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Seasonal Variation in the Expression of Five Subtypes of Gonadotropin-releasing Hormone Receptor Genes in the Brain of Masu Salmon from Immaturity to Spawning

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ABSTRACT—Seasonal variation in the expression of five subtypes of gonadotropin-releasing hormone receptor (GnRH-R) genes, designated as msGnRH-R1, -R2, -R3, -R4, and -R5, was examined in the brain of masu salmon (*Oncorhynchus masou*). In addition, responses of these genes to GnRH were examined in a GnRH analog (GnRHa) implantation experiment. Brain samples were collected one week after the implantation every month from immaturity through spawning. The absolute amount of GnRH-R mRNA in single forebrains was determined by real-time PCR assays. Among the five genes, R4 and R5 were dominantly expressed in both sexes. R1, R4, and R5 mRNAs showed similar changes throughout the experimental period in both sexes. Levels tended to be high in winter and low in the pre-spawning season, followed by elevations in the spawning period. The mRNA levels had weak to moderate negative correlations with the plasma level of estradiol-17 β (E2) in females. The effects of GnRHa on msGnRH-R mRNAs were not apparent for all the subtypes. These results indicate that the msGnRH-R1, -R4, and -R5 genes are synchronously expressed during sexual maturation. There was a trend toward decreased levels of their expression prior to the spawning period and then increased levels at spawning, possibly causing GnRH target neurons to sensitize to a GnRH stimulus. Furthermore, E2 may be involved in msGnRH-R gene expression in the brain of female masu salmon during sexual maturation.

Key words: GnRH receptor, gene expression, brain, seasonal reproduction, spawning behavior, real-time PCR

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) plays a central role in the control of reproductive function in vertebrates. In salmonids, two molecular forms of GnRH, namely salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), have been identified in the brain. sGnRH secreted by preoptic GnRH neurons regulates gonadal maturation through stim-

ulation of synthesis and release of pituitary gonadotropins (GTHs) (Amano *et al.*, 1997; Ando *et al.*, 2005). In addition, there are two other main GnRH neuronal systems in the fish brain, terminal nerve sGnRH neurons and midbrain cGnRH-II neurons, both of which include wide distribution of GnRH fibers in the brain. This wide distribution of GnRH fibers supports the notion that GnRH acts as a neuromodulator that is involved in reproductive behavior (Oka, 2002; Millar, 2003). In fact, in salmonids administration of a GnRH analog (GnRHa) shortened the duration of homing behavior, as well as stimulated gonadal maturation (Sato *et al.*, 1997; Kitahashi *et al.*, 1998b). Therefore, the bifunctional (neuromodulatory and hypophysiotropic) GnRH serves to integrate reproductive behavior with gonadal maturation, and is cru-

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cial for reproductive success. However, molecular mechanisms of action of the multifunctional GnRH remain to be elucidated.

Recently, the existence of multiple types of GnRH receptor (GnRH-R) genes has been demonstrated in various vertebrate species (Lethimonier *et al.*, 2004; Ando *et al.*, 2005). In masu salmon (*Oncorhynchus masou*), five different GnRH-R genes, msGnRH-R1, R2, R3, R4, and R5, are expressed in the brain and the pituitary (Jodo *et al.*, 2003). A splicing variant of R1 (R1-v) is also expressed in these tissues. In mammals, two types of GnRH-R, namely type I and type II receptors, have distinct ligand selectivity: the type I receptor is particularly sensitive to mammalian GnRH, whereas the type II receptor shows a clear preference for cGnRH-II (Millar *et al.*, 2001). In teleosts, however, all the multiple GnRH-Rs show a distinct preference for cGnRH-II, followed by sGnRH and a third endogenous GnRH form (Lethimonier *et al.*, 2004). Therefore, ligand-receptor relationships of the multiple GnRH-Rs in teleosts remain unclear.

The expression of the GnRH-R gene was shown to change during sexual maturation in the pituitary of striped bass (Alok *et al.*, 2000) and European sea bass (González-Martínez *et al.*, 2004). We recently reported that the five subtypes of msGnRH-R genes were differently expressed in the pituitary of masu salmon during growth and sexual maturation, depending on subtype, reproductive stage, and gender (Jodo *et al.*, 2005). Furthermore, GnRHa implantation increased the five subtypes of msGnRH-R mRNAs in the pituitary of females. At present, however, there is a paucity of information concerning changes in expression of GnRH-R gene in the brain. In European sea bass, González-Martínez *et al.* (2004) showed a higher expression of the GnRH-R gene in various brain regions in the spawning and post-spawning periods by quantitative *in situ* hybridization. However, it remains unknown how the expression of multiple types of GnRH-R genes is regulated in the brain during the reproductive cycle.

In the present study, we examined changes in the amounts of five subtypes of msGnRH-R mRNAs in the brain of masu salmon from immaturity to spawning by a real-time PCR technique. The brain samples contained forebrain regions from the posterior half of the telencephalon to the anterior end of the saccus vasculosus, because of the exclusive expression of the GnRH-R gene (Madigou *et al.*, 2000; Peter *et al.*, 2003; González-Martínez *et al.*, 2004; Soga *et al.*, 2005). In addition, the effects of GnRHa implantation on the expression of these msGnRH-R genes were examined at each stage during the experimental period.

MATERIALS AND METHODS

Fish

The fish used in the present study, the procedures for GnRHa implantation, and sample collection were described in detail by Bhandari *et al.* (2003). In brief, masu salmon of the Mori hatchery

strain were reared at the Toya Lake Station for Environmental Biology, Hokkaido University in 1400-L circular outdoor tanks with flowing spring water (9.8–12.1°C) under natural photoperiod. They were fed dry pellets (Oriental Feed Industry, Yokohama) equivalent to 2% of body weight per day. Under these conditions, the salmon generally mature and spawn in late September at the age of 3 years, and most of the males also mature precociously at the age of 2 years. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and also conformed to the guidelines set by the Research Center of Laboratory Animals, Hokkaido University.

GnRHa implantation experiments

Monthly implantation experiments were started at the end of December 1998, when the fish reached 2 years old and were completed at the end of September 1999, when the fish were almost 3 years old. In September 1999, the experiment was performed twice at a 2-week interval. Twenty fish were randomly selected every month and anesthetized with 0.01% tricaine methanesulfonate (MS-222, Sigma) buffered with an equal amount of sodium bicarbonate. They were then divided into two groups. A 2-mm GnRHa capsule containing 75 µg of [Des-Gly¹⁰, D-Ala⁶, Pro⁹]-GnRH ethyl amide in an ethylene vinyl acetate copolymer matrix (Zohar, 1996) was implanted with a needle in the dorsal muscle of 10 fish (implant group), while the other 10 fish received a blank copolymer matrix (control group). The dose of GnRHa was decided by our previous study in which 75 µg of GnRHa significantly elevated expression of luteinizing hormone (LH) subunit genes (Kitahashi *et al.*, 1998a) and the plasma level of 17α, 20β-dihydroxy-4-pregnen-3-one in sockeye salmon (Fukaya *et al.*, 1998). Since the sex was not discernable by appearance until the fish matured, the number of fish in each group varied from 3 to 8, and consequently some data points were not available in December 1998 and June and August 1999 for implanted males, and early September 1999 for the control and implanted females.

Sample collection

One week after implantation, both control and implanted fish were sampled. The fork length and body weight of the fish were measured under MS222 anesthesia. The brains were removed and trimmed to prepare forebrain samples. They were cut frontally at the middle of the telencephalon, and posteriorly at the anterior end of the saccus vasculosus (Fig. 1). Therefore, the forebrain samples contained the posterior half of the telencephalon, the diencephalon, and the mesencephalon, including part of the optic tectum. They were frozen in liquid nitrogen and stored at –80°C. The gonads were weighed to calculate the gonadosomatic index (GSI) as an estimate of gonadal maturity. Blood samples were collected from the caudal

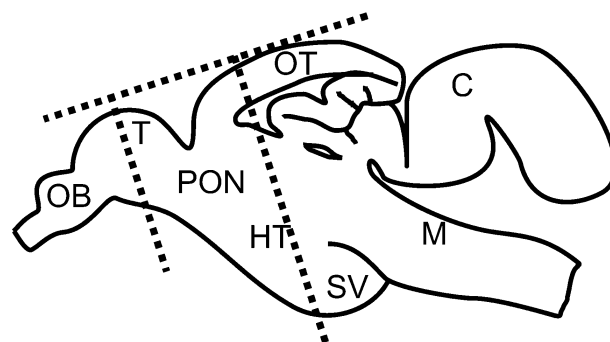


Fig. 1. Schematic illustration of trimming the masu salmon brain. OB, olfactory bulb; T, telencephalon; PON, nucleus preopticus; HT, hypothalamus; OT, optic tectum; SV, saccus vasculosus; C, cerebellum; M, medulla oblongata.

Table 1. Nucleotide sequences of primers and fluorogenic probes for real-time PCR assays.

mRNA	Forward primer	Reverse primer	Probe
R1	5'-CACCTTAATCCTCTTCTATTTGCC-3'	5'-GCTCATGATGAGTGGTCGCAG-3'	5'-TGGCCGGCGCCTGGCTTCTC-3'
R1-v	5'-CAAGTGAGTTGAGCGTTAAACAAGC-3'	5'-GCTCATGATGAGTGGTCGCAG-3'	5'-TGGCCGGCGCCTGGCTTCTC-3'
R2+R3	5'-AGATGGATCAACCGAAACGC-3'	5'-TGATCAGCTCAGCCGTAATGC-3'	5'-CAATTATCTTTGATGCTGCGGCAACTGAAG-3'
R4	5'-CGAGATGGTTAAAAAGTCGCG-3'	5'-AAAGACCAACCTGGCAGCAT-3'	5'-CTTTGGTGCACGGACAGCTCCTCTCTATG-3'
R5	5'-CCAACCTCTCCGTGCTGATT-3'	5'-CACAGCAGCTTGACATGGT-3'	5'-ACGAACGTCATCACCAGGTCTGCAGA-3'

vasculature, kept in ice, and later centrifuged at 3000 rpm for 15 min to obtain plasma samples, which were stored at -30°C until they were assayed.

Preparation of samples

Total RNA was extracted from single brain samples by the guanidium thiocyanate hot phenol-chloroform method (Chirgwin *et al.*, 1979). Recovery of total RNA extracted by this method was $70.6 \pm 4.6\%$. The integrity of total RNA was confirmed by agarose gel electrophoresis. Total RNA (150, 200, or 400 ng) from the brains was used for synthesis of first strand cDNA by reverse transcription (RT) with sequence-specific primers (for R1, R1-v, R2, R3, and R4, 5'-CTGACCACCAGGATGAAGGC-3'; for R5, 5'-GAGTGCATGGCAAACAG-3') as described previously (Jodo *et al.*, 2005).

Real-time PCR

We determined the amount of mRNA for each subtype of msGnRH-R with the exception of R2 and R3. Since R2 and R3 share a high sequence identity, we determined the sum of these mRNAs. Absolute amounts of msGnRH-R mRNAs were determined using standard sense RNAs that were synthesized *in vitro* with MAXIsript (Ambion, Texas) as described previously (Jodo *et al.*, 2005). The serially diluted standard RNAs were reverse transcribed to prepare standard cDNAs.

Each PCR reaction contained $1 \times$ TaqMan Buffer A (PE Applied Biosystems), 5 mM MgCl_2 (for R1, R1-v, and R4), 3.5 mM MgCl_2 (for R2+R3), or 2 mM MgCl_2 (for R5), 0.2 mM dNTP, 100 nM each forward and reverse primer, 50 nM fluorogenic probe, and 0.25 U AmpliTaq Gold DNA polymerase (PE Applied Biosystems). The nucleotide sequences of the primers and probes used are shown in Table 1. Amplification was carried out at 95°C for 10 min, followed by 45 cycles (for R1, R1-v, and R5) or 50 cycles (for R2+R3 and R4) at 95°C for 15 sec and then 59°C (for R1, R1-v, R4, and R5) or 62°C (for R2+R3) for 1 min, using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). In these assays, doses of standard cDNA (20 , 2×10^2 , 2×10^3 , 2×10^4 , 2×10^5 , and 2×10^6 copies) were assayed in triplicate, and one-fifth of the volume of reverse transcription product synthesized from total RNA was assayed in duplicate. The sensitivity of the present assay was 20 copies/well. Cross-reactivity with other subtypes of msGnRH-R mRNAs was less than 0.01%, except for R2 and R3 mRNAs in the R1 assay ($<1.0\%$; Jodo *et al.*, 2005). The range of intra-assay coefficients of variation (CVs) was 2.3–9.4% for R1, 1.1–7.2% for R1-v, 4.4–6.6% for R2+R3, 1.1–5.0% for R4, and 1.3–9.2% for R5. The inter-assay CVs were 6.8% for R1, 7.1% for R1-v, 5.2% for R2+R3, 19.6% for R4, and 10.5% for R5.

Assays of plasma steroid hormones

Plasma levels of testosterone (T), 11-ketotestosterone (11-KT), and estradiol- 17β (E2) were determined by time-resolved fluoroimmunoassays as described previously (Ando *et al.*, 2004). Sensitivities of the present assays were 24 pg/ml for all steroid hormones. The range of intra-assay CVs was 0.6–10.5% for T, 1.1–10.2% for 11-KT, and 0.2–4.2% for E2. The inter-assay CVs were 17.5% for

T, 14.8% for 11-KT, and 19.0% for E2.

Statistical analyses

All data are presented as mean $\pm$ SEM after the elimination of abnormal values by Smirnov-Grubb's test. To test for statistically significant differences among months, the Games-Howell multiple-comparison test was employed. The effects of GnRHa implantation on GnRH-R mRNAs were tested each month with Welch's t-test. The correlation between the amounts of msGnRH-R mRNAs and the logarithm of plasma levels of steroid hormones in the control fish was analyzed by Pearson's correlation test. Statistical analyses were performed using SPSS Version 12.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Growth and sexual maturation

The fork length and body weight of the fish used in this study were described in detail by Bhandari *et al.* (2003). They gradually increased during the experimental period. Briefly, when the experiment started, fork lengths were 16.7 ± 0.7 cm in both sexes ($n=7$ for males, $n=13$ for females), and body weights were 45.8 ± 6.2 g in the males and 46.3 ± 6.2 g in the females. At the end of experiment, fork lengths were 24.7 ± 0.7 cm in the males ($n=9$) and 25.5 ± 0.4 cm in the females ($n=12$), and body weights were 166.4 ± 13.1 g in the males and 177.9 ± 7.4 g in the females. No significant effects on fork length and body weight were induced by GnRHa implantation in either sex.

Sexual maturation assessed by changes in GSI was also described in detail by Bhandari *et al.* (2003) and Jodo *et al.* (2005). In the males, GSI increased from April ($0.6 \pm 0.2\%$, $n=3$) to the pre-spawning period in August ($8.0 \pm 0.8\%$, $n=5$). In the females, GSI increased from May ($0.9 \pm 0.2\%$, $n=6$) and peaked in the spawning period in late September ($13.7 \pm 1.0\%$, $n=7$). Although no significant effects on the changes in GSI were induced by GnRHa implantation in the males, GnRHa increased the proportion of spermiating males in August (60% in the controls and 100% in the implants). In the females, GnRHa significantly increased GSI in August ($11.0 \pm 0.9\%$, $n=6$, $P<0.05$). However, it had no effect on ovulation time. Both the control and implanted females spawned in late September.

Changes in msGnRH-R mRNAs during sexual maturation

In the males, the changes in the amounts of the five subtypes of msGnRH-R mRNAs were similar during the

experimental period (Fig. 2A). The amounts of R4 and R5 mRNAs were 2–10 times those of the other subtypes. All subtypes of msGnRH-R mRNAs tended to be high in winter, and then gradually decreased toward June. The R2+R3 and R4 mRNAs, however, were likely to decrease in March. Thereafter, R1, R1-v, R4, and R5 mRNAs showed a transient peak in July. During the spawning period, all subtypes of mRNAs showed a similar upward trend. Because of the apparently synchronous expression of the five msGnRH-R

Table 2. Correlation coefficients between different subtypes of msGnRH-R mRNAs.

	mRNA	Male				
		R1	R1-v	R2+R3	R4	R5
Female	R1		0.816**	0.172	0.635**	0.635**
	R1-v	0.880**		0.243	0.540**	0.576**
	R2+R3	-0.077	-0.081		0.207	0.455**
	R4	0.497**	0.469**	-0.177		0.393**
	R5	0.299*	0.196	-0.158	0.423**	

*, $P<0.05$; **, $P<0.01$

genes, correlation between the different subtype mRNAs in single pituitaries were examined by calculation of correlation coefficients (Table 2). The amounts of msGnRH-R

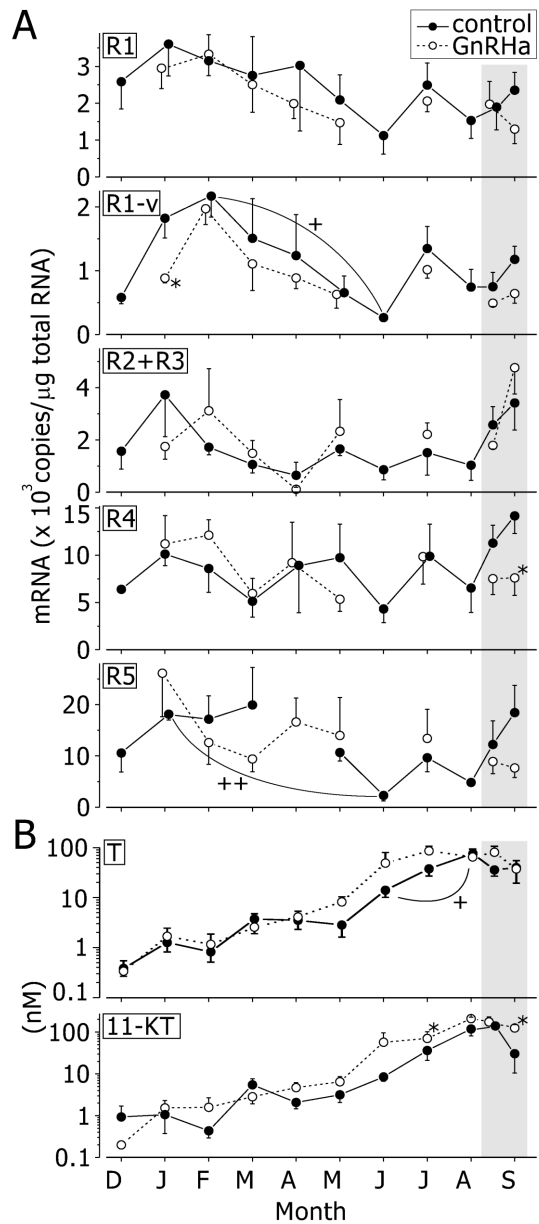


Fig. 2. Changes in (A) the amounts of msGnRH-R mRNAs, and (B) plasma levels of T and 11-KT in male masu salmon. Data are represented as mean±SEM. The amounts of msGnRH-R mRNAs in the GnRH-a implanted fish were not determined in December, June, and August. +, significant difference ($P<0.05$); ++, significant difference ($P<0.01$) among the months studied. *, significant difference ($P<0.05$) between control and implanted groups in the same month. The vertical gray bar indicates the spawning period.

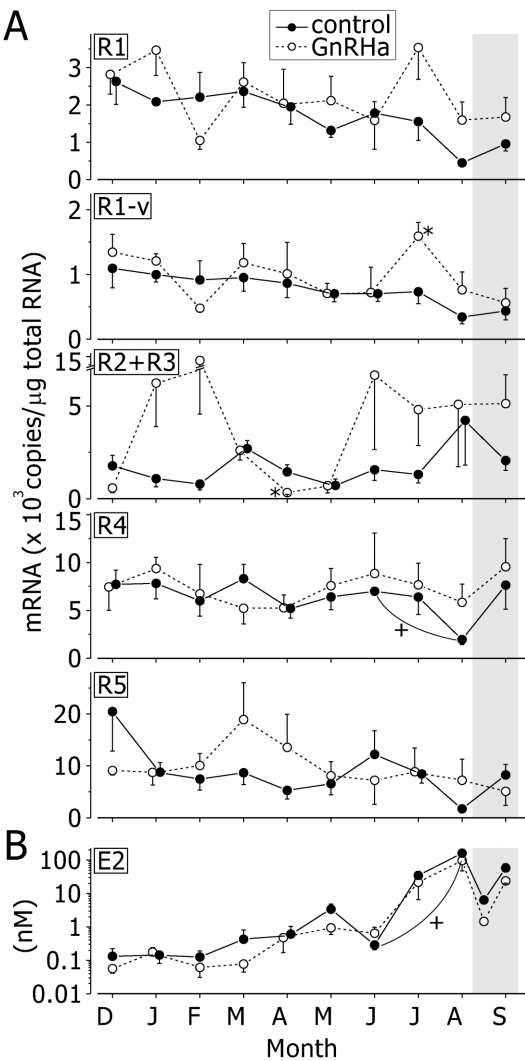


Fig. 3. Changes in (A) the amounts of msGnRH-R mRNAs, and (B) the plasma level of E2 in female masu salmon. Data are represented as mean±SEM. The amounts of msGnRH-R mRNAs in the control and GnRH-a implanted fish were not determined in early September. +, significant difference ($P<0.05$) among the months studied. *, significant difference ($P<0.05$) between control and implanted groups in the same month. The vertical gray bar indicates the spawning period.

except for R2+R3 showed weak to moderate correlations between different subtypes. A strong correlation was observed between R1 and R1-v mRNAs.

In the females, changes in the amounts of msGnRH-R mRNAs except for R2+R3 were similar during the experimental period (Fig. 3A). The amounts of R4 and R5 mRNAs were 2–5 times those of the other subtypes. The R1, R1-v, R4, and R5 mRNAs tended to decrease gradually from December 1998 toward August, when the levels reached their minimums. R4 and R5 mRNAs then increased in late September. The amounts of msGnRH-R mRNAs except for R2+R3 showed weak to moderate correlations between different subtypes (Table 2). A strong correlation was observed between R1 and R1-v mRNAs.

Correlation between msGnRH-R mRNAs and plasma steroid hormones

Plasma levels of T and 11-KT in the males increased gradually until June, increased rapidly to maximum levels in August, and then decreased in September (Fig. 2B). In the

females, plasma levels of E2 tended to increase gradually until June, and then increased sharply to their maximum levels in August, followed by a decrease in early September (Fig. 3B). To examine relevance of the peak level of E2 to the lowest amounts of msGnRH-R mRNAs, a correlation analysis was performed on data from June to September, when the plasma level of E2 varied considerably. Weak to moderate negative correlations were observed between the amounts of R1, R1-v, R4, and R5 mRNAs and the plasma level of E2 (Fig. 4). No significant correlation was observed between msGnRH-R mRNAs and plasma levels of T or 11-KT in the males (data not shown).

Effects of GnRHa on msGnRH-R mRNAs

The administration of GnRHa had almost no effects on the amounts of msGnRH-R mRNAs, except for R1-v in January and R4 in late September in the males. Similarly, it had no significant effects in the females, except for R2+R3 in April and R1-v in July.

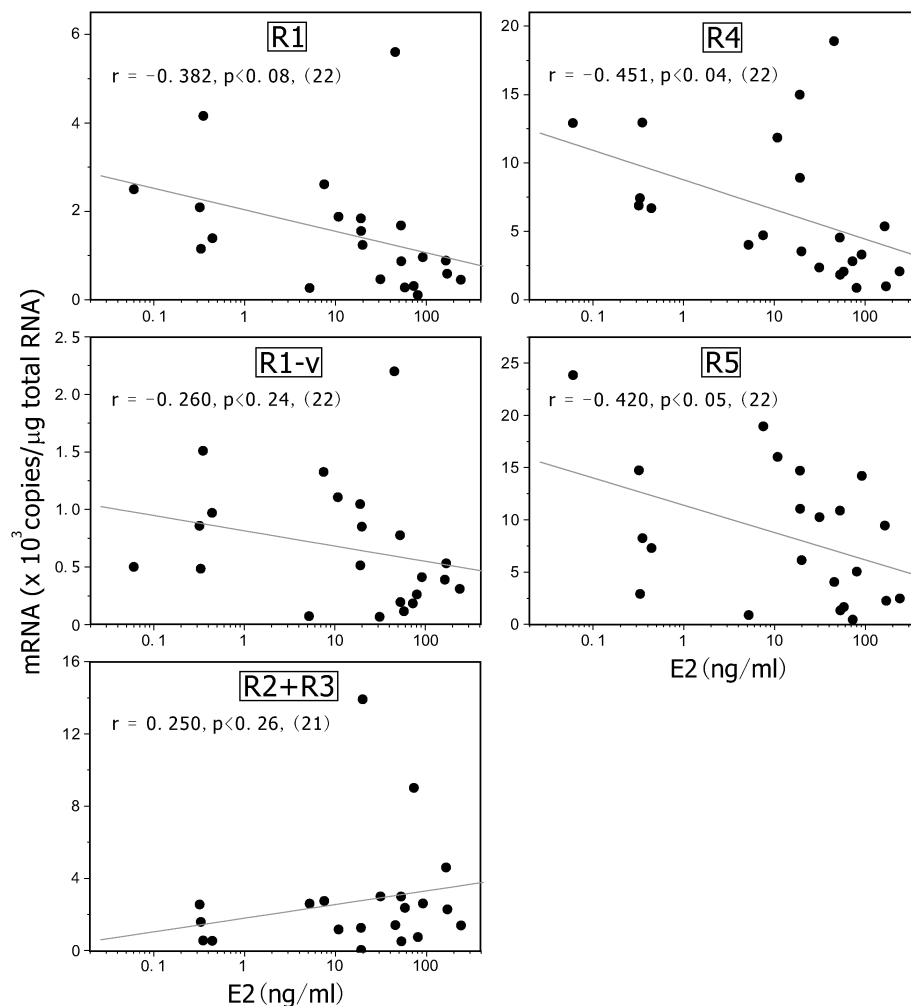


Fig. 4. Correlations between msGnRH-R mRNAs and the plasma level of E2 in the control female masu salmon. The scatter-plot distribution of data during sexual maturation from June to September is shown. The number of data points is shown in parentheses.

DISCUSSION

In the present study, seasonal changes in the amounts of five subtypes of msGnRH-R mRNA were examined in detail by real-time PCR. The subset types showed fairly similar changes from immaturity to spawning. These results exhibit a striking contrast to the pituitary of the same fish samples, in which the five msGnRH-R genes were differently expressed during the course of growth and sexual maturation. Most of them, however, increased considerably in the pre-spawning period, when GnRH stimulates LH subunit gene expression (Jodo *et al.*, 2005). In the brain, there were weak to moderate correlations among the different subtypes of msGnRH-R except R2+R3. Thus, expression of the multiple msGnRH-R genes except R2+R3 may be regulated in common in the masu salmon brain by a mechanism that is different from that in the pituitary.

The brain samples used in the present study contained the posterior half of the telencephalon, the diencephalon, and the mesencephalon including part of the optic tectum (Fig. 1). Several studies in teleosts have demonstrated the exclusive expression of the GnRH-R gene in the telencephalon, preoptic region, and basal hypothalamus (Madigou *et al.*, 2000; Peter *et al.*, 2003; González-Martínez *et al.*, 2004; Soga *et al.*, 2005). Furthermore, sGnRH-immunoreactive fibers densely innervate in these regions in masu salmon (Amano *et al.*, 1991). Thus, the changes in the amounts of msGnRH-Rs are thought to be connected to seasonal variations in the brain responsiveness to GnRH. Although it is of considerable importance to determine expression loci of msGnRH-Rs in the brain, the present results provide prerequisite information for understanding the molecular mechanisms of GnRH action in salmonids.

The present results clearly indicate that expression of the five GnRH-R genes is seasonally variable. The levels of msGnRH-R gene expression tended to decrease prior to spawning in both sexes, then showed upward trends in the spawning period, particularly in the males. This transient decrease in the expression of msGnRH-R genes during the pre-spawning period may act to reset responsiveness to GnRH, so that the latter can subsequently facilitate spawning behavior (Sato *et al.*, 1997; Kitahashi *et al.*, 1998). Indeed, González-Martínez *et al.* (2004) reported elevation of GnRH-R gene expression in the parvocellular preoptic nucleus during the spawning period of the European sea bass. Therefore, our results suggest that the changes in expression of msGnRH-R genes in the masu salmon brain during the pre-spawning and spawning periods result in sensitizing GnRH target neurons to respond more robustly to GnRH stimulation of spawning behavior, although the target neurons need to be determined.

The similar expression pattern of the R1, R4, and R5 genes, with dominant expression of R4 and R5, suggests that they are regulated by a common mechanism and have similar roles in the action of GnRH, such as stimulation of spawning behavior. All these receptors are type I receptors

(Millar *et al.*, 2004), but are divided into two subtypes; one subtype (type Ia) includes R1, R2, and R3 and the other (type Ib) includes R4 and R5 (Jodo *et al.*, 2003). The two subtypes may have arisen from a genomic duplication. They share high identity of nucleotide sequences (59–81%) from the 5' untranslated region to the third transmembrane domain. Although their biochemical properties need to be determined, the multiple msGnRH-Rs in the masu salmon brain may mediate sGnRH signals, so that the stimulatory effects of sGnRH on spawning behavior are strengthened. In contrast, the multiple msGnRH-Rs were differently expressed in the pituitary during growth and sexual maturation, and they may play different roles in the action of GnRH (Jodo *et al.*, 2005). Recently, another type of GnRH-R that is a type III receptor was identified in rainbow trout (Lethimonier *et al.*, 2004). There is probably also a type III GnRH-R in masu salmon. It is of considerable interest to determine if the sixth isoform of GnRH-R is expressed differently during sexual maturation.

The present results suggest that E2 is involved in the regulation of msGnRH-R gene expression in females. In mammals, both positive and negative effects of sex steroids on GnRH-R gene expression have been reported in the pituitary (Kakar *et al.*, 2002). E2 reduces GnRH-R mRNA *in vivo* in rats (Kaiser *et al.*, 1993; Kakar *et al.*, 1994), whereas it increases GnRH-R mRNA in sheep (Turzillo *et al.*, 1994; Adams *et al.*, 1996). On the other hand, much less is known about regulation of GnRH-R genes by steroid hormones in the brain. In the rat hippocampus, gonadectomy increased the amount of GnRH-R mRNA (Jennes *et al.*, 1995). In a teleost, the catfish, sex steroids decreased GnRH binding activity in the pituitary (Habibi *et al.*, 1989). It is thus possible that E2 has a negative effect on GnRH-R genes, and the low amounts of msGnRH-R mRNAs in August are probably due to the peak level of E2. However, at present, we cannot exclude the possibility that both changes are independent or indirectly related during sexual maturation. A possible role of E2 in regulation of msGnRH-R gene expression needs to be further examined.

In addition to the increase in the spawning period, the amounts of msGnRH-R mRNAs tended to be high from winter through spring. In this period, sGnRH content and sGnRH mRNA also remained at high levels in the brain (Ando *et al.*, 2001), suggesting that sGnRH has a physiological role in this prepubertal stage, such as stimulation of neurosecretory cells (Oka, 2002; Saito *et al.*, 2003).

It is generally acknowledged that GnRH acts as a positive regulator of its own receptor. However, GnRHa treatment did not induce any apparent change in the expression of msGnRH-R genes in most seasons. In contrast, GnRHa treatment significantly increased the levels of msGnRH-R mRNAs in the pituitary of the same fish used in the present study (Jodo *et al.*, 2005). The ineffectiveness of GnRHa in the brain may not be the result of impermeability of the blood-brain barrier (BBB) to peripherally administered GnRHa, because GnRH is known to cross the BBB by a sat-

urable transport mechanism (Barrera *et al.*, 1991). In fact, intraperitoneal injection of GnRH α decreased GnRH-R gene expression in the preoptic area of female rat (Roth *et al.*, 2001). However, study of the effects of central administration of GnRH α will be required to further define the role of GnRH on msGnRH-R gene expression in the brain.

In conclusion, the expression of the msGnRH-R1, -R4, and -R5 genes changed similarly from immaturity to spawning. They tended to decrease prior to the spawning period and then showed upward trends at spawning in both sexes. These changes may be related to a stimulatory role of GnRH in reproductive behavior. Furthermore, E2 may be involved in msGnRH-R gene expression in the brain of female masu salmon during sexual maturation.

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