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Changes in Salmon GnRH and Chicken GnRH-II Contents in the Brain and Pituitary, and GTH Contents in the Pituitary in Female Masu Salmon, *Oncorhynchus masou*, from Hatching through Ovulation

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ABSTRACT—Changes in salmon gonadotropin-releasing hormone (sGnRH) and chicken GnRH-II (cGnRH-II) contents in the brain and pituitary, and gonadotropin (GTH) subunits GTH Iβ and GTH IIβ contents in the pituitary of female masu salmon (*Oncorhynchus masou*) were investigated from hatching through ovulation. Gonadosomatic index (GSI) showed a gradual increase until the second summer (two year-olds), and thereafter a rapid increase was observed in accordance with vitellogenesis. Ovulation occurred in autumn. Brain sGnRH was already measurable at hatching, whereas cGnRH-II was first detected two months later. Both GnRHs contents increased during the underyearling phase, and fluctuated thereafter. Pituitary sGnRH contents showed a stepwise increase every summer for three years. sGnRH concentrations in each discrete brain area showed seasonal changes: high during autumn-winter and low in summer. sGnRH concentrations in the olfactory bulbs and telencephalon, and pituitary contents of sGnRH, GTH Iβ and GTH IIβ significantly increased prior to ovulation. Pituitary GTH Iβ contents showed clear seasonal changes for three years-high in autumn and low in winter-regardless of the state of ovarian maturity. Brain cGnRH-II contents were lower than sGnRH contents. Moreover, pituitary cGnRH-II contents were undetectable and no significant changes in concentration in discrete brain areas were observed during vitellogenesis and ovulation. These results suggest that sGnRH is involved in ovarian maturation via the regulation of GTH synthesis and release in this species, whereas cGnRH-II has little or no involvement in reproduction.

INTRODUCTION

Recent studies have shown that more than one type of GnRH exists in the teleost brain [1]. In most teleost species examined including salmonids, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II) have been detected. It is generally accepted that one of the important roles of GnRH is the regulation of the synthesis and release of GTH by the GTH cells in the pituitary. Although both sGnRH and cGnRH-II molecules stimulate the release of GTH in the fish pituitary both *in vivo* and *in vitro* under exogenous administration [2, 3], their physiological roles in the brain and pituitary are not fully understood. These two types of GnRHs exist not only in the hypothalamus but also in the other parts of the brain, and show differential distributions [4, 5]. Furthermore, distribution patterns of both GnRHs are vary according to species. Both sGnRH and cGnRH-II exist in the goldfish pituitary [4], but only sGnRH is detectable in rainbow trout *Oncorhynchus mykiss* pituitary [5].

There are few studies on the changes in brain GnRH contents in relation to gonadal maturation, and such results are not consistent. Gentile *et al.* [6] measured GnRH concentrations in the tel-

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encephalon and hypothalamus of Venezuelan freshwater fish, *Pygocentrus notatus*, using a mammalian GnRH RIA system and found that GnRH concentrations were high in mature fish. Yu *et al.* [7] measured GnRH content in discrete brain areas of female goldfish at different stages of ovarian development. However, brain GnRH content did not show clear parallel changes with seasonal ovarian development. Okuzawa *et al.* [5] measured sGnRH and cGnRH-II contents in discrete brain areas of rainbow trout sampled in September, and found that the pattern of sGnRH distribution differed with age and stage of sexual maturity.

Recently, Suzuki *et al.* [8] reported that two structurally different GTHs, GTH I and GTH II, exist in the chum salmon pituitary. GTH I is considered to be involved in regulation of the early stages of gonadal maturation, and GTH II is considered to mainly regulate ovulation and spermiation [9]. There is, however, no information on the changes in pituitary GTH I and GTH II contents throughout the fishes' life span. Since the release of GTH is regulated by GnRH, it is speculated that changes in pituitary GTH contents are correlated with those in brain GnRH contents.

Therefore, in the present study, we investigated changes in sGnRH and cGnRH-II contents in the brain and pituitary, and GTH Iβ and IIβ subunit contents in the pituitary of female masu salmon (*Oncorhynchus masou*) which were maintained in fresh water for three years. Changes were followed from hatching through ovulation, in order to obtain basic information on annual rhythms of these hormones.

MATERIALS AND METHODS

Fish

For purposes of this study, eggs of masu salmon, *Oncorhynchus masou*, were artificially fertilized in October 1987 at the Nikko Branch, National Research Institute of Aquaculture, Tochigi Prefecture. The eggs hatched in December 1987, and the fish were reared under natural photoperiod in spring water of constant temperature (9–10°C) throughout the experiment. The fish we used for this study were offspring of wild fish which had migrated to the Shiribetsu River (Hokkaido). Wild masu salmon migrate to the sea in the spring (1.5 years-old), and return to the river in May after a one-year stay in the sea; they spawn in autumn and die. The masu salmon kept in the institute also smoltified at 1.5 years-old and matured at three years of age in fresh water, although the growth rate was not very rapid. There is a landlocked form of masu salmon called "yamame". This variety for the most part does not smoltify.

Sampling

Sampling was carried out once a month (twice in May 1988) from December 1987 (month of hatching) through October 1988, and at 2–4 months intervals from January 1989 through October 1990 (month of ovulation). On the day of autopsy, fish were randomly selected and were anesthetized in ethyl-p-aminobenzoate (0.05%). After measurements on body length and weight, brain and pituitary were rapidly removed and frozen on dry-ice intended for the measurement of GnRHs and GTHs. At the first two samplings (December 1987 and January 1988, mean body weight 0.16–0.39 g), the head region was cut off and used for only GnRH measurement as dissection of the brain was difficult. The pituitary was removed on sampling occasions starting from April 1988 (mean body weight 3.21 g). From September 1988 (mean body weight 17.2 g), brain tissue was dissected into five parts: the olfactory bulb, the telencephalon, the hypothalamus, the optic tectum-thalamus and the

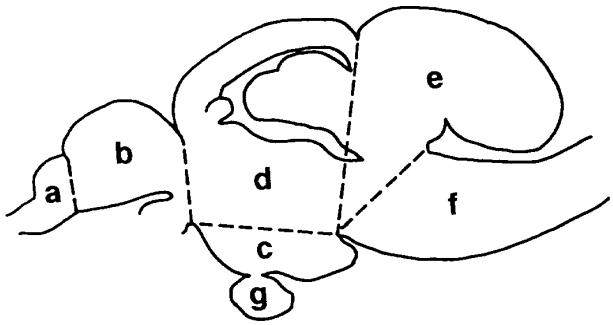


FIG. 1. Schematic diagram of sagittal section of masu salmon brain. Letters represent the following brain areas: a, olfactory bulbs and tracts; b, telencephalon, including preoptic area; c, hypothalamus; d, optic tectum-thalamus, including anterior part of cerebellum; e, cerebellum; f, medulla oblongata; g, pituitary.

cerebellum-medulla oblongata. The cerebellum and medulla oblongata were further differentiated from May 1989 (mean body weight 34.5 g) as shown in Fig. 1. Brain tissues were stored at −30°C until extraction. At the first two samplings, distinction of sex was impossible. From February 1988 (mean body weight 1.35 g), ovaries were fixed with Bouin's fluid for 24 hr and their weights were measured to calculate GSI. The ovaries were embedded in paraffin and sectioned at 5 μm. The sections were stained with hematoxylin and eosin for histological observation. The results pertaining to males will be reported separately. From September 1988, blood was also sampled in order to measure plasma GTH and steroid hormone levels. These data will also be reported elsewhere.

GnRH RIAs

Extraction of GnRH from the brain tissue was done according to Okuzawa *et al.* [5]. sGnRH and cGnRH-II contents were measured by respective RIAs established by Okuzawa *et al.* [5]. Prior to the measurement of sGnRH and cGnRH-II contents, it was confirmed that masu salmon possess both GnRHs in the brain (Fig. 2), by HPLC-RIA analysis according to Okuzawa *et al.* [5]. Moreover, brain extracts of this species showed displacement curves which were parallel to the curves for the sGnRH and cGnRH-II standards in each RIA (Fig. 3). These sGnRH and cGnRH-II RIA were thus validated for application to masu salmon. GnRH was expressed in terms of both content (per region) and concentration (per g tissue).

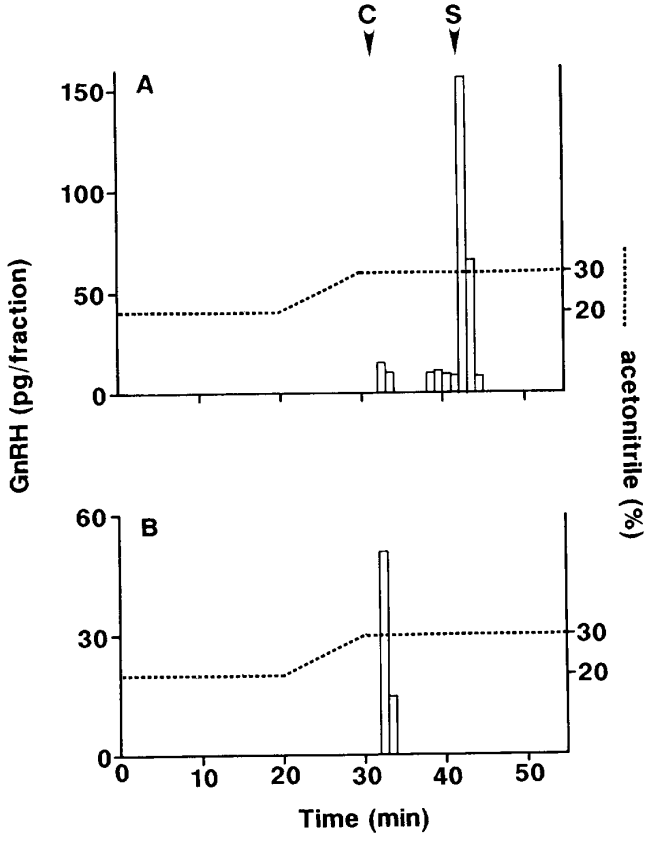


FIG. 2. Reverse-phase HPLC of masu salmon brain extract followed by sGnRH RIA (A) and cGnRH-II RIA (B). Arrows indicate the elution time of synthetic cGnRH-II and sGnRH. The mobile phase was CH_3CN (acetonitrile) containing 0.1% TFA.

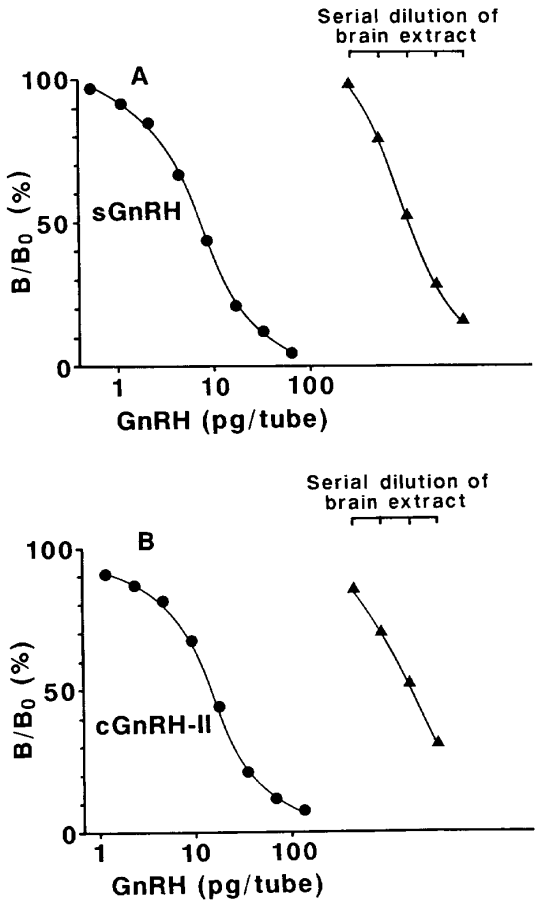


FIG. 3. Competition curves for sGnRH and brain extract of masu salmon in sGnRH RIA system (A), and cGnRH-II and brain extract of masu salmon in cGnRH-II RIA system (B). The scale for dilution of brain extract indicates a two-fold serial dilution. Each point represents the average of duplicate determinations.

GTH RIAs

Pituitary contents of GTHs were measured by two different RIAs using the same samples used

for GnRH measurement. GTH Iβ and GTH IIβ and antisera against GTH Iβ and GTH IIβ were kindly provided by Dr. H. Kawauchi of Kitasato University. GTH Iβ and GTH IIβ were measured by respective RIAs. GTH Iβ and GTH IIβ were purified from chum salmon (*Oncorhynchus keta*) pituitary by Suzuki *et al.* [10], and the antisera against GTH Iβ and GTH IIβ were raised by Suzuki *et al.* [9]. GTH Iβ and GTH IIβ were iodinated according to the method of Kobayashi *et al.* [11]. The procedure of each RIA was the same as that in the sGTH RIA [11]. Displacement curves for pituitary samples were parallel to the standard curves in both GTH Iβ and GTH IIβ RIA. The intra- and inter-assay coefficients of variation in GTH Iβ RIA were 10.9% (n=4) and 23.0% (n=4), respectively, at about 50% binding. The sensitivity of the assay, defined as twice the standard deviation at zero dose, was 62.5 pg/tube (n=5). The antiserm 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 intra- and inter-assay coefficients of variation in GTH IIβ RIA were 12.0% (n=4) and 11.7% (n=4), respectively. Sensitivity was 5 pg/tube (n=8). 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.

Statistics

The Student's *t*-test and Cochran-Cox test were employed in statistical analysis.

RESULTS

GSI

Changes in body weight and GSI are shown in Fig. 4. GSI gradually increased from 0.18% (February 1988) to 0.52% (May 1990), showing a small peak in September 1989. GSI rapidly increased from July (0.73%) through October 1990 (12.6%), in accordance with the advancement of vitellogenesis and ovulation. Ovulation was observed in 9 out of 12 individuals in October 1990.

Pituitary GTH contents

Changes in pituitary GTH Iβ and GTH IIβ

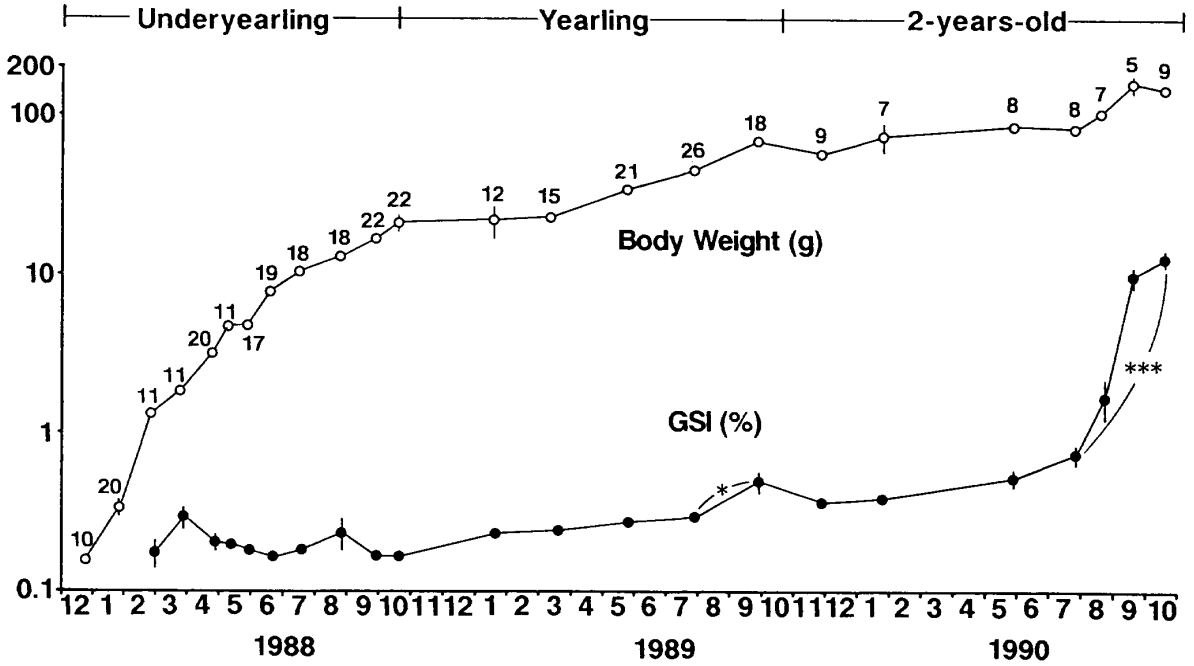


FIG. 4. Seasonal changes in body weight and gonadosomatic index (GSI) of masu salmon from February 1988 to October 1990. Numbers beside each symbol indicate the number of fish employed. Each value is expressed as the mean (point) and the standard error (bar). * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$) indicate the levels of significant differences.

contents are shown in Fig. 5A. Pituitary GTH Iβ contents showed clear seasonal changes: high in autumn after increases from spring, and low in winter. A distinct decrease was seen in January 1989 and a distinct increase was observed during vitellogenesis and ovulation in 1990.

GTH IIβ showed a gradual increase until July 1990. Thereafter the contents increased rapidly until October 1990, in accordance with the advancement of vitellogenesis and ovulation.

Changes in pituitary GTH Iβ and GTH IIβ concentrations were similar to those in contents

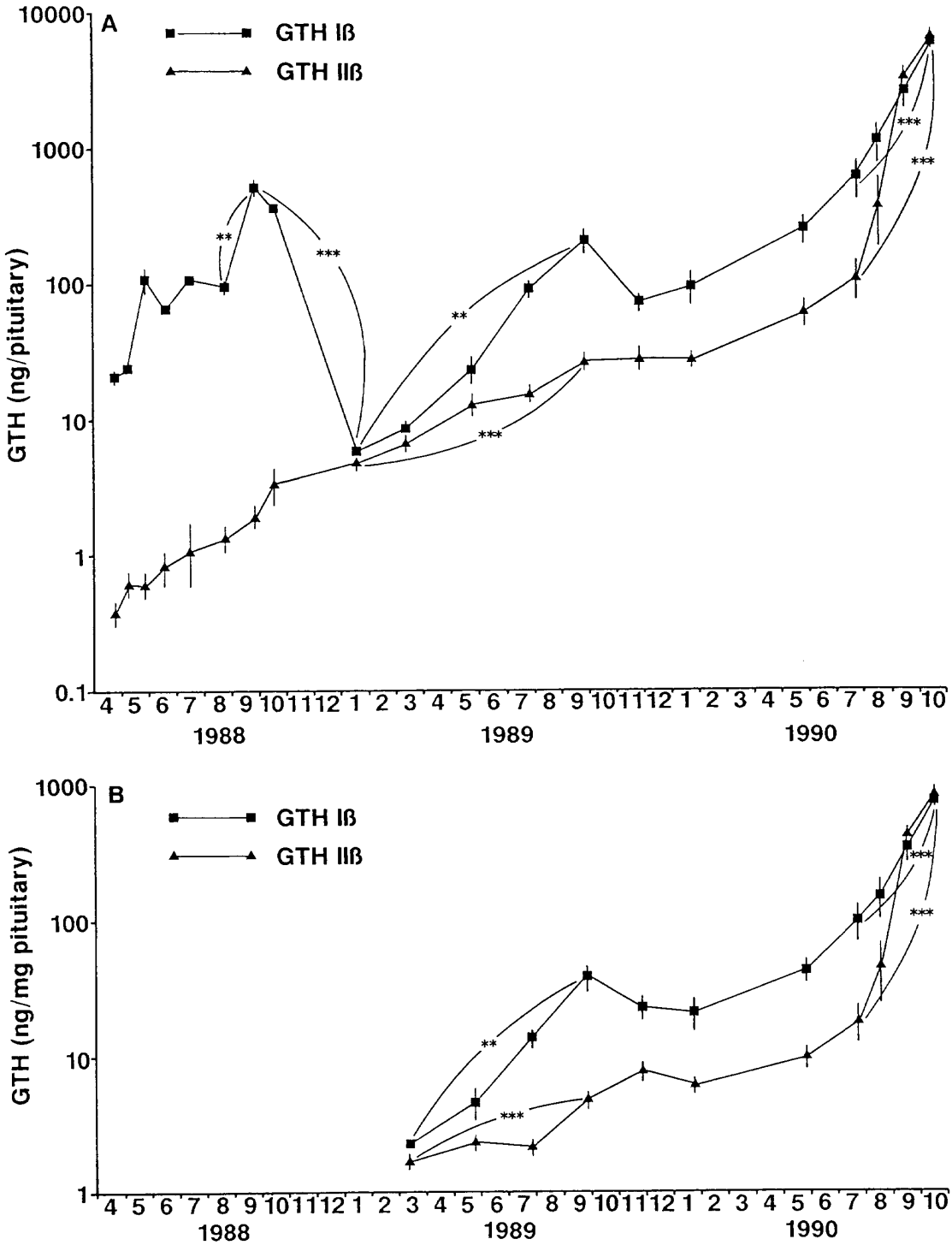


FIG. 5A and B. Seasonal changes in pituitary GTH Iβ (square) and GTH IIβ (triangle) contents (A) and concentrations (B) of the masu salmon from April 1988 to October 1990 and from March 1989 to October 1990, respectively. Presentation of statistical data is as in Fig. 4.

(Fig. 5B).

sGnRH in the brain and pituitary

Brain sGnRH was already detectable at hatching, and total brain content increased with growth during the underyearling stage (Fig. 6A). Thereafter, increases were observed from March to May 1989 and from September to January 1990. A decrease was observed from May to September 1989.

Changes in brain sGnRH concentrations are shown in Fig. 6B. A significant decrease was observed from April to August 1988, and a significant increase was seen in September 1988. Thereafter, concentrations decreased until September 1989, increased again until January 1990, and then decreased until July 1990.

sGnRH concentrations in the discrete brain areas are shown in Figs. 7A-E. They showed a tendency to decrease from winter through summer and to increase from autumn through winter. sGnRH concentrations in the olfactory bulbs and telencephalon increased significantly during vitellogenesis and ovulation. sGnRH concentrations in the hypothalamus also showed a similar tendency. On the contrary, no significant changes were seen in the cerebellum and medulla oblongata during vitellogenesis and ovulation.

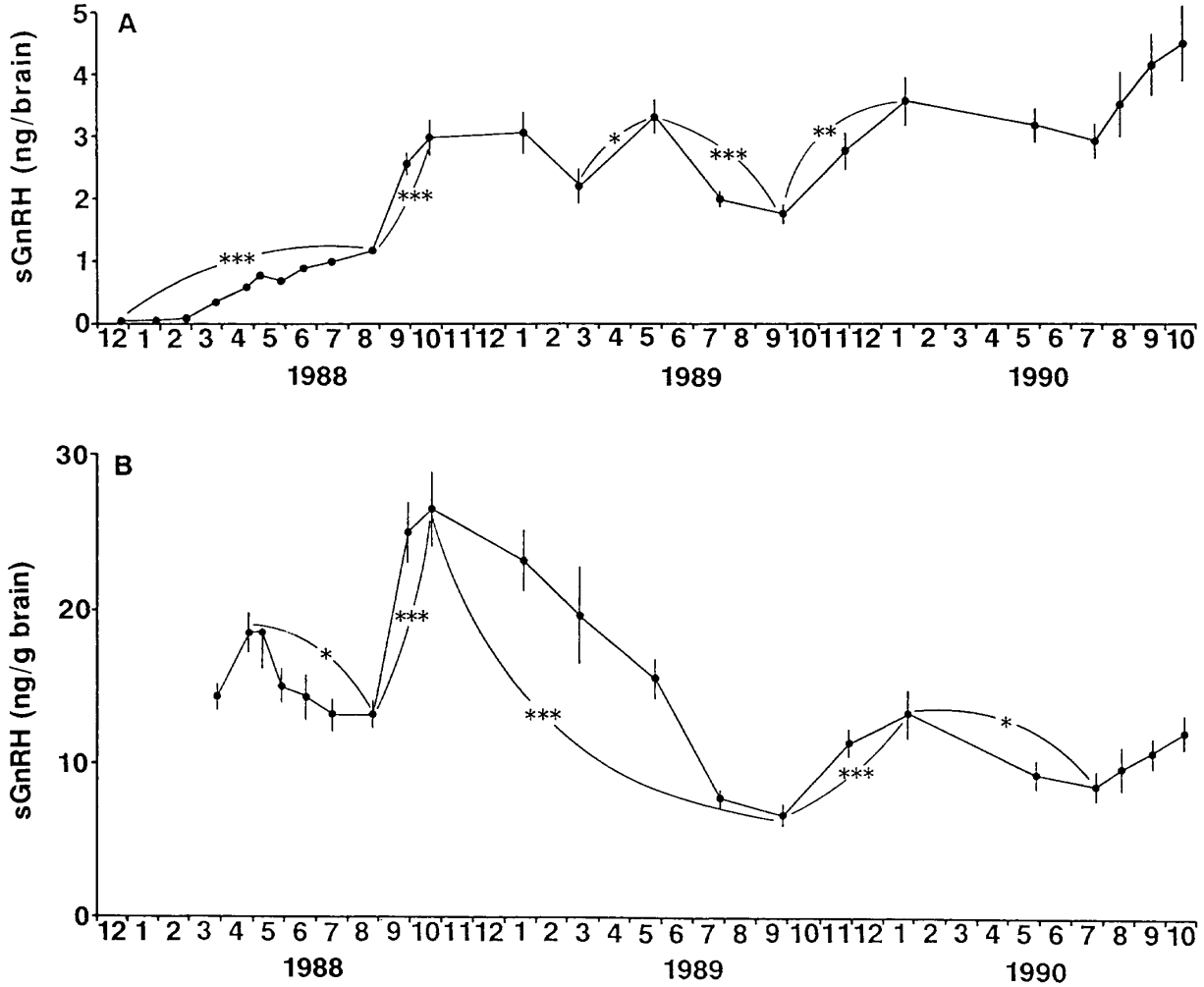
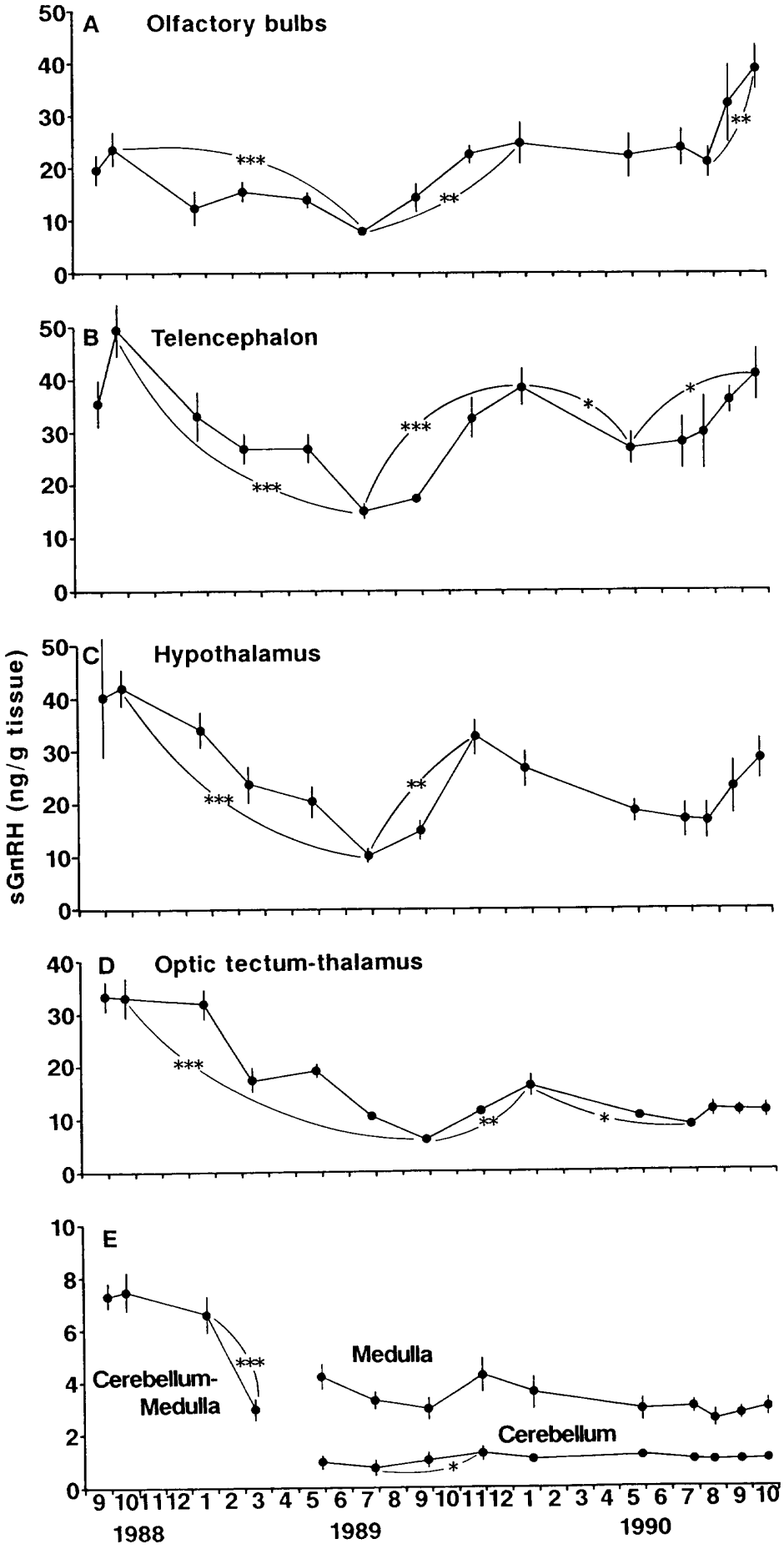


FIG. 6A and B. Seasonal changes in brain sGnRH contents (A) and concentrations (B) of the masu salmon from December 1987 to October 1990 and from March 1988 to October 1990, respectively. Presentation of statistical data is as in Fig. 4.

FIG. 7A-E. Seasonal changes in sGnRH concentrations in olfactory bulbs (A), telencephalon (B), hypothalamus (C), optic tectum-thalamus (D) and cerebellum and medulla oblongata (E) of the masu salmon from September 1988 to October 1990. Presentation of statistical data is as in Fig. 4.



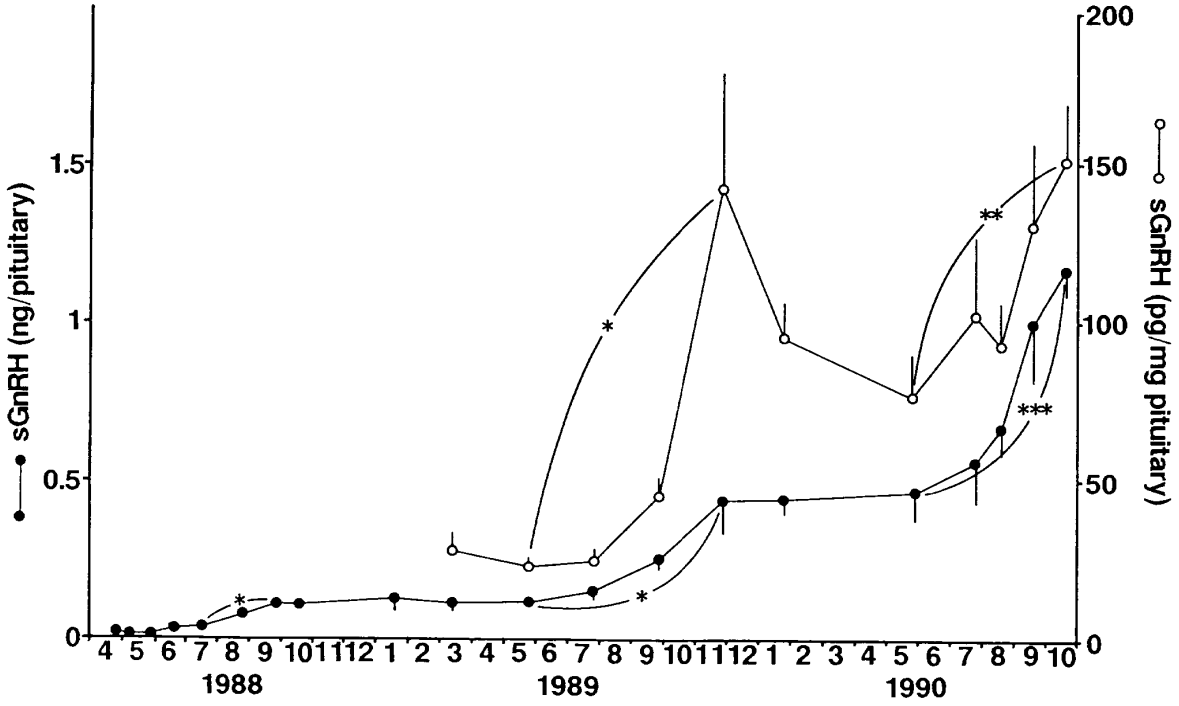


FIG. 8. Seasonal changes in sGnRH contents and concentrations in the pituitary of the masu salmon from April 1988 to October 1990. Presentation of statistical data is as in Fig. 4.

Pituitary sGnRH contents significantly increased from July through September 1988, from May through November 1989 and from May through October 1990 as shown in Fig. 8. Pituitary sGnRH concentrations also increased significantly from May through November 1989 and from May through October 1990.

cGnRH-II in the brain and pituitary

cGnRH-II was undetectable at the first two samplings (December 1987 and January 1988) and became detectable from February 1988. Brain cGnRH-II contents increased as fish grew, but were lower than those of sGnRH (Fig. 9A).

Brain cGnRH-II concentrations increased significantly from August 1988 through January 1989 and from September 1989 through January 1990. On the contrary, concentrations decreased from January 1989 through September 1989 (Fig. 9B).

cGnRH-II contents in the olfactory bulbs and pituitary were below the detectable limit in almost all individuals throughout the experiment.

cGnRH-II concentrations in discrete brain areas are shown in Figs. 10A-D. They showed a tendency to decrease from winter through summer and to increase from autumn through winter as those of sGnRH did, especially in optic tectum-thalamus. However, in contrast to sGnRH, no remarkable changes were observed during vitellogenesis and ovulation.

DISCUSSION

This is the first report which shows long term changes in two types of GnRH, sGnRH and cGnRH-II, in the brain and pituitary, and GTHs in the pituitary in fish. The observation period covers nearly the entire life cycle from hatching through ovulation.

sGnRH was detected in the brain just after hatching, whereas cGnRH-II was first detected two months later. It is remarkable that both GnRHs appear during early developmental stages when the ovary was still in an immature stage. Both GnRH contents increased with a growth during underyearling stage. The time lag between the appearance of sGnRH and cGnRH-II, and subsequent development of a differential distribution of sGnRH and cGnRH-II suggest the difference in their physiological function.

sGnRH concentrations in the olfactory bulbs and the telencephalon, and pituitary sGnRH con-

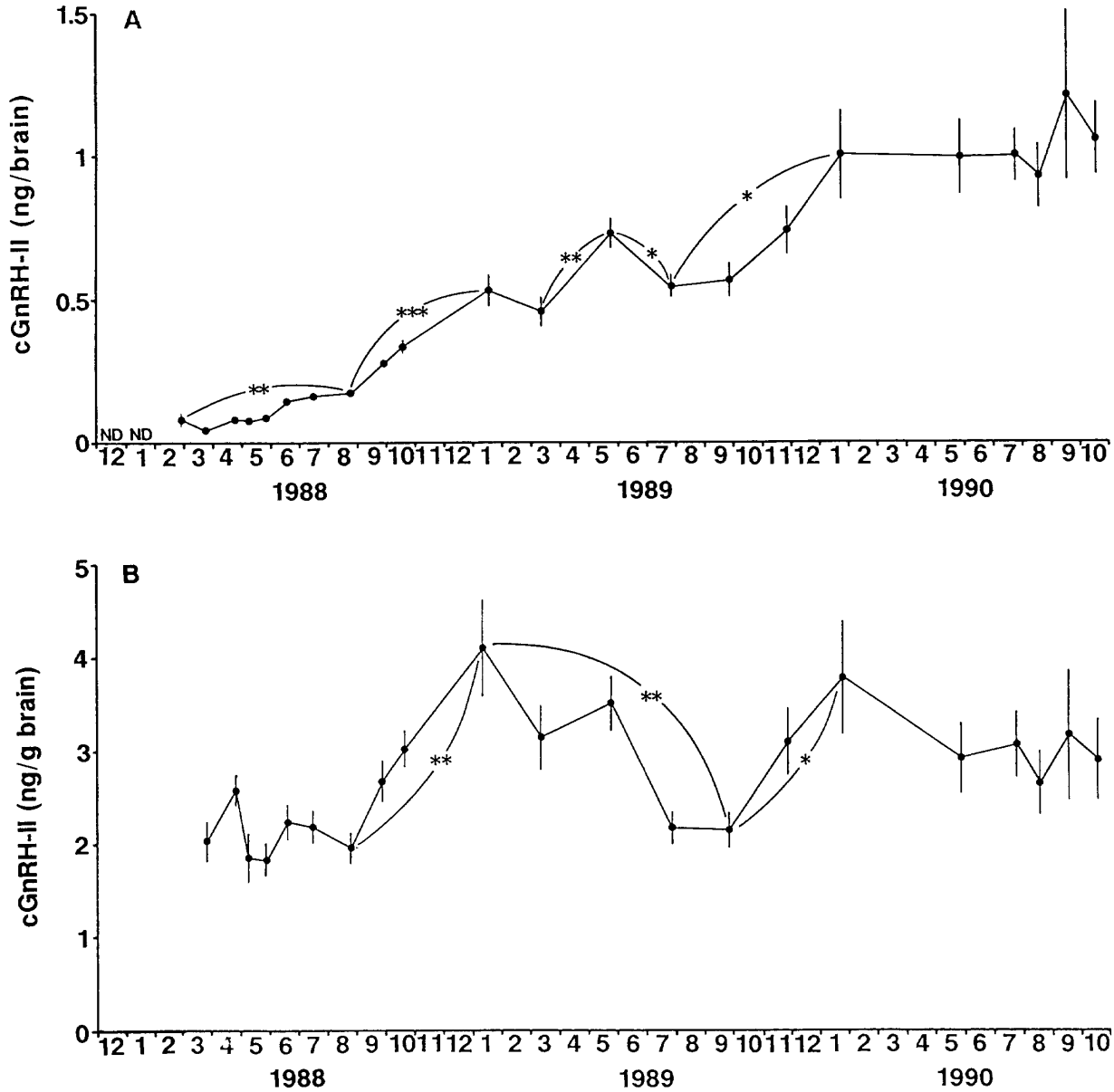


FIG. 9A and B. Seasonal changes in brain cGnRH-II contents (A) and concentrations (B) of the masu salmon from December 1987 to October 1990 and from March 1988 to October 1990, respectively. Presentation of statistical data is as in Fig. 4.

tents increased significantly during vitellogenesis and ovulation (Figs. 7, 8). Moreover, sGnRH concentrations in the hypothalamus showed a tendency to increase. These changes may be related to gonadal maturation, since pituitary GTH Iβ and GTH IIβ contents and GSI also increased significantly in this period (Figs. 4, 7). On the other hand, no significant changes in cGnRH-II concentration in the discrete brain areas were observed (Fig. 10). These results suggest that sGnRH participates mainly in vitellogenesis and ovulation of this species possibly through the regulation of GTH synthesis and release.

GTH I is considered to regulate the early stages of gonadal maturation, and GTH II is considered mainly to regulate ovulation and spermiation [9]. Pituitary GTH Iβ contents showed clear seasonal changes: contents increased from spring to autumn and decreased in winter (Fig. 5A). Pituitary sGnRH contents showed stepwise increases from spring to autumn for three years (Fig. 8). Such increases in pituitary GTH Iβ contents were likely correlated with the increase in pituitary sGnRH

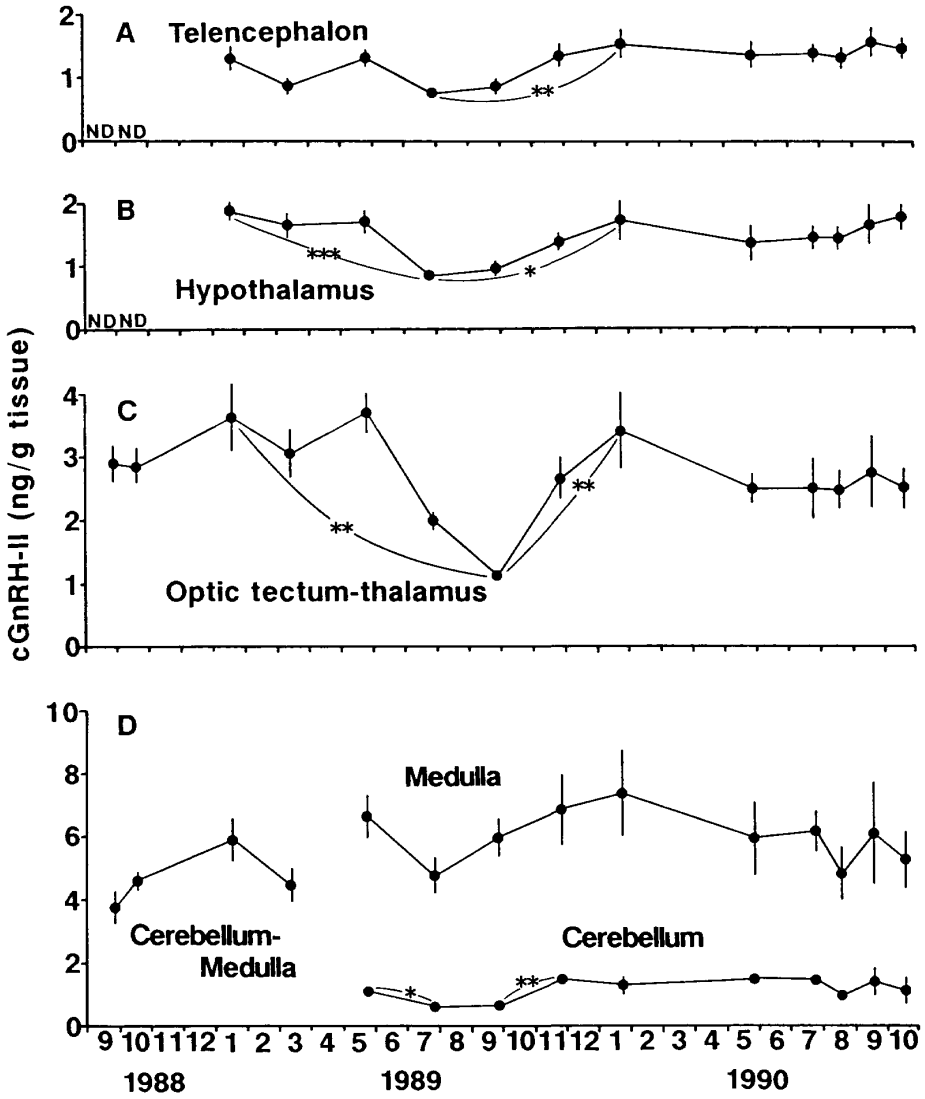


FIG. 10A-D. Seasonal changes in cGnRH-II concentrations in telencephalon (A), hypothalamus (B), optic tectum-thalamus (C) and cerebellum and medulla oblongata (D) of the masu salmon from September 1988 to October 1990. Presentation of statistical data is an in Fig. 4.

contents. Two types of GTH I (stable and unstable) are known to exist in the chum salmon pituitary [10]. Since GnRH extraction was undertaken under acidic conditions, unstable GTH I is considered to have dissociated to its α and β subunits. Therefore, changes in GTH Iβ may reflect those of unstable GTH I. In order to confirm this hypothesis, GTH I should be measured by RIA; however, at present, a specific RIA for GTH I has not been established in our laboratory. Since no significant histological changes of ovaries were observed when pituitary GTH Iβ contents were high in underyearling and yearling autumn, the complete form of GTH I may not be produced or secreted during first two years. Changes in pituitary GTH IIβ contents were similar to those of GSI: after gradual increases until the second spring (two year-old fish), rapid increases occurred in accordance with vitellogenesis and ovulation. Changes in GTH IIβ may reflect those of GTH II, since GTH II is considered to dissociate under the acidic conditions employed in GnRH extraction.

sGnRH and cGnRH-II concentrations in the brain showed a tendency to increase from autumn through winter and to decrease from winter through summer (Figs. 6B, 7, 10). Since water temperature was constant throughout the experiment (9–10°C), photoperiod may be involved in the regulation of the synthesis and release of

sGnRH and cGnRH-II. Takashima and Yamada [12] reported that although maturation is initiated under long photoperiod, rapid vitellogenesis is induced by short photoperiod in the landlocked masu salmon "yamame."

Pituitary sGnRH contents increased significantly from July through September in underyearling, from May through November in yearling and from May through October in two year-old females, suggesting that the increase occurs under shortening day length (Fig. 8). On the contrary, no significant change was observed during other periods. It may be possible to speculate from the present results that synthesis of sGnRH in the brain increases under shortening day length and sGnRH produced is transported from cell bodies to the pituitary from summer to autumn. Changes in sGnRH concentrations in the olfactory bulbs, the telencephalon and the hypothalamus support this hypothesis.

cGnRH-II was mostly undetectable in the pituitary and no significant changes of cGnRH-II concentrations in the discrete brain areas were observed during vitellogenesis and ovulation. cGnRH-II may not have a function of regulating GTH synthesis and release. It may function only as a neuromodulator in the brain, although cGnRH-II has the same potency to induce GTH release from the pituitary of hime salmon, *Oncorhynchus nerka, in vitro* (unpublished data).

We have previously examined the distribution of sGnRH and cGnRH-II in the brain of masu salmon [13]. sGnRH-immunoreactive (-ir) cell bodies were scattered in the transitional areas between the olfactory nerve and the olfactory bulb and between the olfactory bulb and the telencephalon, the ventral telencephalon, and the preoptic area, and sGnRH-ir fibers were distributed in various regions of the brain, as well as in the pituitary. On the other hand, cGnRH-II-ir cell bodies were found in the midbrain tegmentum, and cGnRH-II-ir fibers were distributed in various brain regions but not in the pituitary. The present results are in correspondence with our previous results.

Gentile *et al.* [6] measured GnRH contents using antibody against mammalian GnRH in the telencephalon and hypothalamus of Venezuelan freshwater fish, "caribe colorado", *P. notatus*. They found that changes of GnRH corresponded to those of GSI, especially in sexually mature female-high in May when maximal GSI is achieved. Our results nearly correspond to their results. Yu *et al.* [7] reported that the sGnRH contents in the hypothalamus and pituitary were higher in sexually regressed fish compared with those in sexually mature fish under certain conditions of temperature acclimation (18°C). Since female masu salmon die after spawning, it is impossible to compare the data at sexually regressed conditions. The difference of most significance is that cGnRH-II exists in the pituitary of goldfish, whereas in the pituitary of masu salmon, cGnRH-II contents are below detectable limits. Okuzawa *et al.* [5] measured sGnRH and cGnRH-II contents in the discrete brain areas of rainbow trout and found that the pattern of sGnRH distribution changed with age and stage of sexual maturity, but they had measured GnRH levels in 1-year-old and 3-year-old fish sampled in September. Therefore, annual changes in sGnRH levels in rainbow trout are still unknown.

GnRH may also stimulate growth hormone release, since sGnRH stimulates growth hormone (GH) release both *in vivo* and *in vitro* in goldfish [2, 3]. In salmonid fish, both sGnRH and cGnRH-II have a potency to stimulate GH release from the pituitary *in vitro* in hime salmon, *Oncorhynchus nerka* (unpublished data). However, cGnRH-II contents were below detectable limits by RIA and undetectable by immunocytochemistry. Therefore, if GH release is regulated by GnRH, only sGnRH is involved in this release in masu salmon.

Future investigation on seasonal changes in the expression of GnRH and GTH genes will be required.

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