 2005). IGF-I has been shown to stimulate the gonadotropic axis at both the hypothalamic and pituitary levels. For example, intraventricular administration of IGF-I in female rats increased plasma luteinizing hormone (LH) and

advanced the onset of puberty (Hiney *et al*., 1996). The administration of IGF-I to rat pituitary cell cultures increased basal secretion of follicle-stimulating hormone (FSH) and LH and gonadotropin-releasing hormone (GnRH)-induced LH secretion (Kanematsu, *et al*., 1991; Soldani *et al*., 1994, 1995; Xia *et al*., 2001). Moreover, in male rats, IGF-I stimulated release of FSH and LH, with increased expression of the glycoprotein α (GPα) subunit gene but not of the FSHβ, nor LHβ subunit genes (Pazos *et al*., 2004).

In teleosts, there is also evidence for stimulatory roles of IGF-I in the gonadotropic axis. In the pituitary cells of European eels (*Anguilla anguilla*), IGF-I increased the release and cell content of LH in a time- and dose-dependent manner (Huang *et al*., 1998, 1999). In salmonids, co-administration of IGF-I and salmon GnRH (sGnRH) to the pituitary cells of rainbow trout elevated sensitivity to sGnRH, as measured by release of FSH and LH at both immature and mature stages (Weil *et al*. 1999). Baker *et al*. (2000) reported that IGF-I increased GnRH-stimulated FSH release and the cell content of FSH and LH in the pituitary cells of coho salmon (*Oncorhynchus kisutch*) early in gametogenesis. Furthermore, the elevation of plasma IGF-I levels during the onset of puberty was one of the important endocrine changes linked to advanced development of the gonads in both sexes (Campbell *et al*., 2003; 2006). All these observations

at the pituitary level suggest that IGF-I has a key role in controlling synthesis and release of FSH and LH through interaction with GnRH in salmonids. However, it is not clear whether IGF-I directly stimulates synthesis and release of FSH and LH at puberty in salmonids. Furthermore, the molecular mechanism of the functional interaction between IGF-I and GnRH remains to be determined.

In masu salmon (*Oncorhynchus masou*), temporal expression patterns of GPα, FSHβ, and LHβ subunit genes vary during sexual maturation. FSHβ mRNA starts to increase in the early stages of gametogenesis in March, whereas LHβ mRNA starts to increase at a later stage in June (Kitahashi *et al*., 2004). In masu salmon pituitary cells, GnRH differently stimulates expression of the three subunit genes, depending on the reproductive stage (Ando *et al*. 2004; Ando and Urano, 2005). Recently, we examined effects of co-administration of IGF-I and sGnRH on the amounts of the three subunit mRNAs in masu salmon pituitary cells at three different reproductive stages, including the early maturing stage in April, the maturing stage in June, and spawning in September (Ando *et al*., 2006). IGF-I had a synergistic interaction with sGnRH, depending on the reproductive stage. However, IGF-I alone had no stimulatory effect on the subunit mRNAs. Therefore, the possible role of IGF-I as a signal molecule for induction of puberty remained

Table 1. Body weight (BW), fork length (FL), and gonadosomatic index (GSI) of masu salmon (means±SEM).

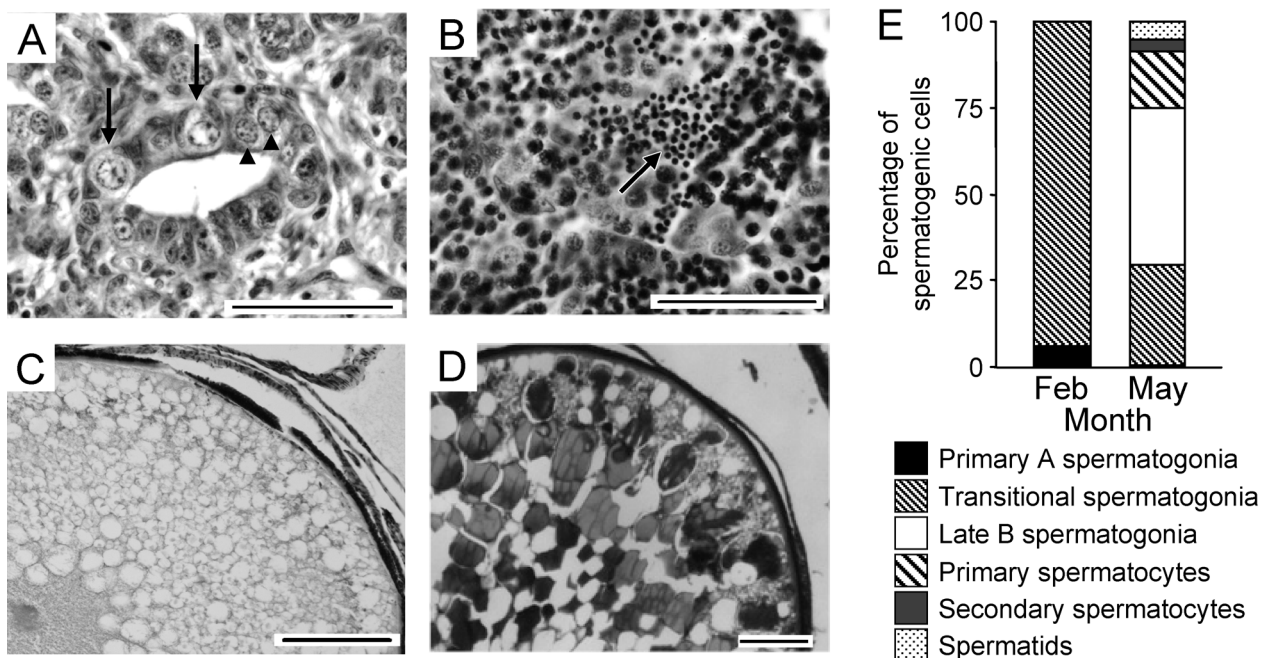| Stage	n	BW (g)	FL (cm)	GSI (%)	n	BW (g)	FL (cm)	GSI (%)
	Male				Female			
Early gametogenesis	47	364±11	30.7±0.3	0.11±0.00	54	421±15	32.1±0.4	0.71±0.02
Maturing	53	660±24	36.3±0.4	0.55±0.04	48	716±22	37.3±0.4	2.02±0.09
Spawning	39	873±30	43.4±0.6	7.22±0.13	35	730±33	39.8±0.6	31.5±0.37

Fig. 1. Germ cell differentiation in masu salmon. **(A)** Testicular cells in February. The arrows indicate primary A spermatogonia. The arrowheads indicate transitional spermatogonia. Bar=50 μm. **(B)** Testicular cells in May. The arrow indicates spermatids. Bar=50 μm. **(C)** Oocyte in February. Bar=200 μm. **(D)** Oocyte in May. Bar=200 μm. **(E)** Relative proportion of spermatogenic cells in February and May.

Table 2. Plasma hormone levels of masu salmon (means±SEM, ng/ml).

Male	Stage	FSH	LH	IGF-I
	Early gametogenesis	0.41±0.05[a] (10)	0.50±0.05[a,b] (5)	29.1±0.3[a] (3)
	Maturing	0.59±0.06[a] (10)	0.50±0.03[a] (10)	57.6±18.3[a] (3)
	Spawning	0.81±0.07[b] (10)	0.67±0.06[b] (9)	15.2±4.2[a] (3)
		T	11-KT	E2
	Early gametogenesis	1.34±0.22[a] (10)	1.44±0.31[a] (9)	0.02±0.01[a] (9)
	Maturing	2.99±0.33[b] (10)	2.66±0.36[b] (9)	1.32±0.84[a] (10)
	Spawning	–	–	–
Female	Stage	FSH	LH	IGF-I
	Early gametogenesis	0.56±0.04[a] (10)	0.45±0.04[a] (5)	35.3±6.9[a] (3)
	Maturing	0.63±0.06[a] (10)	0.52±0.03[a] (10)	49.5±8.4[a] (3)
	Spawning	1.07±0.07[b] (10)	2.23±0.33[b] (10)	4.53±0.5[b] (3)
		T	11-KT	E2
	Early gametogenesis	3.45±0.80[a] (10)	0.71±0.27[a] (5)	0.27±0.15[a] (9)
	Maturing	2.68±0.51[a] (8)	1.35±0.63[a] (5)	1.70±0.28[b] (9)
	Spawning	–	–	–

[a,b] Values with different characters are significantly different among stages ($p<0.05$).
The numbers of data are shown in parentheses.

to be determined.

In the present study, we examined direct effects of IGF-I on the expression of GPα, FSHβ, and LHβ subunit genes and the release of FSH and LH by using the primary pituitary cells of masu salmon, and we also examined the cooperative effects of IGF-1 with sGnRH. Fish was assessed at three reproductive stages, including the early gametogenesis stage in February, the maturing stage in May, and spawning in September. Maturational stages of experimental fish in February and May were determined by histological diagnosis of their gonadal development and plasma levels of the sex steroid hormones FSH, LH, and IGF-I.

MATERIALS AND METHODS

Fishes and sample collection

Masu salmon of the Mori hatchery strain were used in the present study. Fish were maintained in outdoor tanks with spring water (9–14°C) under natural photoperiod at the Mori branch of Hokkaido Fish Hatchery. They were fed with dry pellets equivalent to 1.7–2.0% of body weight every two days until the end of July, after which they did not eat any more. Under these conditions, gonadal development was initiated in spring, and the fish spawned in early September.

Fish were sampled in February, May, and September 2003. Under anesthesia with 0.01% tricaine methanesulfonate (MS222), fork length and body weight were measured. Blood samples of ten fishes were then collected from the caudal vasculature, kept on ice, and later centrifuged at 3,000 rpm for 15 min to obtain plasma samples, which were stored at –30°C until they were assayed. Whole pituitaries of 35–54 fishes were removed immediately after decapitation, collected in ice-cold RPMI medium containing 20 mM HEPES, 9 mM sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 U/ml), and fungizone (0.25 mg/ml). Finally, gonads were removed and weighed to calculate the gonadosomatic index (GSI=gonad weight/body weight×100). Gonads of five fishes were fixed in Bouin fixative for 24 hr before storage in 70% ethanol. Experimental procedures followed the guidlines for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University. Changes in body weight, fork length, and GSI are shown in Table 1.

Hormones

Human recombinant IGF-I (Sigma-Aldrich, Tokyo, Japan) and
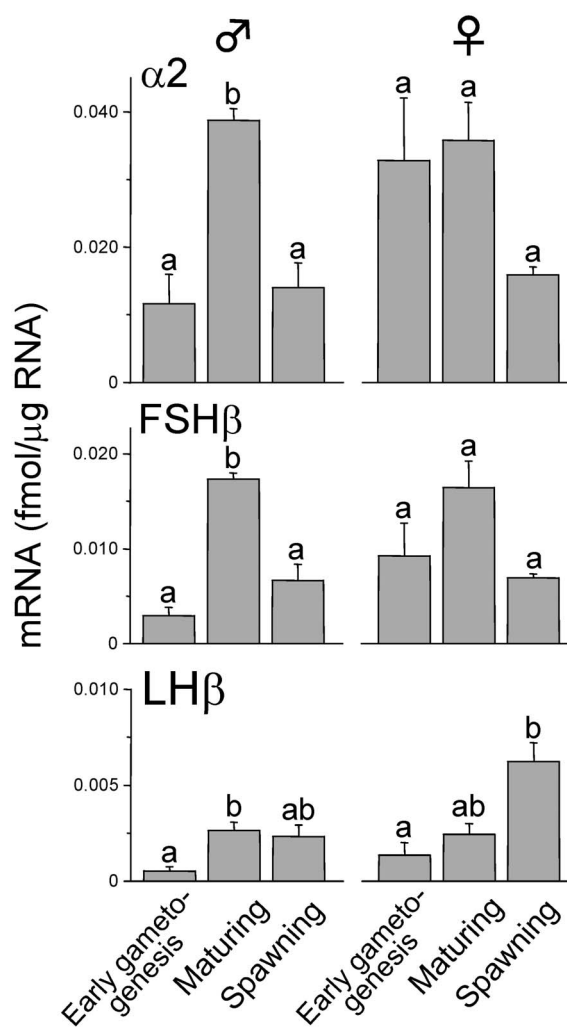

Fig. 2. Changes in the amounts of GTH subunit mRNAs during sexual maturation. The amounts of GTH subunit mRNAs in the control cell cultures on day 0 are indicated (n=4–6). Values with different characters are significantly different among reproductive stages ($P<0.05$).

[Trp[7], Leu[8]]GnRH (sGnRH, Peptide Institute, Inc. Osaka, Japan) were solubilized in 0.1 M acetic acid at a concentration of 0.5 g/l and stored at −80°C. Hormone solutions were further diluted with culture medium to obtain appropriate final concentrations and immediately used in experiments.

Static cell culture

Preparation of primary pituitary cultures was described in detail previously (Ando et al. 2004; 2006). Briefly, pooled pituitaries were dispersed by treatment with 0.1% collagenase (GIBCO Invitrogen Corp., Carlsbad, CA) and 3% bovine serum albumin (BSA, GIBCO Invitrogen Corp.) at 12°C for 20 h in RPMI medium containing 20 mM HEPES, 9 mM sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 U/ml), and fungizone (0.25 mg/ml). After washing with the RPMI medium supplemented with Serum Replacement 2, which did not contain steroid hormones, glucocorticoids, and growth factors (Sigma-Aldrich, Tokyo, Japan), dispersed cells were immediately plated on 12-well polylysine-coated culture plates at a density of 3×10^5 cells/1.3 ml medium/well and were incubated at 18°C for 2 days. The medium was then changed and hormones were included in the fresh medium. IGF-I (0.01, 0.1, 1, 10 and 100 nM) alone or IGF-I (100 nM) and sGnRH (100 nM) were added to the medium in order to examine the effects of IGF-I in different doses or in combination with sGnRH. Cells were successively incubated for 1, 2, and 3 days. After incubation, the media were collected and frozen at −80°C until they were assayed by RIA for FSH and LH. Cells were harvested by scraping in 0.7 ml of medium twice and were then collected by centrifugation at 5,000 rpm for 10 min and frozen at −80°C until they were assayed by real-time PCR for GPα, FSHβ, and LHβ mRNAs.

Quantitation of GPα, FSHβ, and LHβ mRNAs by real-time PCR

RNA extraction, reverse transcription (RT), and real-time PCR were previously described in detail (Ando et al. 2004; 2006). Briefly, total RNAs (50 or 200 ng) were used for synthesis of first strand cDNAs by RT reaction using Multiscribe Reverse Transcriptase (PE Applied Biosystems, California) according to the manufacturer's instructions. To determine absolute amounts of GPα, FSHβ, and LHβ mRNAs, standard sense RNAs were synthesized in vitro by MAXIscript (Ambion Inc, Texas) using chum salmon GPα, FSHβ, and LHβ cDNAs (Sekine et al. 1989), and they were used for RT reactions to prepare standard cDNAs. Real-time PCR was carried out with an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). For GPα and FSHβ mRNAs, each PCR reac-
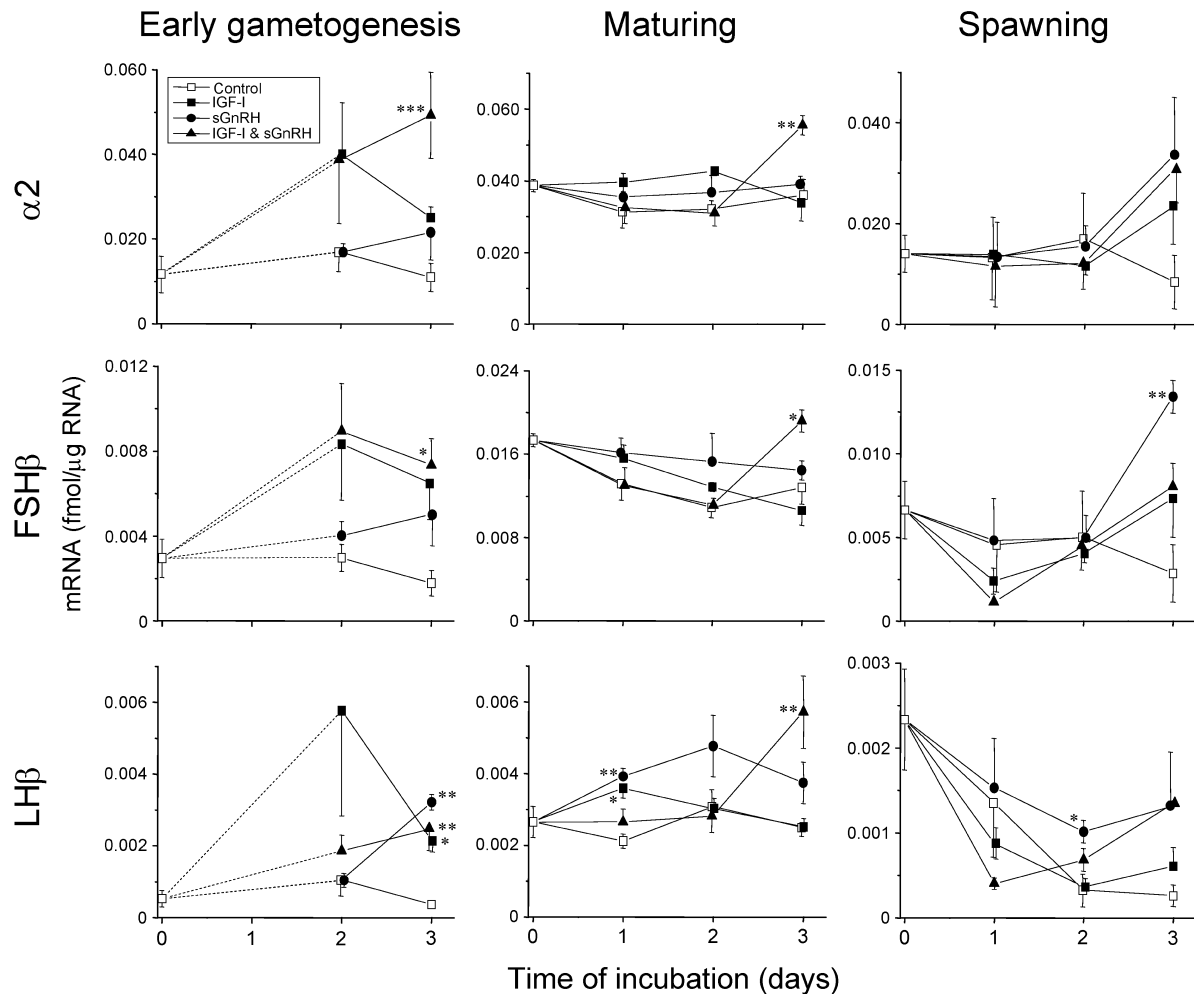

Fig. 3. Effects of IGF-I and sGnRH on the amounts of GTH subunit mRNAs during sexual maturation in males. After pre-incubation at 18°C for 2 days, cells were treated with IGF-I (100 nM) with or without sGnRH (100 nM) for 1–3 days, and the amounts of GTH subunit mRNAs were assayed by real-time PCR (n=3–6). In the early gametogenesis stage, the amounts on day 1 could not be determined due to the low number of cells available. Asterisks denote a significant difference between the control and hormone-treated groups at each time of incubation (* $P<0.05$, ** $P<0.01$).

tion mixture contained 1×TaqMan Buffer A (PE Applied Biosystems), 25 mM MgCl₂, 2.5 mM dNTPs, 72 nM each of forward and reverse primers for GPα and FSHβ, 130 nM fluorogenic probes, and 0.25 U AmpliTaq Gold DNA polymerase. Amplification was carried out at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 59°C for 1 min. For LHβ mRNA, PCR reaction conditions were the same as those for GPα and FSHβ, except for 100 nM primers, and annealing and extension temperatures at 57°C. In each assay, a standard sample (chum salmon pituitary cDNA) was subjected to amplification in triplicate to estimate coefficients of variation (CV) within and between runs. For the GPα mRNA assay, intra-assay CV was 4.6±0.4% and inter-assay CV was 9.7%; for the FSHβ mRNA assay, intra-assay CV was 5.6±0.5% and inter-assay CV was 9.7%. Finally, for the LHβ mRNA assay, intra-assay CV was 6.9±0.7% and inter-assay CV was 13.0%.

Determination of FSH, LH, and IGF-I levels by RIA

Levels of FSH and LH in the plasma and culture medium were determined using heterologous RIAs for coho salmon GTHs, as described previously (Swanson *et al.*, 1989). Serial dilutions of masu salmon plasma and cell culture medium showed displacement curves parallel to those observed with purified coho salmon GTHs in both assays. The plasma sample and cell medium were diluted in assay buffer (0.2M barbital buffer, pH 8.6, 1.0% BSA) prior to assay. For the FSH assay, the range of intra-assay CV was 3.6–6.3% and inter-assay CV was 1.9%; for the LH assay, the range of intra-assay CV was 0.6–11.5% and inter-assay CV was 15.7%.

The plasma IGF-I levels of three samples in each group were determined by RIA using commercially available components (GroPep, Adelaide, Australia) (Shimizu *et al.* 1999). For the IGF-I assay, the range of intra-assay CV was 3.5–8.3% and inter-assay CV was 14.9%.

Assay of Plasma Steroid Hormones

Plasma levels of estradiol-17β (E2), testosterone (T), and 11-ketotestosterone (11-KT) were determined by enzyme immunoassays as described in detail by Onuma *et al.* (2003). In brief, standard hormones and samples were incubated with anti-steroid antiserum and HRP-labeled steroid (Cosmo Bio, Tokyo) in wells of 96-well microtiter plates (Sumitomo Bakelite, Tokyo), in which anti-rabbit IgGs (ICN Pharmaceuticals, Aurora) were immobilized by physical adsorption. After an overnight incubation at 4°C, wells were washed with 0.9% NaCl, a substrate solution (0.5 g/l *o*-phenylenediamine, 0.01% H₂O₂ in 0.2 M citrate buffer, pH 4.5) was added, and mixed solutions were incubated at room temperature for 30 min. Optical densities at 492 nm were then measured by using a microplate reader (MTP-300, Corona Electric, Hitachinaka). Sensitivities of assays were: E2, 0.5 pg/tube; T, 1.5 pg/tube; and 11-KT, 1.5 pg/tube. Intra-assay CVs were: E2, 4.5±0.9%; T, 6.6±2%; and 11-KT, 2.6±0.5%. Inter-assay CVs were: E2, 7.0%; T, 13.3%; and 11-KT, 9.8%.

Histology

Fixed gonads in each group were dehydrated through a graded series of ethanol and embedded in paraffin wax, sectioned (4–8 μm thickness), and stained with hematoxylin-eosin. Stages of spermatogenesis were determined by light microscopy, according to Loir (1999) and Campbell *et al.* (2003). Cross sections of the testes were viewed under a microscope and two randomly selected areas (160 μm×210 μm) in each sample were photographed. Testicular development was estimated by calculating the proportion of each spermatogenic cell type relative to the total number of spermatogenic cells. Stages of oogenesis were determined according to Schulz (1984) and Campbell *et al.* (2006).

Statistical analysis

Data were presented as means±SEM. The amounts of GPα, FSHβ, and LHβ mRNAs and medium FSH and LH in control cells and plasma hormone levels were analyzed by ANOVA to test for statistically significant differences among months; when appropriate, Tukey's post-hoc test was applied. Levels of plasma steroid hormones were analyzed by a Student t-test for statistically significant differences between February and May. Statistical analyses of effects of IGF-I and sGnRH on expression of GPα, FSHβ, and LHβ genes and release of FSH and LH were performed using the Dunnett pairwise multiple comparison t-test. Statistical analyses were performed using SPSS Version 12.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Gonadal development and plasma hormones during sexual maturation

In both sexes, gonadal development was initiated in spring and progressed in May, and the fish finally spawned in early September. The GSI of both sexes increased from the early gametogenesis stage from February onward, and
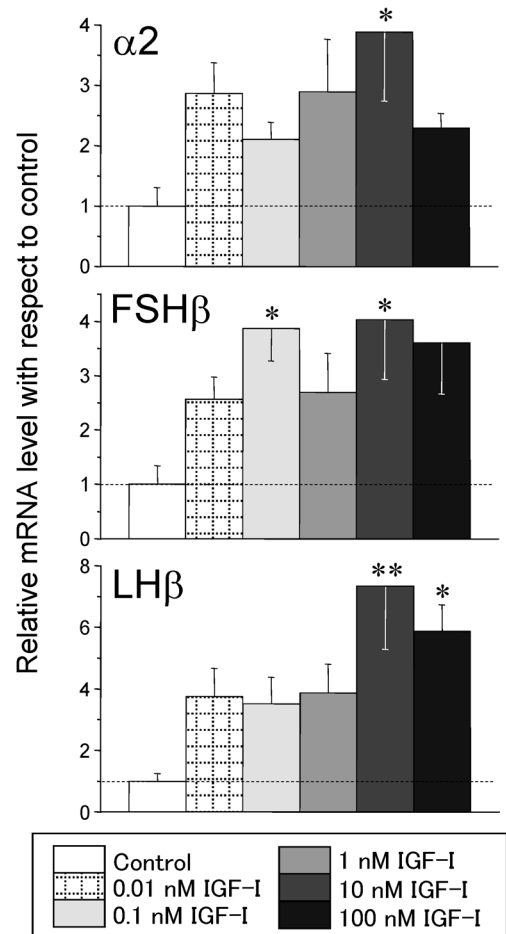

Fig. 4. Dose-dependent effects of IGF-I on the amounts of GTH subunit mRNAs on day 3 in the early gametogenesis stage in males. After pre-incubation at 18°C for 2 days, cells were treated with IGF-I (0.01–100 nM) for 3 days, and the amounts of GTH subunit mRNAs were assayed by real-time PCR (n=4 or 5). Asterisks denote a significant difference between the control and hormone-treated groups (* *P*<0.05, ** *P*<0.01).

reached the maximum during spawning (Table 1). These changes were consistent with those determined in our previous experiments in 2001 (Ando *et al*., 2004) and 2002 (Ando *et al*., 2006).

Histological analyses of gonadal development are showed in Fig. 1. In males, only two categories of spermatogenic cells, primary A spermatogonia and transitional spermatogonia, were identified in February. In the maturing stage in May, the majority of spermatogenic cells of all fish were late B spermatogonia, whereas the testes of three of five individuals had advanced to the spermatid stage. In females, the oocytes had yolk vesicles and lipid droplets in February and yolk globules in the cytoplasm in May. Thus, vitellogenesis of oocytes was initiated during the months of February to May.

Plasma hormone levels at the three reproductive stages are shown in Table 2. The levels of FSH and LH increased from the maturing stage to spawning in both sexes. The levels of plasma IGF-I tended to increase from the early gametogenesis stage to the maturing stage in both sexes,

followed by declines during spawning. The levels of plasma T and 11-KT in males and E2 in females also increased from the early gametogenesis stage to the maturing stage. These changes in the sex steroid hormone levels were consistent with those determined in our previous experiments (Ando *et al*., 2004, 2006).

Changes in basal amounts of GPα, FSHβ and LHβ mRNAs during sexual maturation

The amounts of the three subunit mRNAs in the male control cell cultures significantly increased from the early spermatogenesis stage to the maturing stage, and thereafter GPα and FSHβ mRNAs declined during spawning (Fig. 2). In females, there were no significant changes in the levels of GPα and FSHβ mRNAs, whereas those of LHβ mRNA were higher during spawning than the early gametogensis stage.

Seasonal changes in effects of IGF-I and sGnRH on GPα, FSHβ, and LHβ mRNA levels

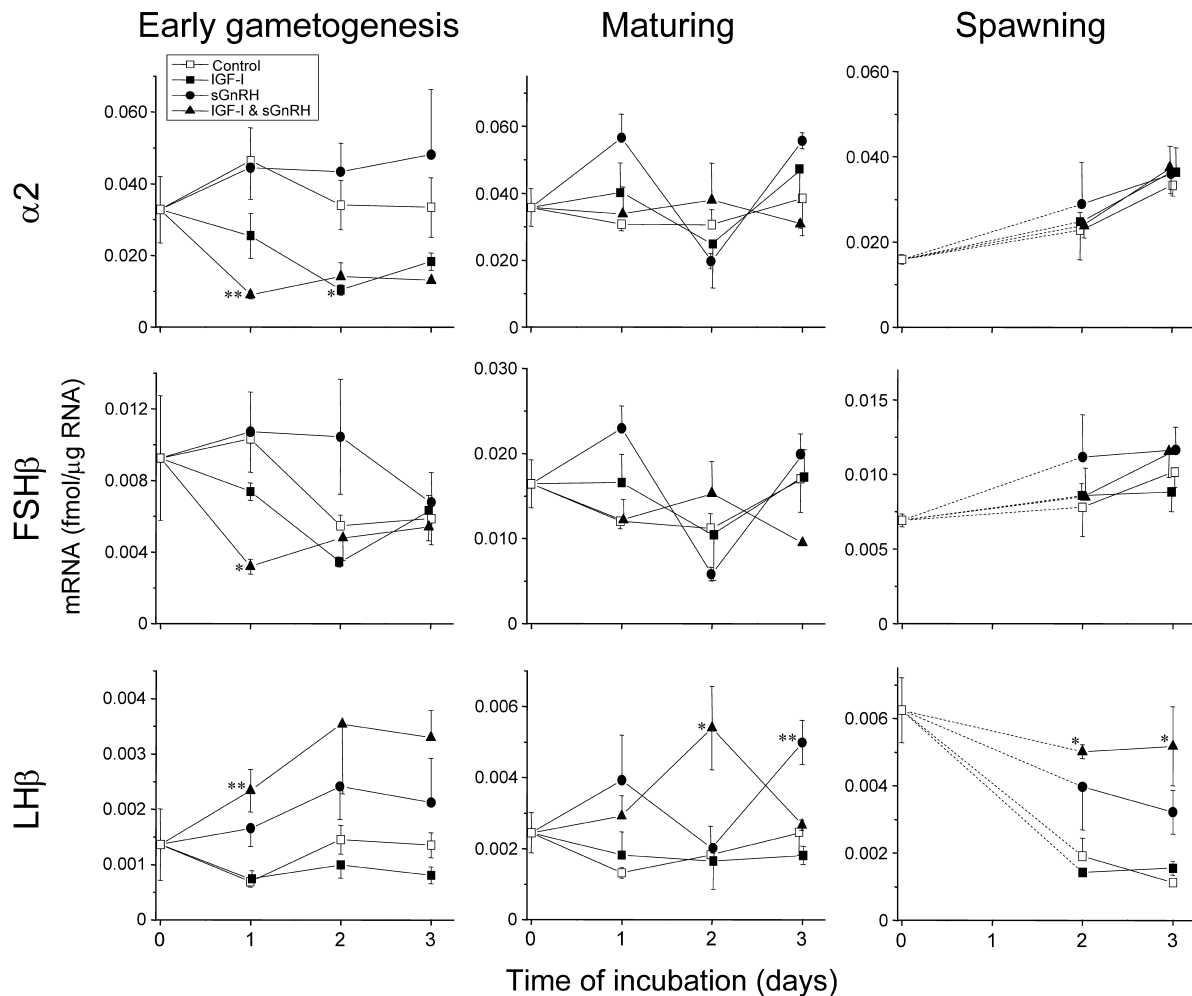The effects of IGF-I alone and IGF-I in combination with

Fig. 5. Effects of IGF-I and sGnRH on the amounts of GTH subunit mRNAs during sexual maturation in females. After pre-incubation at 18°C for 2 days, cells were treated with IGF-I (100 nM) with or without sGnRH (100 nM) for 1–3 days, and the amounts of GTH subunit mRNAs were assayed by real-time PCR (n=3–5). In the spawning stage, the amounts on day 1 could not be determined due to the low number of cells available. Asterisks denote a significant difference between the control and hormone-treated groups at each time of incubation (* $P<0.05$, ** $P<0.01$).

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sGnRH on the levels of the three subunit mRNAs were examined at the three different reproductive stages. The primary pituitary cell cultures were incubated with various doses of IGF-I (0.01, 0.1, 1, 10 and 100 nM) alone or IGF-I (100 nM) + sGnRH (100 nM) for 1, 2, and 3 days. The effects of IGF-I and IGF-I + sGnRH at 100 nM during three days of incubation are shown in Fig. 3 for males and Fig. 5 for females. In males, IGF-I significantly increased the LHβ mRNA levels on day 3 in the early spermatogenesis stage (Fig. 3). Stimulatory trends were also noted for GPα and FSHβ mRNAs. These stimulatory effects of IGF-I were dependent on doses of IGF-I in the range of 0.01–100 nM (Fig. 4). IGF-I at 10 nM significantly increased the three subunit mRNAs. In addition, IGF-I + sGnRH significantly increased the three subunit mRNAs on day 3 in the early spermatogenesis stage (Fig. 3). In the advanced stages in May and September, IGF-I had almost no effects on the three subunit mRNAs, while IGF-I + sGnRH significantly increased the three subunit mRNAs on day 3 in the maturing stage. There were no significant effects of IGF-I at low

doses in any of the experimental conditions in May and September (data not shown).

In females, IGF-I decreased GPα mRNA regardless of sGnRH on day 2 in the early oogenesis stage, whereas it had no effects on FSHβ and LHβ mRNAs (Fig. 5). This negative effect on GPα mRNA was dependent on the dose of IGF-I (data not shown). IGF-I + sGnRH significantly reduced GPα and FSHβ mRNAs but increased LHβ mRNA on day 1. In the later stages, almost no response to IGF-I was observed. IGF-I + sGnRH significantly increased LHβ mRNA on day 2 in the maturing stage. Stimulatory effects of IGF-I + sGnRH were also observed in the spawning stage.

Changes in basal release of FSH and LH during sexual maturation

The amounts of 3 days of accumulated FSH and LH secreted from the control cell cultures were determined to examine basal activity of GTH release in the primary pituitary cells (Fig. 6). Changes in the activity of FSH release during sexual maturation were different from those of LH release in both sexes. The medium FSH levels remained unchanged in the early gametogenesis and maturing stages and slightly increased in spawning. In contrast, the medium LH levels extensively increased during spawning in both sexes. These changes were consistent with those determined in our previous study (Ando *et al*., 2004).

Seasonal changes in effects of IGF-I and sGnRH on release of FSH and LH

Effects of IGF-I alone and IGF-I + sGnRH on FSH and LH release were examined at the three different reproductive stages. In males, IGF-I at 0.01–100 nM did not have any effects on the levels of medium FSH and LH, except for stimulation on day 1 in the maturing stage (Fig. 7). IGF-I + sGnRH synergistically stimulated release of FSH in the early spermatogenesis and spawning stages and release of LH in all stages.

In females, IGF-I at 100 nM was effective to stimulate release of FSH and LH on day 1 in the early oogenesis stage (Fig. 8). The stimulatory effects were dependent on the doses of IGF-I and showed maximum responses at 0.1 nM in both FSH and LH release (Fig. 9). In the maturing stage, IGF-I stimulated LH release on days 1 and 2 (Fig. 8). IGF-I + sGnRH synergistically stimulated release of FSH and LH in the maturing and spawning stages.

DISCUSSION

The present study examined both the direct effects of IGF-I on the synthesis and release of FSH and LH in masu salmon pituitary cells during sexual maturation and its cooperative effects with sGnRH. In our previous study in 2002, IGF-I alone had no stimulatory effects during sexual maturation from the early maturing stage in April to spawning (Ando *et al*., 2006). In April, gonadal maturation had already progressed during postpubertal development. We therefore examined the effects of IGF-I in the earlier stage of gonadal development in February 2003. In the present study, IGF-I alone stimulated expression of the GPα, FSHβ, and LHβ genes in males and release of FSH and LH in females in the early gametogenesis stage. These stimulatory effects were dependent on the dose of IGF-I. Although there are reports
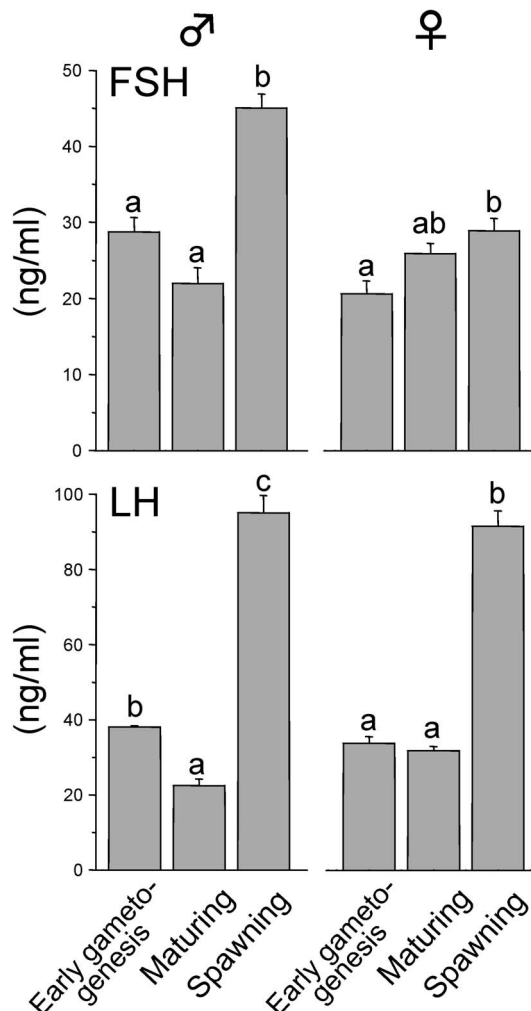

Fig. 6. Changes in the levels of medium FSH and LH during sexual maturation. The levels of medium FSH and LH in the control cell cultures on day 3 are indicated (n=4 or 5). Values with different characters are significantly different among reproductive stages (*P*<0.05).
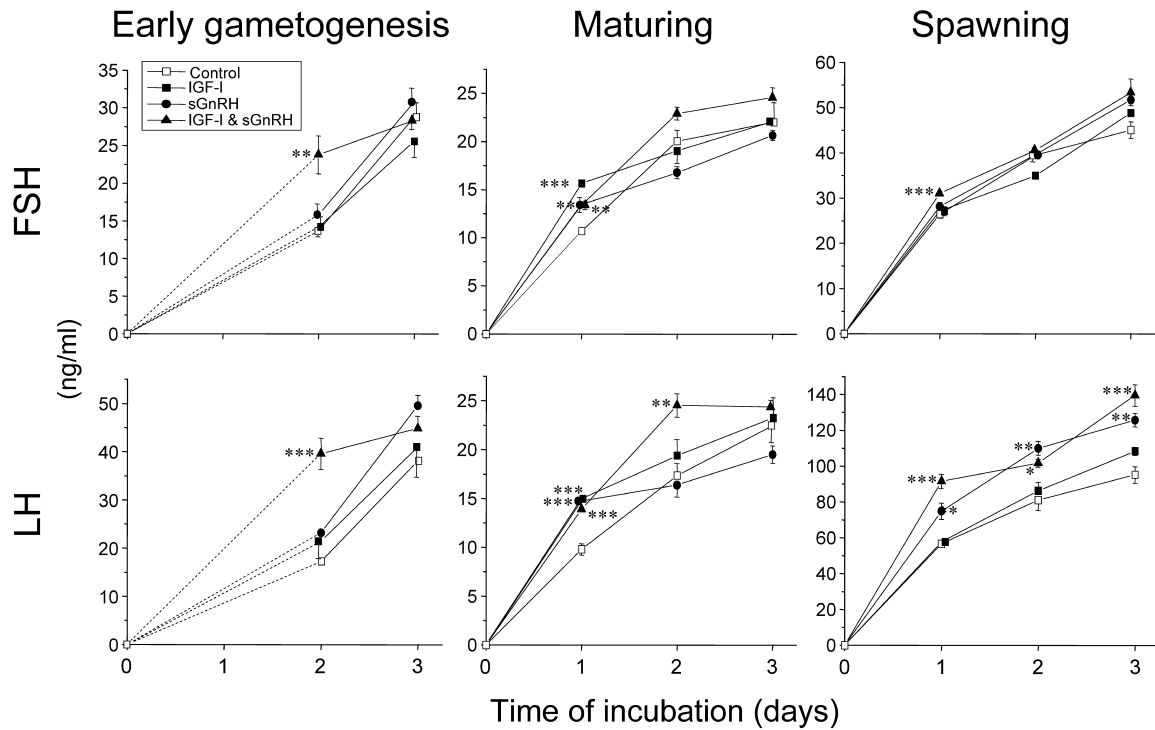
Fig. 7. Effects of IGF-I and sGnRH on the levels of medium FSH and LH during sexual maturation in males. After pre-incubation at 18°C for 2 days, cells were treated with IGF-I (100 nM) with or without sGnRH (100 nM) for 1–3 days, and the levels of medium GTHs were assayed by RIA (n=4 or 5). In the early gametogenesis stage, the levels on day 1 could not be determined due to the low number of cells available. Asterisks denote a significant difference between the control and hormone-treated groups at each time of incubation (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).
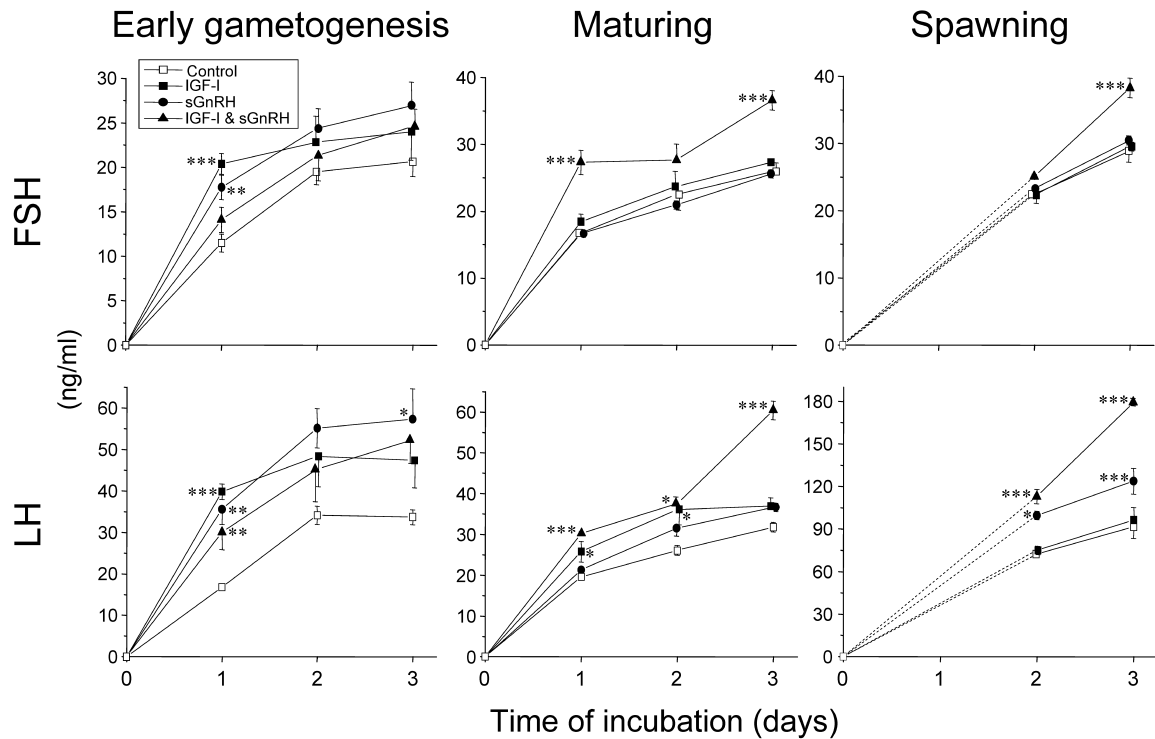

Fig. 8. Effects of IGF-I and sGnRH on the levels of medium FSH and LH during sexual maturation in females. After pre-incubation at 18°C for 2 days, cells were treated with IGF-I (100 nM) with or without sGnRH (100 nM) for 1–3 days, and the levels of medium GTHs were assayed by RIA (n=4 or 5). In the spawning stage, the levels on day 1 could not be determined due to the low number of cells available. Asterisks denote a significant difference between the control and hormone-treated groups at each time of incubation (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).
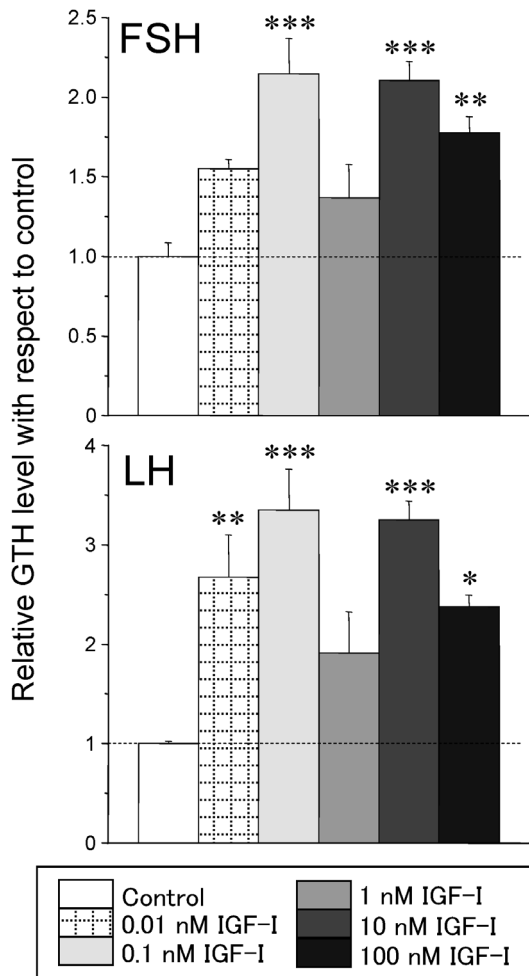
Fig. 9. Dose-dependent effects of IGF-I on the levels of medium FSH and LH on day 1 in the early gametogenesis stage in females. After pre-incubation at 18°C for 2 days, cells were treated with IGF-I (0.01–100 nM) for 1 day, and the levels of medium GTHs were assayed by RIA (n=5). Asterisks denote a significant difference between the control and hormone-treated groups (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

on the direct effect of IGF-I on the release of GTH, the present findings are the first evidence for a direct role of IGF-I in stimulating GTH subunit gene expression.

In males, the gonadal stage was significantly different between the early spermatogenesis and the maturing stages. The majority of spermatogenic cells comprised transitional spermatogonia in the former stage, but late B spermatogonia, with subpopulations of more advanced cells such as spermatocytes and spermatids, in the latter stage. These features were consistent with gonadal development in the chinook salmon (*Oncorhynchus tshawytscha*), in which the relative proportions of germ-cell types changed markedly from the early stage of gametogenesis in January to the maturing stage in June (Campbell *et al*., 2003). Therefore, the differentiation of spermatogonia progresses markedly from winter to spring. Accordingly, the plasma levels of T and 11-KT significantly increased from the early spermatogenesis stage to the maturing stage in the present study (Table 2). During this period, the circulating levels of IGF-I

increased with gonadal development (Table 2, Campbell *et al*., 2003). Larsen *et al*. (2004) showed that the treatment of coho salmon with T or 11-KT increased the plasma levels of IGF-I. Moreover, IGF-I mRNA levels in the liver and pituitary increased from winter through early spring in masu salmon (Yokota *et al*., in preparation). These data support the notion that the increase in plasma IGF-I levels from winter through spring is an important endocrine event for the early development of gametogenesis.

Campbell *et al*. (2003) demonstrated that in male chinook salmon, there was a critical period when growth affected the onset of puberty, which occurred during premeiotic stages from July to December one year in advance of spawning. Then, during winter and spring, reproductive parameters such as GSI and plasma levels of FSH, 11-KT, and IGF-I increased in fish that were expected to spawn in the autumn, but they remained low in fish that were not expected to spawn in the autumn. Therefore, it is possible that 11-KT and IGF-I stimulate FSH synthesis, and that the increased expression of FSH activates spermatogenesis. The present results provide direct evidence that IGF-I can stimulate GTH subunit gene expression during this period. Since the circulating levels of IGF-I highly correlate with growth rates in salmonids (Beckham *et al*., 1998; Campbell *et al*., 2003), IGF-I may therefore act as a signaling molecule that transmits growth and nutritional status to the reproductive axis at the onset of puberty.

In contrast to males, IGF-I had no effects on the levels of GPα, FSHβ, and LHβ mRNAs in females. However, it stimulated release of FSH and LH early in oogenesis, when oocytes were in the lipid droplet stage. Campbell *et al*. (2006) showed in female coho salmon that reproductive parameters including plasma FSH levels started to increase in the cortical alveoli stage, and that significant increases in GSI and plasma FSH and IGF-I levels were already prominent in the lipid droplet stage. Therefore, the reproductive stage in February in the present study may be too late to determine the stimulatory effects of IGF-I on GPα, FSHβ, and LHβ gene expression. Since the stimulatory effect of IGF-I on GTH subunit gene expression is considered to be highly dependent on gonadal stage, IGF-I might stimulate these genes at an earlier stage of oogenesis in the females.

It is also possible that the kinetics of GTH synthesis and release are different between males and females. In the present study, the effects of IGF-I were examined in the pituitary cell cultures after incubation for 1-3 days. In the pituitary of tilapia, IGF-I stimulated prolactin (PRL) release after incubation for 2 h without any increase in the levels of PRL mRNA, and it suppressed release of GH after 8–24 h, with decreased levels of GH mRNA after 24 h (Kajimura *et al*., 2002). Therefore, it is conceivable that IGF-I may influence release first, and then change de-novo synthetic activity. In the pituitary of females, longer incubation might be required for the stimulation of GTH subunit gene expression. In long-term pituitary cell cultures (incubation for 10 days), IGF-I markedly increased release and the cell content of LH in European eels (Huang *et al*., 1998, 1999), and the cell content of FSH and LH in coho salmon (Baker *et al*. 2000). Although further study of long-term cell cultures is needed to clarify the effects of IGF-I on GTH genes in females, the present results suggest that IGF-I most probably stimulates

release and synthesis of FSH and LH in the early stages of gametogenesis to trigger the onset of puberty.

In our previous study in 2002, IGF-I modulated sGnRH-induced expressions of GPα, FSHβ, and LHβ genes at particular stages (Ando *et al.*, 2006). In the present study, the cooperative effects on those mRNAs were also variable, depending on the stage, subunit, and gender. In males, IGF-I + sGnRH elevated the three subunit mRNAs on day 3 in the maturing stage, although neither IGF-I nor sGnRH had any effects (Fig.3). In spawning, IGF-I counteracted sGnRH-induced FSHβ gene expression, as reported previously (Ando *et al.*, 2006). In females, the co-administration decreased GPα and FSHβ mRNAs in the early oogenesis stage, but increased LHβ mRNA in all stages. Similar inhibitory effects on GPα and FSHβ mRNAs by IGF-I + sGnRH were observed in April 2002 (Ando *et al.*, 2006). Interestingly, IGF-I + sGnRH stimulated FSH release from the same cell cultures. Although the physiological significance of these adverse effects is not clear at present, the present results clearly indicate that IGF-I and sGnRH differentially regulate GTH synthesis and release.

In contrast to the variable effects on GTH genes, co-administration of IGF-I and sGnRH mostly stimulated release of FSH and LH during sexual maturation. These results are consistent with the previous study of Weil *et al*. (1999) showing that IGF-I enhanced sGnRH-induced release of FSH and LH at both immature and mature stages in rainbow trout. Thus, IGF-I has an important role in stimulation of GTH release in cooperation with sGnRH during sexual maturation.

In conclusion, IGF-I alone directly stimulated expression of GPα, FSHβ, and LHβ genes in males and release of FSH and LH in females early in gametogenesis. The co-administration of IGF-I and sGnRH stimulated release of FSH and LH during sexual maturation, although it regulated GTH subunit gene expression differently depending on the reproductive stage, subunit, and gender. The present results suggest that IGF-I has an important role in the onset of puberty through directly stimulating GTH synthesis. Furthermore, in cooperation with sGnRH, it regulates the synthesis and release of FSH and LH during sexual maturation.

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