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Variations of Gonadotropin Subunits mRNA Levels at Different Stages of Ovarian Development in Masu Salmon, *Oncorhynchus masou.*

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The variations of gene expression and pituitary contents of GTH subunits during the ovarian development of masu salmon, *Oncorhynchus masou*, were investigated. The pituitary GTHs contents was measured by radioimmunoassays (RIAs) using purified GTH subunits and their antibodies. Pituitary contents of GTH Iβ gradually increased from April through August, and reached the maximum in October. On the other hand, pituitary contents of GTH IIβ remained low until August, but they rapidly increased in October. Total RNAs were prepared from pooled pituitaries and the GTH subunits mRNA in pituitaries was quantified by Northern blot hybridization using masu salmon cDNA probes for each GTH subunit. GTH Iβ mRNA level increased with the progression of ovarian maturity. However, GTH IIβ mRNA was detected only at a more advanced stage, and were extremly high at ovulation. A high levels for GTH α mRNA was detected only at ovulation stage. The synchronous increase in pituitary contents and mRNA levels suggested that ovarian maturity in masu salmon was regulated by both GTH I and GTH II.

Key words: masu salmon, ovarian development, GTH subunits mRNA, RIA, Northern blot analysis

Introduction

Control of gonadal function by pituitary gonadotropins (GTHs) is a general feature of vertebrate reproduction. In most tetrapods, gonadal function has long been known to be regulated by two GTHs: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Licht et al., 1977). FSH, LH, and a third pituitary hormone, thyroid-stimulating hormone (TSH), are chemically related. All three consist of glycosylated α and β subunit, which interact noncovalently. In mammals it has been found that the α subunits of TSH, LH, and FSH are identical within a species, whereas the β subunits are hormone specific and structurally conserved between species (Pierce and Parsons, 1981).

The question of whether fish reproduction is regulated by one or two pituitary GTHs has been controversial for nearly two decades. Recently, two pituitary GTHs, GTH I and GTH II, which are distinctly different from each other in chemical characteristics and structurally homologous to tetrapod FSH and LH, have been isolated from chum salmon (*Oncorhynchus keta*) (Suzuki et al., 1988a, b) and coho salmon (*O. kisutch*) (Swanson et al., 1991). In salmonid species, GTH I and GTH II appear to have similar steroidogenic activities when tested *in vitro*. The blood and pituitary levels of these two GTHs vary significantly during reproductive development. GTH I was predominant in the plasma and pituitary of vitellogenic/spermatogenic, whereas GTH II was predominant at the time of final reproductive maturation (Suzuki et al., 1988c; Swanson et al., 1989; Prat et al., 1996).

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However, the physiological distinction between GTH I and GTH II in fish is not as clear as that of FSH and LH in tetrapods.

Recently, GTHβ subunit mRNA levels were monitored during the reproductive cycles in rainbow trout, *Oncorhynchus mykiss*: GTH Iβ gene expression was predominant during the early gonadal development stage, whereas GTH IIβ gene expression increased during the spawning period (Naito et al., 1991; Weil et al., 1995). However, both GTH Iβ and GTH IIβ subunit gene expression in gilthead seabream (*Sparus aurata*) and goldfish (*Carassius auratus*) were high at the time of spawning (Elizur et al., 1996; Sohn et al., 1999). The variable patterns of GTH subunit expression in teleosts may be responsible for the different reproductive strategies as well as their high fecundity.

The objective of this study was to obtain basic information on GTH subunit synthesis using Northern blot analysis coupled with laser densitometry and radioimmunoassay during the ovarian development of masu salmon, *Oncorhynchus masou*, reared in fresh water.

Materials and Methods

Fish

Two-year old masu salmons (*Oncorhynchus masou*) at different stages of ovarian development were used in this study. The fish were reared at the National Research Institute of Aquaculture (Japan), Nikko, Tochigi prefecture under natural conditions. Body weight of female fish were 84.5 ± 5.4 g (April), 83.6 ± 5.4 g (June), 138 ± 4.5 g (August) and 152 ± 4.1 g (October). Ovaries were fixed with Bouin's fluid for 24 hr and their weights were measured to calculate a gonadosomatic index (GSI; gonad weight/body weight×100). To observe the stages of ovarian development, the ovaries were embedded in paraffin and sections of 5 μm were prepared. The sections were stained with hematoxylin and eosin for histological observation (data not shown).

GTH radioimmunoassays (RIAs)

Extraction of GTH from the pituitary gland was done according to Amano et al. (1992). Chum salmon GTH Iβ and IIβ and antisera against GTH Iβ and IIβ were kindly provided by Dr. H. Kawauchi of Kitasato University. GTH Iβ and IIβ were iodinated according to the methods of Kim (1997). The procedure of each RIA was the same as that in the salmon GTH RIA (Kim, 1997). Displacement curves for pituitary samples were parallel to the standard curves in both GTH Iβ and IIβ RIA (Amano et al., 1992). The antiserum against GTH Iβ cross-reacted with GTH I, GTH II and GTH IIβ at 1.7%, 4.0% and 4.4%, respectively, at 50% binding. The antiserum against GTH IIβ was found to cross-react with GTH I, GTH II and GTH Iβ at 0.22%, 3.7% and 1.0%, respectively, at 50% binding.

RNA preparation and Northern blot analysis

Fish were rapidly anesthetized in 2-phenoxyethanol (0.5 mℓ/ℓ). Pituitary glands were removed and frozen immediately by immersion in liquid nitrogen. Frozen pituitary glands were pooled into two tubes, and stored at −80℃ until required for use. RNA extraction and Northern blot analysis were performed as described previously by Yoshiura et al. (1997). Total RNA was extracted from the pooled pituitaries with RNA extraction solution, hyIIsogen (Nippon Gene). Extracted total RNA was about 8.7 μg/pituitary (April), 11.5 μg/pituitary (June), 13 μg/pituitary (August) and 16.2 μg/pituitary (October), respectively. The total RNAs (each 5 μg for α, Iβ and IIβ) were denatured at 65℃ for 15 min in 50% formamide and subjected to electrophoresis on a 0.9% agarose gel in 0.2% MOPS, pH 7.0, containing 2.2 M formamide, 0.05 M sodium acetate, and 5 mM EDTA, then transfered to Hybond N^+ Nylon membrane (Amersham Corp.). The membranes were air-dried and baked at 80℃ for 15 min prior to hybridized with randomly labeled identical [^{32}P] dCTP cDNA probes encoding masu salmon pituitary GTH α, Iβ and IIβ. The masu salmon cDNA probes for α, Iβ and IIβ were provided by Dr. K. Gen (National Research Institute of Aquaculture, Japan). The membranes were washed at room temperature for 15 min in 2X SSC and at 65℃ for 15 min in 1X SSC containing 0.5% SDS. The hybridized membranes were scanned by a Fujix BAS 1000 Mac Bio-Imaging Analyzer (Fuji Film, Tokyo, Japan) to count the hybridization signals. The quantified α, Iβ, IIβ mRNA levels were represented with respect to total RNA.

Statistics

Data were analyzed for significance ($P<0.05$) using one-way ANOVA and Duncan's new multiple range test.

Results

GSI

Changes in GSI are shown in Fig. 1A. GSI rapidly increased from April (0.5%) through October (12.8%), in accordance with vitellogenesis and ovulation. Ovulation was observed in October.

Changes in pituitary GTH Iβ and GTH IIβ contents

Changes in pituitary GTH Iβ and GTH IIβ content (μg/pituitary) are shown in Fig. 1B. Pituitary contents of GTH Iβ measured by RIA gradually increased from April through August, and reached the maximum in October. Pituitary contents of GTH IIβ remained low until August, but they rapidly increased in October.

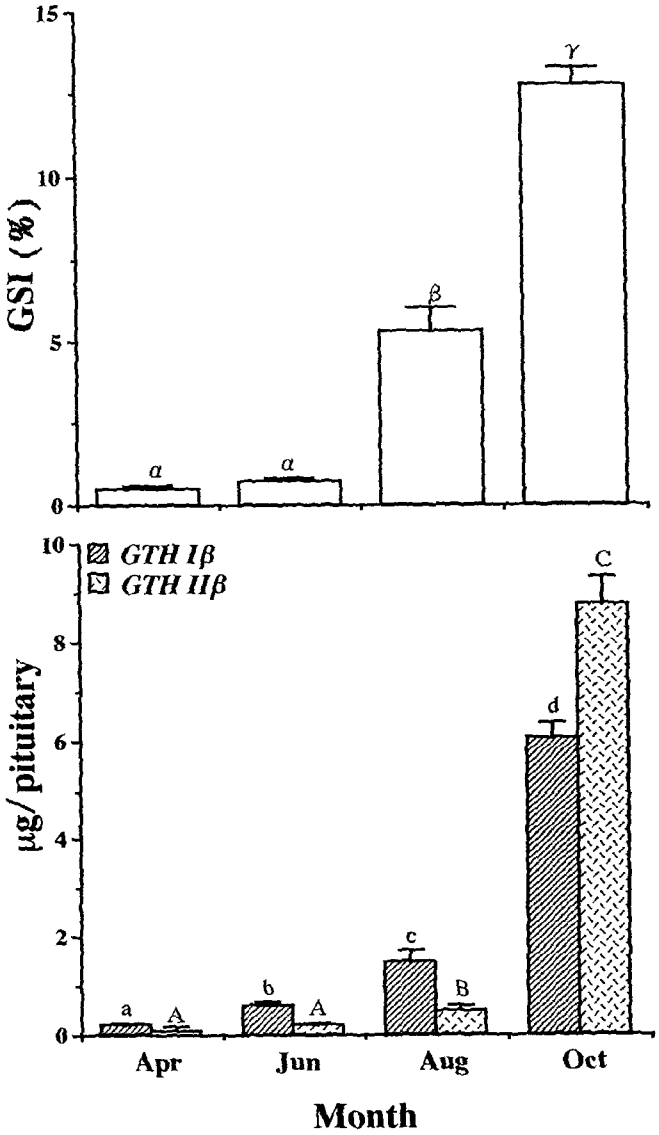

Fig. 1. Seasonal changes in GSI (A) and pituitary GTHs contents (B) of the female masu salmon from April to October. A significant difference ($p<0.05$) was observed between columns to indicated by different letters.

Changes in α, GTH Iβ and GTH IIβ mRNA levels

In order to examine the pituitary GTH α, GTH Iβ and GTH IIβ mRNA levels in masu salmon of differing maturity, we carried out Northern blot hybridization with the respective masu salmon cDNA probes (Fig. 2). The quantitative changes in mRNA encoding for GTH α, GTH Iβ and GTH IIβ with the ovarian development are shown in Fig. 3. Although GTH Iβ mRNA levels increased with the progression of ovarian maturity, GTH IIβ mRNA were observed only at a more advanced stage (end of vitellogenesis: August), and were extremly high at ovulation (October). A high levels for GTH α mRNA were detected only at the ovulation stage.

Discussion

The variations of pituitary GTHs contents were examined by radioimmunoassays (RIAs) using purified GTH subunits and their antibodies, as well as quantifies the GTH subunits mRNA in pituitaries by the Northern blot analysis coupled with laser densitometry using masu salmon cDNA probes for each GTH subunit during ovarian development in masu salmon, *Oncorhynchus masou*.

GTH Iβ and GTH IIβ contents increased with

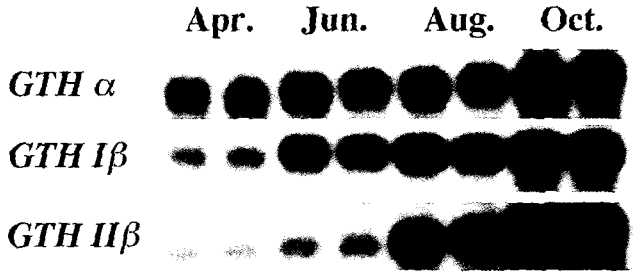

Fig. 2. Representative results of Northern blot hybridization of GTH α, Iβ and IIβ subunit mRNA of pituitary gland in masu salmon. Pituitary total RNA was extracted, and approximately 5 μg was loaded per lane. The quantified data are shown in fig. 3. The mRNA levels were measured by Northern blot analysis and quantified using a computerized densitometer, Fujix BAS 1000 Bio-Imaging Analyzer (Fuji Film, Tokyo, Japan).

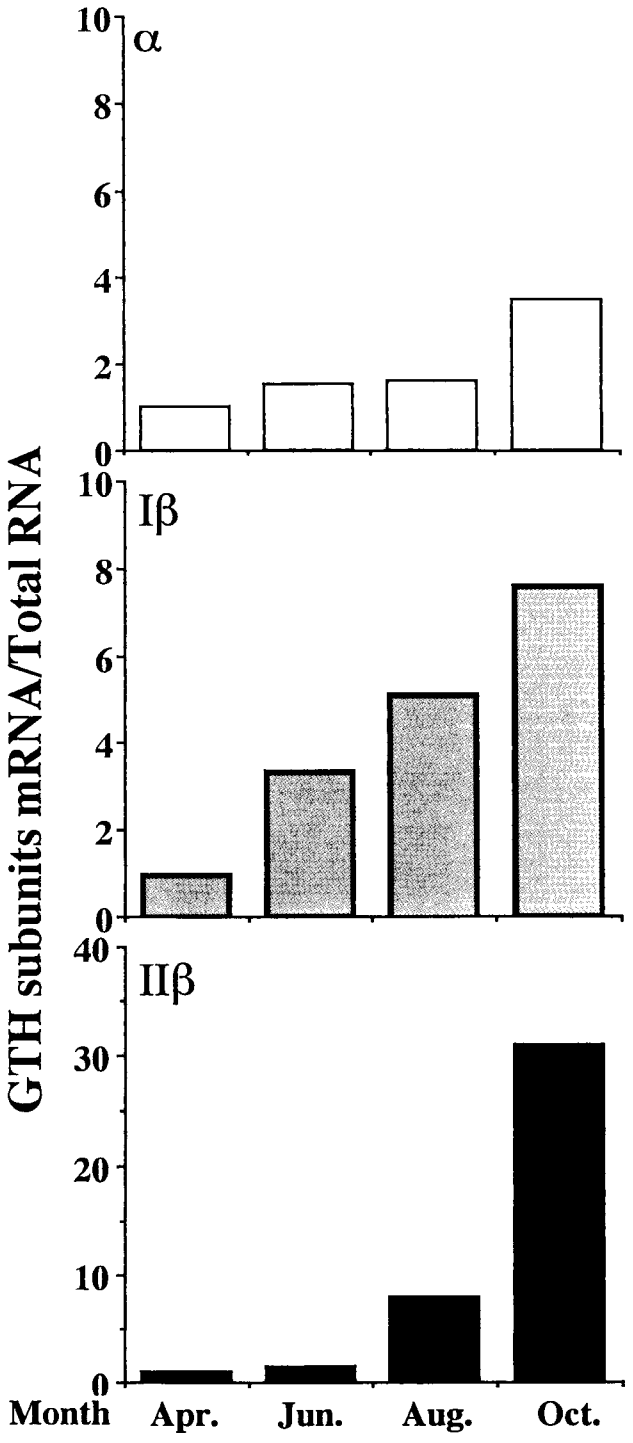

Fig. 3. Seasonal changes in relative mRNA levels of the α subunit of GTH and the β subunits of GTH I and GTH II in female masu salmon. The mRNA levels were measured by Northern blot analysis and quantified using a computerized densitometer, Fujix BAS 1000 Bio-Imaging Analyzer (Fuji Film). The quantified mRNA levels were standardized by pituitary total RNA and given a value of 1 to the levels of samples of April.

the progression of ovarian maturity in masu salmon. Two types of GTH I (stable and unstable) are known to exist in chum salmon pituitary (Suzuki et al., 1988 a, b). Unstable GTH I is considered to be dissociated to its α and β subunits under acidic conditions. Therefore, changes in GTH Iβ may reflect those of unstable GTH I. In the present study, however, GTH I (stable GTH I) contents were not measured by RIA. Amano et al. (1992) reported that pituitary GTH Iβ contents were high in underyearling and yearling autumn, but the complete form of GTH I may not be produced or secreted during the first two years in masu salmon. Further, they reported that pituitary GTH Iβ contents showed clear seasonal changes, high in autumn and low in winter, regardless of the state of ovarian maturity.

The changes in pituitary GTH IIβ contents were similar to those of GSI: rapid increases occurred in accordance with the end of vitellogenesis and ovulation. Changes in GTH IIβ may reflect those of GTH II, since GTH II is considered to dissociate under acidic conditions. In rainbow trout, pituitary GTH II levels rose dramatically, and reached the peak at the beginning of spawning, but were extremely low in immature fish (Sumpter and Scott, 1989). Pituitary GTH IIβ contents in masu salmon also showed clear seasonal fluctuations i.e., high in autumn and low in winter. These results were agree with some features of the seasonal pituitary GTHs contents reported by other authors (Sumpter and Scott, 1989; Amano et al., 1992). Therefore, these changes might be related to ovarian maturation, since pituitary GTH Iβ and IIβ contents and GSI also gradually increased in this period.

In the Northern blot hybridization analyses of total RNA preparations of masu salmon, GTH Iβ mRNA strongly expressed with the progression of ovarian maturity. In rainbow trout, however, GTH Iβ predominated in the early stage of ovarian development (previtellogenesis), with GTH IIβ being weakly expressed (Naito et al., 1991; Weil et al., 1995). The patterns of masu salmon GTH Iβ gene expression were different from those of rainbow trout. This may reflect the difference in reproductive strategy between masu salmon and rainbow trout. Masu salmon spawn only once and then die, whereas rainbow trout may spawn annually over a number of years, so that at the time of ovulation the ovary contains not only fully matured oocytes but also primary oocytes that have already begun growth (Lou et al., 1984; Prat et al., 1996).

The patterns of masu salmon GTH α and GTH IIβ gene expression were in accord with results previously reported for GTH α and GTH IIβ gene expression for female rainbow trout (Naito et al., 1991; Weil et al., 1995). A slight increase in GTH IIβ mRNA levels was observed at the end of the vitellogenesis stage in masu salmon. This increase in GTH IIβ mRNA levels might be induced by the increased estradiol-17β production observed at this stage (Amano et al., 1995). Indeed, a positive effect of testosterone or estradiol-17β *in vivo* treatments on GTH IIβ mRNA levels was observed in immature fish (Trinh et al., 1986; Querat et al., 19 91). Plasma estradiol-17β levels decrease from the end of vitellogenesis to ovulation (Amano et al., 19 95) but GTH α and GTH IIβ mRNA levels remain high. These levels could be due to the presence at these stages of elevated synthesis and release of endogenous GnRH (Amano et al., 1992), since exogenous GnRH has recently been shown to increase GTH α and GTH IIβ mRNA levels (data not shown) in testosterone-treated immature rainbow trout.

In summary, mRNA levels and pituitary contents of GTH subunits in masu salmon synchronously increased with the progression of ovarian maturity. These results suggested that both GTH I and GTH II were involved in the regulation of ovarian development in masu salmon, although the biological functions of the two masu salmon GTHs remained to be elucidated.

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