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Effects of insulin-like growth factor I on GnRH-induced gonadotropin subunit gene expressions in masu salmon pituitary cells at different stages of sexual maturation

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## **Abstract**

Effects of insulin-like growth factor I (IGF-I) and salmon gonadotropin-releasing hormone (sGnRH) on expression of gonadotropin (GTH) subunit genes were examined using primary pituitary cell cultures of masu salmon (*Oncorhynchus masou*). Fishes were assessed at three reproductive stages, i.e., in April (early maturation), in June (maturing), and in September (spawning). Amounts of GTH subunit mRNAs in pituitary cells were determined using real-time PCR after incubation with IGF-I and/or sGnRH. IGF-I alone had almost no effects on three GTH subunit mRNAs in both sexes, except for decrease in follicle-stimulating hormone (FSH)  $\beta$  mRNA in males in June. sGnRH alone was effective in stimulation of FSH $\beta$  and luteinizing hormone (LH)  $\beta$  gene expression in males in April. Thereafter it had no significant effects on GTH subunit mRNAs, although in September it tended to increase FSH $\beta$  and LH $\beta$  mRNAs in females. Co-administered IGF-I counteracted the sGnRH-induced expression of FSH $\beta$  and LH $\beta$  genes in males in April, but not in females in September. These results suggest that IGF-I is involved in direct regulation of GTH subunit genes during sexual maturation. In particular, IGF-I differently modulates sGnRH-induced GTH subunit gene expression, depending on reproductive stages.

**Keywords:** Gonadotropin, Gonadotropin-releasing hormone, Insulin-like growth factor I, Pituitary, Gene expression, Puberty, Salmon

## 1. Introduction

Several lines of evidence showed that insulin-like growth factor-I (IGF-I) played a pivotal role in interactions between somatotrophic and gonadotropic axes. IGF-I has metabolic and mitogenic activities, and acts as a mediator of somatotrophic actions of growth hormone. In addition, it activated directly the hypothalamic-pituitary-gonadal axis to promote onset of puberty (Hiney et al., 1991, 1996; Huang et al., 1998). IGF-I stimulated the release of gonadotropin-releasing hormone (GnRH) in rats at puberty, and GnRH in turn elevated plasma levels of luteinizing hormone (LH) (Hiney et al., 1991, 1996). IGF-I also functioned directly at the pituitary level. Addition of IGF-I to rat pituitary cell cultures increased basal secretions of follicle-stimulating hormone (FSH) and LH, and GnRH-induced LH secretion (Kanematsu, et al., 1991; Soldani et al., 1994, 1995; Xia et al., 2001; Pazos et al., 2004). Similarly, IGF-I enhanced GnRH-stimulated LH release from bovine pituitary cells (Hashizume et al., 2002). Furthermore, Pazos et al. (2004) showed that in male rats, IGF-I stimulated basal release of FSH and LH with increased expression of glycoprotein (GP)  $\alpha$  but not FSH $\beta$ , nor LH $\beta$  subunit genes.

There is also evidence for roles of IGF-I in stimulating the gonadotropic axis in teleosts. IGF-I increased release and cell content of LH in pituitary cells of European eels in a time- and dose-dependent manner (Huang et al., 1998, 1999). In salmonids, co-administration to rainbow trout pituitary cells of IGF-I and salmon GnRH (sGnRH) elevated sensitivity to sGnRH as measured by FSH and LH releases (Weil et al., 1999). IGF-I increased GnRH-stimulated FSH release and cell content of FSH and LH in coho salmon pituitary cells (Baker et al., 2000). All these observations support the idea that IGF-I has an important role in controlling GTH production through interactions with GnRH. Since peripheral levels of IGF-I highly correlate with growth rate in salmonids (Beckham et al., 1998, 2001; Campbell et al., 2003), IGF-I is

thought to act as a signaling molecule that transmits growth and nutritional status to the gonadotropic axis at the onset of puberty. However, little is known about its mechanisms of action in functional interactions with GnRH to control GTH production. In particular, no information is available on effects of IGF-I on expression of GTH subunit genes in teleosts.

Expressions of GTH subunit genes vary during reproductive cycles in salmonids. Amounts of their mRNAs increased with sexual maturation, and considerably rose at spawning (Gomez et al., 1999; Kitahashi et al., 2004). In masu salmon, FSH $\beta$  mRNA started to increase at early stages of gametogenesis in March, whereas LH $\beta$  mRNA started to increase at a later stage of gametogenesis in June (Kitahashi et al., 2004). GnRH has been shown to influence GTH subunit gene expression in a subunit- and reproductive stage-dependent manner (Kitahashi et al., 1998b; Dickey and Swanson, 2000; Ando et al., 2004; Ando and Urano, 2005). In masu salmon, GnRH analog (GnRH $\alpha$ ) that was implanted into the dorsal muscle increased GP $\alpha$ 2 and LH $\beta$  mRNAs in June onwards, while it had no effects on FSH $\beta$  mRNA during sexual maturation (Kitahashi et al., 2004). Therefore, sGnRH has a pivotal role in increasing expressions of LH subunit genes during sexual maturation. However, mechanisms underlying the earlier increase of FSH $\beta$  gene expression remain unknown.

One of the candidates responsible for upregulation of FSH $\beta$  gene expression is IGF-I as described above. Therefore, we examined effects of IGF-I alone and in combination with sGnRH on expression of GTH subunit genes using masu salmon pituitary cell cultures. Since responses to IGF-I and sGnRH were expected to depend on reproductive stages, pituitary cell cultures were prepared from fishes at three different reproductive stages, i.e., early maturation in April, maturing stage in June, and spawning in September. Levels of plasma sex steroid hormones were also determined using enzyme immunoassays to confirm levels of sexual maturation in the experimental fishes.

## **2. Materials and methods**

### *2. 1. Experimental design*

On the basis of our previous studies in which the effects of sGnRH on expression of GTH subunit genes were clarified (Ando and Urano, 2005), we carried out three experiments to examine effects of IGF-I, sGnRH, and E2 on GTH subunit gene expression. In experiment I, effects of different doses of IGF-I (1, 10, 100 nM) alone or in combination with sGnRH (100 nM) were examined in female fishes in April. Cells were incubated with these hormones for 1 day. In experiment II, the kinetics of effects of IGF-I (100 nM) and sGnRH (100 nM) were examined in male and female fishes in April. Cells were incubated with appropriate hormones for 1, 2, and 3 days. In experiment III, seasonal changes in effects of IGF-I (100 nM) and sGnRH (100 nM) at three days after treatment were examined in males in June and in females in June and September. Because of limited number of fish available, we could not examine the effects in males in experiment I and in September in experiment III.

### *2. 2. Fishes*

Experiment I was conducted in April 2002 using masu salmon of the Shiribetsu strain, reared at the Kumaishi branch of Hokkaido Fish Hatchery; and experiments II and III were conducted in April, June, and September 2002, using the Mori hatchery strain reared at the Mori branch of Hokkaido Fish Hatchery. Fishes of both strains retained the ability of homing migration, nonetheless they could grow, mature, and spawn in a freshwater environment. In our previous experiments in 2001, levels of GTH subunit mRNAs in pituitary cells from the two strains showed similar changes during sexual maturation and also in response to sGnRH and sex steroid hormones (Ando et al. 2004).

Fishes were maintained in outdoor tanks with a flow of spring water (8 °C in experiment I, and 9-14 °C in experiments II and III) under natural photoperiod. They were fed with dry pellets equivalent to 1.7-2 % 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 Shiribetsu strain at the Kumaishi branch spawned in late September, and the Mori hatchery strain at the Mori branch spawned in early September.

### *2. 3. Sample collection*

Under anesthesia with 0.01 % tricaine methanesulfonate (MS222), fork length and body weight of fishes were measured. Then, blood samples of ten fishes were collected from the caudal vasculature, kept on ice, and were later centrifuged at 3,000 rpm for 15 min to obtain plasma samples, which were stored at -30 °C until assayed. Whole pituitaries of 34-61 fishes were removed immediately after decapitation, collected in ice-cold RPMI 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 ( $GSI = \text{gonad weight/body weight} \times 100$ ) as an estimate of gonadal maturity. In addition to fishes used in the three experiments, ten males and ten females from the same stock were removed in August 2002, and blood samples were collected to determine plasma sex steroid levels. Changes in body weight, fork length, and GSI are shown in Table 1.

### *2. 4. Hormones*

Human recombinant IGF-I (Sigma-Aldrich, Tokyo, Japan) and [Trp<sup>7</sup>, Leu<sup>8</sup>]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.

### *2. 5. Primary cell culture*

Preparation of primary pituitary cultures was described previously (Ando et al., 2004). Pooled pituitaries were minced with a surgical blade, and transferred to RPMI medium containing 20 mM HEPES, 9 mM sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 U/ml), and fungizone (0.25 mg/ml), supplemented with 0.1 % collagenase (GIBCO Invitrogen Corp., Carlsbad, CA) and 3 % bovine serum albumin (BSA, GIBCO Invitrogen Corp.). After incubation at 12 °C for 20 h, cells were mechanically dissociated by aspiration for several times through a pipette. Then, they were washed twice with RPMI medium supplemented with Serum Replacement 2 (1x), which did not contain steroid hormones, glucocorticoids, and growth factors (Sigma-Aldrich, Tokyo, Japan). We verified that Serum Replacement 2 had no effects on the basal expression of GTH subunit mRNAs (data not shown). Dispersed cells were immediately plated on 12-well polylysine-coated culture plates at a density of  $3 \times 10^5$  cells/1.3 ml medium/well (n = 3-6), and were incubated at 18 °C for 2 days. Then, medium was changed, and hormones were included into the fresh medium. Cells were successively incubated for 1-3 days as described above. After incubation, the medium was removed, and cells were harvested by scraping in 0.7 ml of medium twice, then collected by centrifugation at 5,000 rpm for 10 min, frozen at -80 °C until assayed by real-time PCR for GTH subunit mRNAs.

### *2. 6. Quantitation of GTH subunit mRNAs by real-time PCR*

RNA extraction, reverse transcription (RT), and real-time PCR were previously described

in details by Ando et al. (2004). Briefly, total RNAs (50, 100, 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. Reactions were performed at 25 °C for 10 min, at 48 °C for 30 min, and finally at 95 °C for 5 min.

To determine absolute amounts of GTH subunit mRNAs, standard sense RNAs were synthesized *in vitro* by MAXIscript (Ambion Inc, Texas) using GP $\alpha$ 2, FSH $\beta$ , and LH $\beta$  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). GP $\alpha$ 2 and FSH $\beta$  mRNAs were determined by multiplex PCR. Each PCR reaction mixture (10  $\mu$ l) contained 1 x TaqMan Buffer A (PE Applied Biosystems), 25 mM MgCl<sub>2</sub>, 2.5 mM dNTPs, 72 nM each forward and reverse primers for GP $\alpha$ 2 and FSH $\beta$ , 130 nM fluorogenic probes, and 0.25 U AmpliTaq Gold DNA polymerase. Amplification was carried out at 95 °C for 10 min, for 40 cycles at 95 °C for 15 s, and 59 °C for 1 min. For LH $\beta$  mRNA, conditions of PCR reactions were the same as those for GP $\alpha$ 2 and FSH $\beta$ , 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 triplicates to estimate coefficients of variation (CV) within and between runs. For GP $\alpha$ 2 assay, intra-assay CV was  $7.2 \pm 1.3$  %, and inter-assay CV was 13 %; for FSH $\beta$  assay, intra-assay CV was  $5.5 \pm 1$  %, and inter-assay CV was 11.8 %. Finally, for LH $\beta$  assay, intra-assay CV was  $6 \pm 1$  %, and inter-assay CV was 8.8 %.

## 2. 7. Assays of plasma steroid hormones

Plasma levels of E2, testosterone (T), 11-ketotestosterone (11-KT), and 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (DHP) were determined by enzyme immunoassays as

described in details by Onuma et al. (2003). In brief, ether-extracted samples were dissolved in assay buffer containing 0.2 % bovine serum albumin, 0.01 % thimerosal in 0.05 M borate buffer, pH 7.8. 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. Following an overnight incubation at 4 °C, wells were washed with 0.9 % NaCl, and a substrate solution (0.5 g/l *o*-phenylenediamine, 0.01 % H<sub>2</sub>O<sub>2</sub> in 0.2 M citrate buffer, pH 4.5) was added, and mixed solutions were incubated at room temperature for 30 min. Then, optical densities at 492 nm were measured using a microplate reader (MTP-300, Corona Electric, Hitachinaka). Sensitivities of assays were: E2, 0.5 pg/tube; T, 1.5 pg/tube; 11-KT, 1.5 pg/tube; and DHP, 0.5 pg/tube. Intra-assay CVs were: E2, 4.5 ± 0.9 %; T, 6.6 ± 2 %; 11-KT, 2.6 ± 0.5 %; and DHP, 3.9 ± 1.3 %. Inter-assay CVs were: E2, 7 %; T, 13.3 %; 11-KT, 9.8 %; and DHP, 14.6 %.

## 2. 8. *Statistical analysis*

Data were presented as means ± SEM. In experiment I, the Games-Howell's multiple comparisons test was applied to test for statistically significant differences of GTH subunit mRNAs after incubation with IGF-I and/or sGnRH. In experiment II, statistical analysis of the effect of time of incubation was performed by ANOVA. In experiment II and III, the effects of IGF-I and/or sGnRH were tested by the Dunnett's pairwise multiple comparison t-test. The basal expression levels of GTH subunit genes in female control cells and plasma steroid levels in both sexes were analyzed by ANOVA to test for differences among months; and when appropriate, the Tukey's post hoc test was applied. GTH mRNAs in males were analyzed by a Student t-test for statistically significant differences between April and June. Statistical

analyses were performed using SPSS Version 12.0 for Windows (SPSS Inc., Chicago, IL).

### **3. Results**

#### *3. 1. Plasma steroid hormones during reproductive cycle*

Adult female masu salmon at early sexual maturation were used in experiment I in April. GSI and plasma levels of all steroid hormones examined were as low as those of females of the Mori hatchery strain used in experiments II and III in April (Tables 1 and 2). In experiments II and III, gonadal maturation of both sexes progressed in June, as assessed by changes in GSI and plasma levels of steroid hormones. GSI of both sexes increased to about 30 % of that in August, the pre-spawning period. T and 11-KT in males and E2 in females increased in June, and thereafter reached their maximum in August. In the spawning period in September, plasma E2 levels in females declined, and plasma DHP levels increased, although this increase was not statistically significant due to high variations in the plasma DHP levels. These changes were consistent with those determined in masu salmon from immature to spawning fishes (Munakata et al., 2001; Ando et al., 2004).

#### *3. 2. Experiment I: Dose-response relationship of effects of IGF-I in April*

Three doses of IGF-I (1, 10, 100 nM) were added to cultures with or without sGnRH (100 nM) and were incubated for 1 day (Fig. 1). sGnRH alone did not alter amounts of the three subunit mRNAs. Furthermore, IGF-I of any concentration had no significant effects on these GTH subunit mRNAs, regardless of sGnRH.

#### *3. 3. Experiment II: Kinetics of effects of IGF-I in April*

In experiment II, kinetics of effects of IGF-I was examined in April. Since no

dose–response relationship was observed in experiment I, IGF-I was used at a high concentration (100 nM), and incubated for 1, 2, and 3 days. There were no significant changes in GTH subunit mRNAs during three days of incubation in any groups of treatment in both sexes, whereas a trend to decrease in FSH $\beta$  mRNA was noted in males. In males, IGF-I alone did not change levels of the three subunit mRNAs during three days. In contrast, sGnRH (100 nM) alone significantly increased FSH $\beta$  and LH $\beta$  mRNAs at day 3 (Fig. 2). However, co-administered IGF-I completely counteracted the sGnRH-induced FSH $\beta$  and LH $\beta$  gene expression at day 3. In females, IGF-I alone or sGnRH alone, and their combination had no significant effects on the three subunit mRNAs during three days (Fig. 3).

#### *3. 4. Experiment III: Seasonal changes in effects of IGF-I*

Effects of IGF-I and sGnRH were examined at three stages of sexual maturation. Basal amounts of GP $\alpha$ 2 and FSH $\beta$  mRNAs in males in June were significantly higher than levels in April (Fig. 4). Similarly, GP $\alpha$ 2 and FSH $\beta$  mRNAs in females significantly increased from April to June, and remained high in September. LH $\beta$  mRNA showed a similar trend, although changes were not statistically significant. These changes were consistent with those determined in our previous study, in which fishes were sampled in May, July, and September 2001 (Ando et al., 2004).

In males, in contrast to the stimulatory effects in April, sGnRH alone did not affect the three subunit mRNAs in June (Fig. 5). IGF-I alone had little effects in April, but decreased amounts of FSH $\beta$  mRNA in June. Co-administration of IGF-I with sGnRH decreased FSH $\beta$  mRNA to the same levels as in IGF-I-treated cells. In females, IGF-I and sGnRH by themselves had little effects on the three subunit mRNAs in June and September (Fig. 6). However, the combination of IGF-I and sGnRH significantly increased FSH $\beta$  mRNA in

September.

#### 4. Discussion

The present study examined *in vitro* effects of IGF-I in combination with sGnRH on expression of GTH subunit genes in masu salmon pituitary cells at three stages of sexual maturation. Our results suggested that IGF-I is directly involved in regulation of expression of three GTH subunit genes at particular stages in both sexes. IGF-I alone had no stimulatory effects during sexual maturation, but had an inhibitory effect of IGF-I on FSH $\beta$  mRNA in males in June. Furthermore, IGF-I differentially modulated sGnRH-induced expression of GTH subunit genes. In particular, our results clearly showed that these effects were dependent on reproductive stages.

These findings are the first evidence for the effects of IGF-I on expressions of FSH $\beta$  and LH $\beta$  subunit genes in any vertebrates, although there were many studies reporting stimulatory effects of IGF-I on FSH and LH contents and release in mammals (Kanematsu, et al., 1991; Soldani et al., 1994, 1995; Xia et al., 2001; Hashizume et al., 2002) and fishes (Huang et al., 1998, 1999; Weil et al., 1999; Baker et al., 2000), and on expression of  $\alpha$  subunit gene in rats (Pazos et al., 2004).

A dose of IGF-I at 100 nM was used in experiments II and III. Although this dose seems to be high, it was chosen because of the following reasons. Similar and even higher doses of IGF-I were previously demonstrated by other authors to stimulate FSH and LH release with high efficiency based on dose-response experiments. In coho salmon pituitary cells, 10-day pre-incubation with IGF-I at 100 nM was most effective for intracellular accumulations of FSH and LH, and for potentiation of GnRH-stimulated FSH release (Baker et al., 2000). IGF-I at 1  $\mu$ M was effective to sensitize rainbow trout pituitary cells to GnRH in terms of releases of FSH

and LH (Weil et al., 1999). Similarly, European eel pituitary cells maximally responded to IGF-I at the concentration of 100 nM in LH release and cell content (Huang et al., 1998, 1999).

In addition, the present dose of IGF-I was of similar order to circulating levels in salmonids. Plasma levels of total IGF-I in coho salmon were 50-250 ng/ml, that is 6.5-32.5 nM (Moriyama et al., 1994). Thus, the present dose of IGF-I was 3 times the maximum circulating level. In fact, at concentrations in the range of 4-250 ng/ml, salmon IGF-I significantly stimulated sulfate uptake by cultured branchial cartilages of coho salmon (Moriyama et al., 1993). Also it should be noted that human recombinant IGF-I, but not salmon IGF-I, was used in the present study. Human IGF-I and salmon IGF-I were equipotent in stimulating sulfate uptake in salmon branchial cartilages (Moriyama et al., 1993). Receptor binding studies indicated that 50 % of displacement of <sup>125</sup>I-IGF-I was achieved by the same dose (1.3 nM) of human IGF-I and salmon IGF-I in salmon embryo cells (CHSE-214) (Upton et al., 1998). Thus, these results indicate that effects of human IGF-I at 100 nM observed in the present study were most probably physiological and maximum responses of masu salmon pituitary cells.

IGF-I did not increase amounts of GTH subunit mRNAs at three reproductive stages. On the contrary, it decreased FSH $\beta$  mRNA in males in June, and had similar inhibitory effects in females in April and September. Furthermore, co-administered IGF-I completely counteracted sGnRH-induced FSH $\beta$  and LH $\beta$  gene expression in males in April. Therefore, the present results suggested that IGF-I has moderate but circumstantially inhibitory effects on GTH subunit gene expression. This is a striking contrast to stimulatory effects of IGF-I on GTH release. Weil et al. (1999) reported in rainbow trout that IGF-I potentiated GnRH-induced releases of FSH and LH in maturing and fully matured fishes. Although effects of IGF-I on GTH release were not determined in the present study, stimulatory effects of IGF-I on GTH release might not parallel changes in their gene expression. In rat pituitary cells, IGF-I

stimulated basal FSH and LH releases, while the amounts of their mRNAs remained unchanged (Pazos et al., 2004). Since we determined steady-state mRNA levels, we cannot neglect the possibility that IGF-I stimulated transcriptional activities of GTH subunit genes as well as release of GTH. However, we currently conclude that IGF-I had little or inhibitory effects on GTH synthesis, at least in terms of amounts of mRNA at the three stages of sexual maturation.

IGF-I has been considered to play an important role in endocrine regulation during the onset of puberty. Circulating levels of IGF-I highly correlate with growth rates in salmonids (Beckham et al., 1998; Campbell et al., 2003), and IGF-I may therefore act as a signaling molecule that transmits growth and nutritional status to the reproductive axis. 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 of spermatogenesis, from July to December one year in advance of spawning. Correlations between plasma 11-KT and plasma FSH, plasma IGF-I and GSI were observed. Therefore, IGF-I in addition to 11-KT may have a pivotal role in stimulation of FSH secretion, which in turn leads to activation of spermatogenesis. Indeed, Baker et al. (2000) showed that pre-incubation with IGF-I for 10 days increased FSH and LH cell contents in the pituitary cells of coho salmon at early maturational stages (GSIs of males and females were <0.10 % and 0.43 %, respectively), suggesting that IGF-I was effective in stimulation of GTH synthesis as well as GTH release in such early stage of sexual maturation. In the present study in April, we used fish at early maturational but later stages (GSI of female was 0.95 % in experiment I, and those of males and females were 0.16 % and 0.73 %, respectively, in experiment III). It is thus possible that IGF-I stimulates GTH gene expression at an earlier stage of sexual maturation, and in the later stages it turns to lose this effect, but still continues to stimulate GTH release.

It is interesting to note that, in September, IGF-I in combination with sGnRH significantly increased FSH $\beta$  mRNA in females. Although we did not examine effects of IGF-I and sGnRH in males in September due to the limited number of available fish, the modulatory effect of IGF-I on sGnRH-induced GTH gene expression may vary during sexual maturation. In spawning in September, loss of suppressive action of IGF-I is highly likely to lead to activate GTH subunit genes (Kitahashi et al., 1998a, b).

Based on the present results, we propose that the effect of IGF-I on GTH gene expression is variable during the postpubertal development and is different from that on GTH release. In addition, cooperative interaction with sGnRH also changes during sexual maturation. How are these stage-dependent changes in IGF-I actions on GTH synthesis and release achieved? IGF-I may differentially influence sGnRH-induced intracellular signals that lead to activation of GTH synthesis or release. GnRH itself is known to differently regulate synthesis and release of GTH, depending on reproductive stages in salmonids (Ando et al., 2004; Ando and Urano, 2005). Stimulations of GTH subunit genes by GnRH are mediated through activation of GnRH receptors that are linked to two major intracellular signaling pathways: protein kinase C/mitogen-activated protein kinase (MAPK) pathway, and Ca<sup>2+</sup> signaling pathway (Ando et al., 2001). Activation of IGF-I receptors also leads to activate the MAPK pathway (Duan, 2002). It is thus possible that a 'cross-talk' between IGF-I and GnRH signals is involved in their functional interactions. It is tempting to speculate that some gonadal factors such as sex steroid hormones and activin may influence the intracellular signals involved in IGF-I and sGnRH actions and also expression of receptors for IGF-I and sGnRH. Further studies on molecular interaction of these factors will provide valuable information on the roles of IGF-I in modulation of gonadotropic function during onset and progression of sexual maturation.

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## Figure legends

Fig. 1. Experiment I: Effects of IGF-I and sGnRH on amounts of GTH subunit mRNAs in females in April. Pituitary cells were plated in culture plates at a density of  $3 \times 10^5$  cells/1.3 ml medium/well ( $n = 3-4$ ). Cells were treated with IGF-I (1, 10, 100 nM) in combination with sGnRH (100 nM) at 2 days after plating and successively incubated for 1 day. After incubation, cells were collected, and the amounts of GTH subunit mRNAs were assayed by real-time PCR. Results are expressed as mean  $\pm$  SEM. No statistically significant changes in three GTH subunit mRNAs were observed in response to any combination of hormones (Games-Howell's test,  $P < 0.05$ ).

Fig. 2. Experiment II: Effects of IGF-I and sGnRH on amounts of GTH subunit mRNAs in males in April. Pituitary cells were plated in culture plates at a density of  $3 \times 10^5$  cells/1.3 ml medium/well ( $n = 4-5$ ). Cells were treated with IGF-I (100 nM) with or without sGnRH (100 nM) at 2 days after plating and successively incubated for 1, 2, and 3 days. After incubation, cells were collected, and the amounts of GTH subunit mRNAs were assayed by real-time PCR. Results are expressed as mean  $\pm$  SEM. Asterisks denote significant difference between the control (without hormones, open square) and hormone-treated groups at each time of incubation (\*  $P < 0.05$ , Dunnett's pairwise multiple comparison t-test). No statistically significant changes in three GTH subunit mRNAs were observed during the three days of incubation (ANOVA,  $P < 0.05$ ).

Fig. 3. Experiment II: Effects of IGF-I and sGnRH on amounts of GTH subunit mRNAs in females in April. Pituitary cells were plated in culture plates at a density of  $3 \times 10^5$  cells/1.3 ml medium/well ( $n = 3-4$ ). Cells were treated with IGF-I (100 nM) with or without sGnRH (100

nM) at 2 days after plating and successively incubated for 1, 2, and 3 days. After incubation, cells were collected, and the amounts of GTH subunit mRNAs were assayed by real-time PCR. Results are expressed as mean  $\pm$  SEM. No statistically significant changes in three GTH subunit mRNAs were observed during the three days of incubation (ANOVA,  $P < 0.05$ ) and in response to hormones (Dunnett's pairwise multiple comparison t-test,  $P < 0.05$ ).

Fig. 4. Experiment III: Changes in basal amounts of GTH subunit mRNAs during sexual maturation. Pituitary cells were collected in April, June and September, and plated in culture plates at a density of  $3 \times 10^5$  cells/1.3 ml medium/well ( $n = 3-6$ ). After incubation at  $18^\circ\text{C}$  for 5 days, cells were collected, and the amounts of GTH subunit mRNAs were assayed by real-time PCR. Results are expressed as mean  $\pm$  SEM. Asterisks denote significant difference between months (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , Student t-test for males and Tukey's post hoc test for females).

Fig. 5. Experiment III: Effects of IGF-I and sGnRH on amounts of GTH subunit mRNAs in males in April and June. Pituitary cells were plated in culture plates at a density of  $3 \times 10^5$  cells/1.3 ml medium/well ( $n = 4-5$ ). Cells were treated with IGF-I (100 nM) with or without sGnRH (100 nM) at 2 days after plating and successively incubated for 3 days. After incubation, cells were collected, and the amounts of GTH subunit mRNAs were assayed by real-time PCR. The data in April were the same as that in experiment II (Figure 2, 3 days). Results are expressed as mean  $\pm$  SEM. Asterisks denote significant difference between the control (without hormones) and hormone-treated groups (\*  $P < 0.05$ , Dunnett's pairwise multiple comparison t-test).

Fig. 6. Experiment III: Effects of IGF-I and sGnRH on the amounts of GTH subunit mRNAs in females in April, June and September. Pituitary cells were plated in culture plates at a density of  $3 \times 10^5$  cells/1.3 ml medium/well (n = 3-6). Cells were treated with IGF-I (100 nM) with or without sGnRH (100 nM) at 2 days after plating and successively incubated for 3 days. After incubation, cells were collected, and the amounts of GTH subunit mRNAs were assayed by real-time PCR. The data in April were the same as that in experiment II (Figure 3, 3 days). Results are expressed as mean  $\pm$  SEM. Asterisks denote significant difference between the control (without hormones) and hormone-treated groups (\* P < 0.05, Dunnett's pairwise multiple comparison t-test).

TABLE 1. Body weight (BW), fork length (FL) and gonadosomatic index (GSI) in masu salmon (means  $\pm$  SEM).

	Month	Sex	n	BW (g)	FL (cm)	GSI (%)
Exp. 1	April	Female	61	458 $\pm$ 16	33.1 $\pm$ 0.4	0.95 $\pm$ 0.02
Exp. 2 and 3	April	Male	41	479 $\pm$ 17	33.4 $\pm$ 0.4	0.16 $\pm$ 0.01
		Female	35	506 $\pm$ 22	34.0 $\pm$ 0.5	0.73 $\pm$ 0.02
	June	Male	48	824 $\pm$ 36	38.6 $\pm$ 0.5	3.27 $\pm$ 0.20
		Female	52	784 $\pm$ 31	38.4 $\pm$ 0.5	5.46 $\pm$ 0.24
	August	Male	10	868 $\pm$ 44	42.1 $\pm$ 0.6	9.15 $\pm$ 0.28
		Female	10	657 $\pm$ 59	38.4 $\pm$ 1.2	17.5 $\pm$ 0.78
September	Female	34	700 $\pm$ 38	39.4 $\pm$ 0.6	28.4 $\pm$ 0.41	

TABLE 2. Plasma steroid hormone levels during sexual maturation (means  $\pm$  SEM, ng/ml).

Sex	Hormone	Exp. 1	Exp. 2 and 3			
		April	April	June	August	September
Male	T	-	0.80 $\pm$ 0.13 <sup>a</sup>	4.71 $\pm$ 0.62 <sup>b</sup>	10.06 $\pm$ 1.32 <sup>c</sup>	-
	11-KT	-	0.80 $\pm$ 0.31 <sup>a</sup>	1.90 $\pm$ 1.00 <sup>a</sup>	5.45 $\pm$ 2.19 <sup>a</sup>	-
	E2	-	0.15 $\pm$ 0.04 <sup>a</sup>	2.60 $\pm$ 0.69 <sup>b</sup>	2.28 $\pm$ 0.83 <sup>ab</sup>	-
	DHP	-	0.05 $\pm$ 0.01 <sup>ab</sup>	0.02 $\pm$ 0.01 <sup>a</sup>	0.10 $\pm$ 0.02 <sup>b</sup>	-
Female	T	0.70 $\pm$ 0.11	1.20 $\pm$ 0.40 <sup>a</sup>	3.72 $\pm$ 0.87 <sup>ab</sup>	6.39 $\pm$ 1.36 <sup>b</sup>	6.26 $\pm$ 1.30 <sup>b</sup>
	11-KT	0.59 $\pm$ 0.11	0.10 $\pm$ 0.06 <sup>a</sup>	0.70 $\pm$ 0.21 <sup>a</sup>	0.17 $\pm$ 0.06 <sup>a</sup>	2.43 $\pm$ 0.81 <sup>b</sup>
	E2	0.30 $\pm$ 0.10	0.29 $\pm$ 0.06 <sup>a</sup>	0.43 $\pm$ 0.14 <sup>a</sup>	7.16 $\pm$ 2.51 <sup>b</sup>	1.26 $\pm$ 0.44 <sup>a</sup>
	DHP	0.26 $\pm$ 0.16	0.05 $\pm$ 0.01 <sup>a</sup>	0.03 $\pm$ 0.01 <sup>a</sup>	0.05 $\pm$ 0.01 <sup>a</sup>	18.74 $\pm$ 9.48 <sup>a</sup>

<sup>a,b,c</sup> Values with different characters are significantly different among months ( $p < 0.05$ ).

Fig. 1 H. Ando

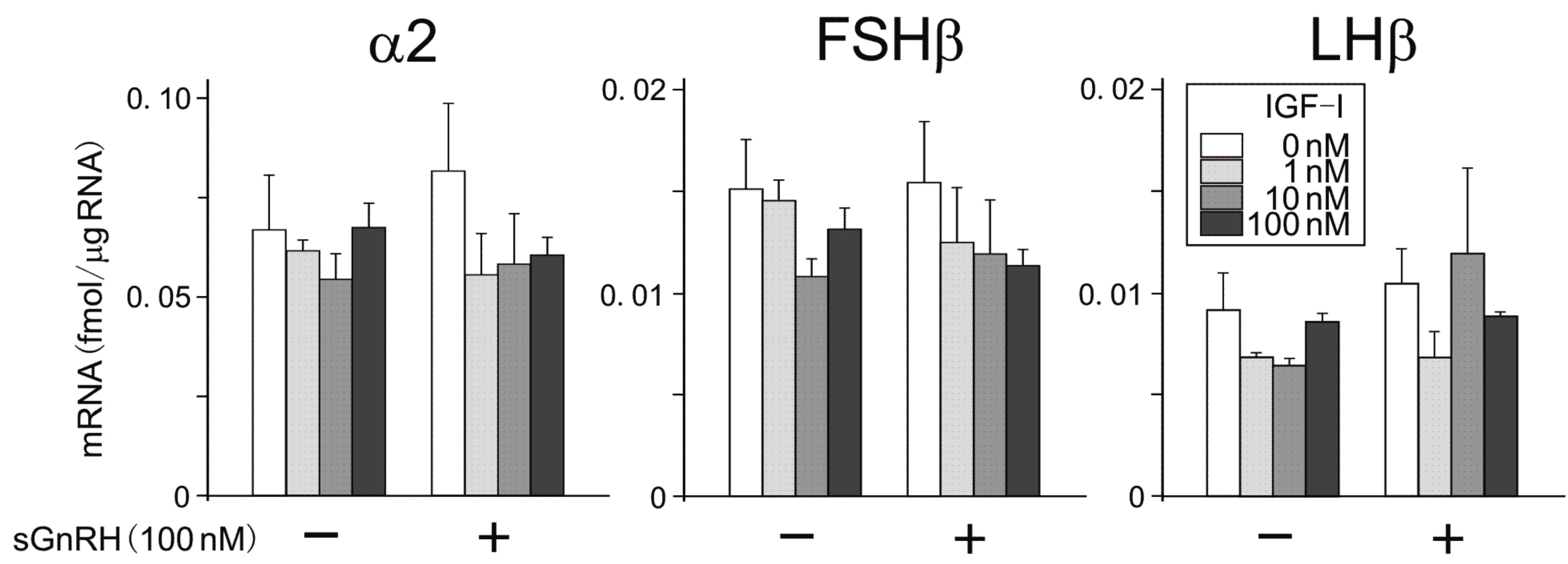


Fig. 2 H. Ando

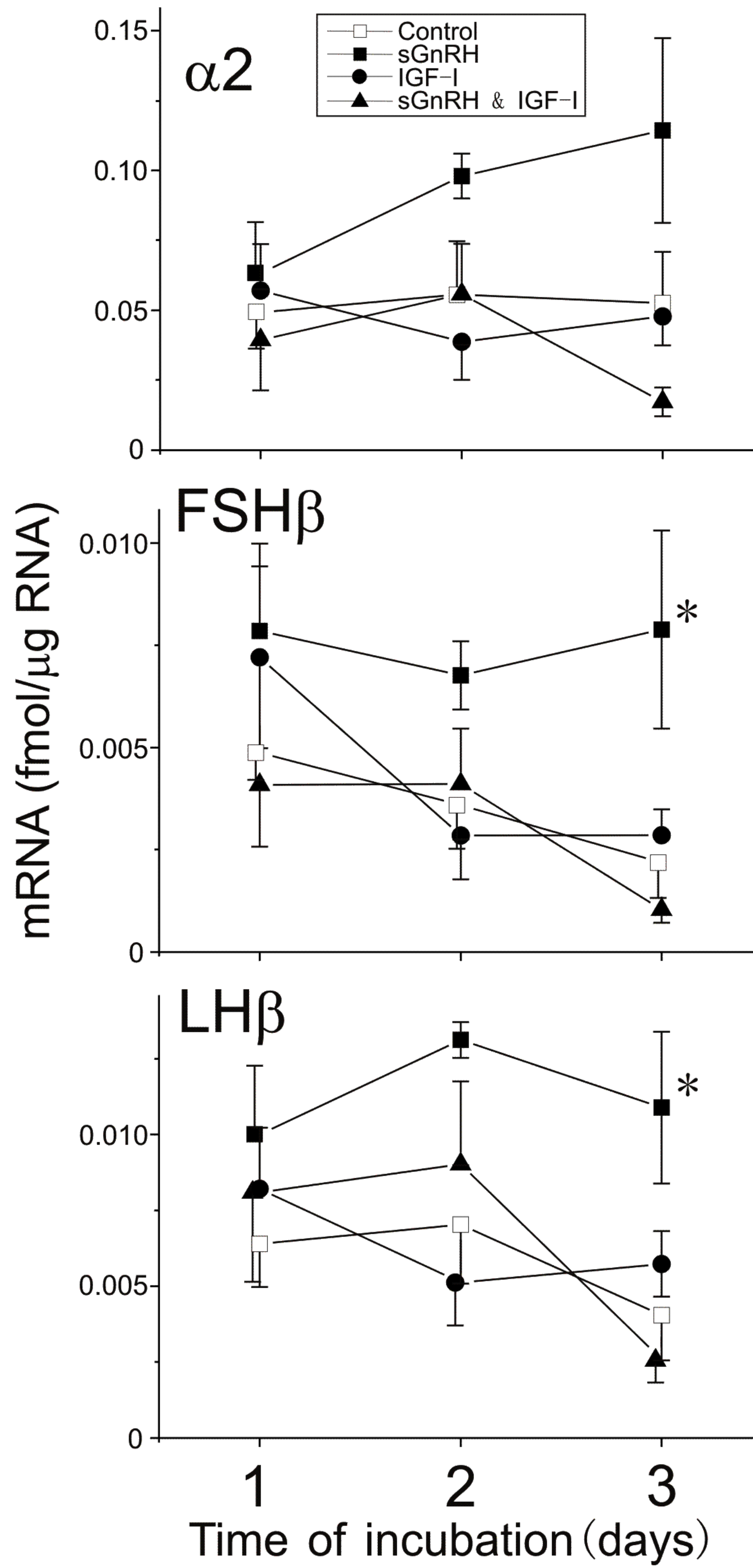


Fig. 3 H. Ando

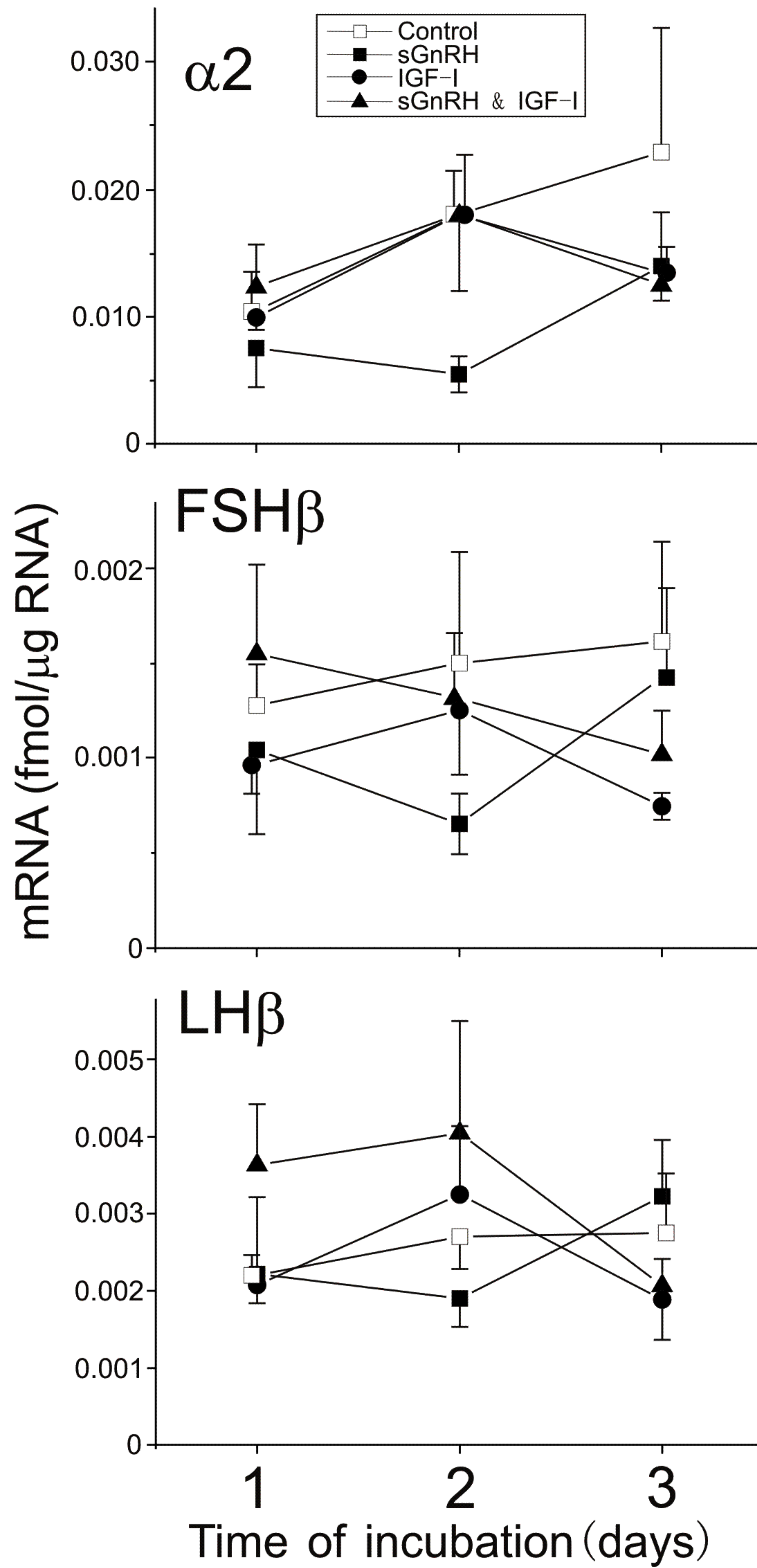


Fig. 4 H. Ando

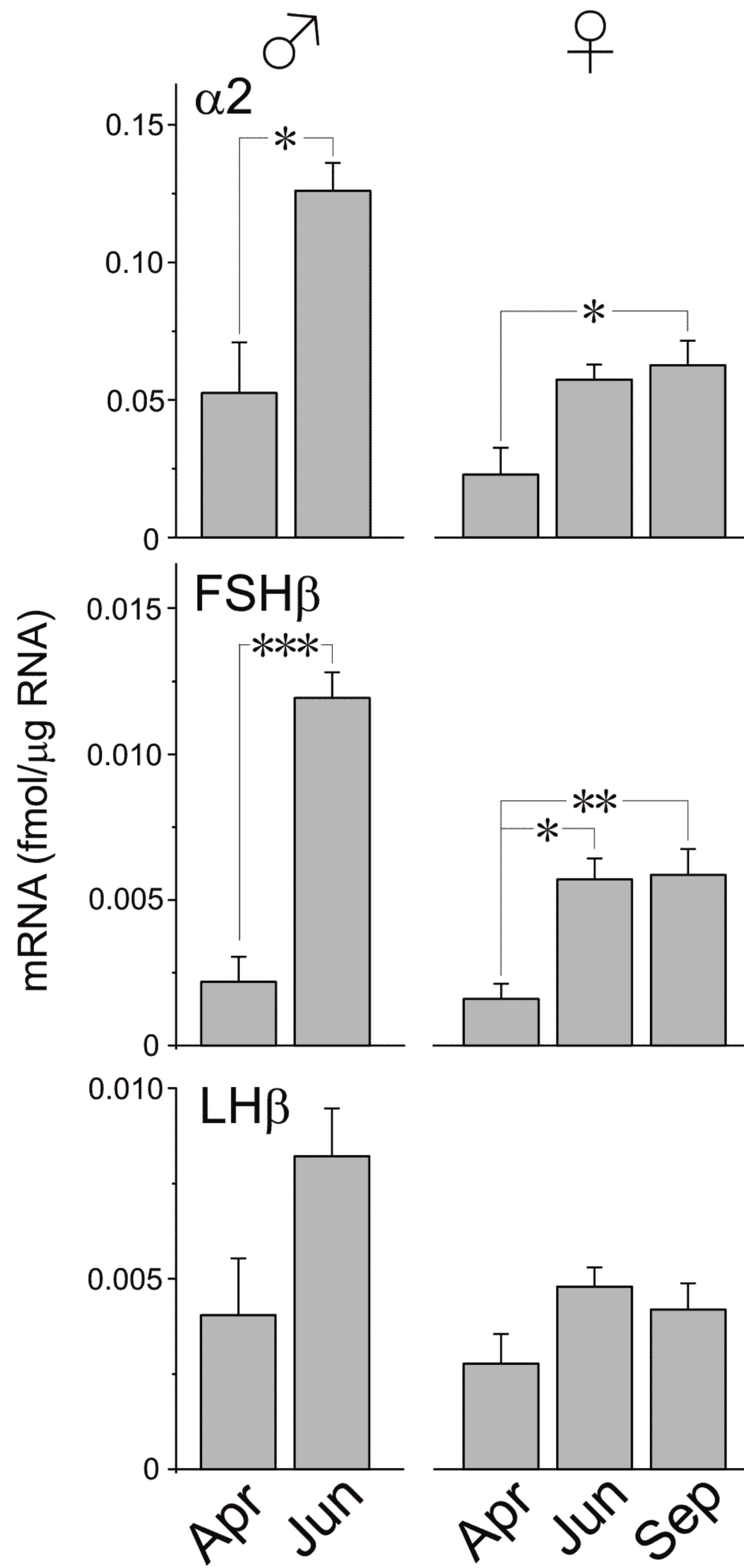


Fig. 5 H. Ando

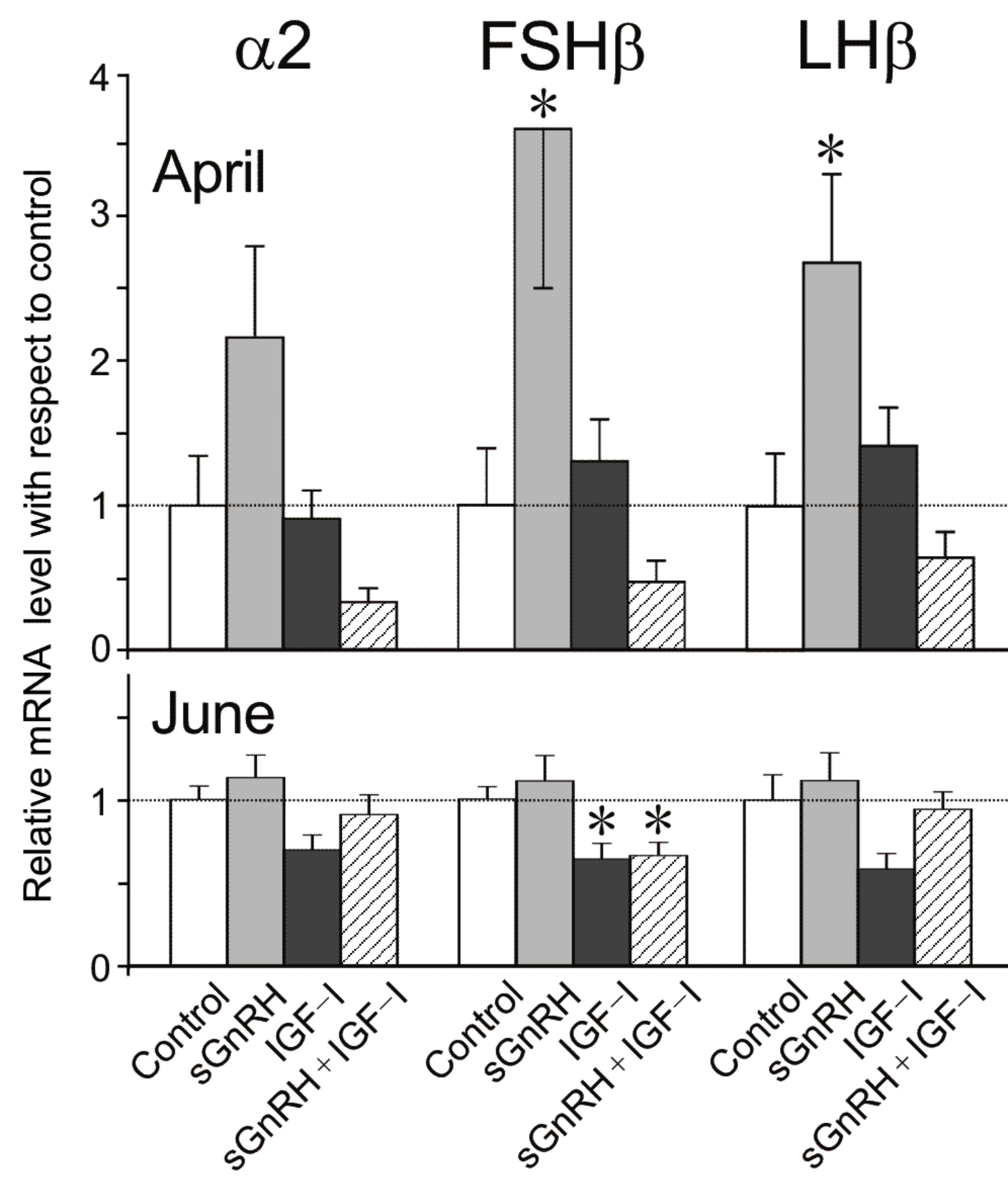


Fig. 6 H. Ando

