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Molecular Aspects of Reproductive Neuroendocrinology in Salmon

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ABSTRACT

Pacific salmon are anadromous fish, which return to their natal river for spawning after oceanic migration. Accumulation of genetic information on hormone precursors and their receptors enabled us to examine various neuroendocrine events that regulate salmon reproduction including spawning migration at a molecular level. We mainly used two salmonid species: masu salmon (*Oncorhynchus masou*) as model fish and chum salmon (*O. keta*) as wild fish, and assessed changes in expression of particular genes in relation to physiological events using a real-time PCR method. Regulatory mechanisms of hormonal gene expression were also examined to some extent, particularly of subunits of gonadotropins.

Cloning and sequencing studies indicated the presence of two salmon (s) GnRH and five GnRH receptor (GnRH-R) genes in masu salmon. Seasonal variation of gene expression for sGnRH and GnRH-R were then estimated through determination of the absolute amounts of mRNAs by the real-time PCR in the brains and the pituitaries of growing and maturing fish, in association with changes in expression of pituitary hormone genes. The expression of two sGnRH genes in the ventral telencephalon and the preoptic area elevated twice in late spring to

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early summer and in breeding season in autumn, whereas patterns of changes in expression of GnRH-R genes in the pituitaries differed among different types. Elevation of gene expression for LH subunits in the pre-spawning season coincided well with that for two sGnRH precursors and some types of GnRH-R. These coincident molecular events may be important for control of final maturation accompanied by upstream migration.

Introduction

The essential requirements for reproductive success are that the partners of different sexes are at the same place at the same time in the same state of reproductive readiness. Spawning migration, which is seen in varieties of animal species including many fishes, is one of evolutionally successful strategies that fulfill the above-mentioned requirements. In vertebrates, it is principally the important part of life history in individual migratory animals. Spawning migration of anadromous salmon is a part of large scale oceanic migration, and considered as a typical stereotyped instinctive behavior which is believed to be genetically programmed.

Migration of salmon is composed of several phases: downstream migration of juveniles following par-smolt transformation; seawater adaptation at the entrance of estuarine habitat; offshore migration for feeding and growth; and then spawning migration to the natal river. The spawning migration includes homing, fresh-water adaptation and upstream migration in maturing adults. Each phase of migration is controlled by the particular neuroendocrine system (Figure 15.1). Par-smolt transformation and downstream migration are under the control of the brain-pituitary-thyroid axis, although roles of thyrotropin-releasing hormone (TRH) are ambiguous in the control of secretion of thyroid-stimulating hormone (TSH). The initial step of seawater adaptation is promoted by cortisol, and then growth hormone (GH) functions in both seawater adaptation and somatic growth through production of insulin-like growth factor I (IGF-I).

Following feeding migration the duration of which is largely different among salmonid species, salmon initiate sexual maturation and spawning migration in late spring probably under the control of the brain-pituitary-gonadal axis (see Urano *et al.*, 1999). When fish arrive at the coastal area or their natal river, prolactin (PRL) and vasotocin serve to adapt fresh-water environment, and sex steroids may stimulate upstream migration. Meantime, final maturation is prompted by luteinizing hormone (LH), which is previously termed as gonadotropin II (GTH II). The neuroendocrine systems are thus involved in the controls of various phases of migratory behavior in salmon, so that we presumed that hypothalamic neurosecretory neurons have crucial roles in the control of salmonid migration.

It is well known that hypothalamic neurosecretory neurons have important roles in the control of instinctive behavior in vertebrates, probably because they have appropriate neuroanatomical features to coordinately control functions of both the central nervous system and the endocrine system (Urano, 1984 and 1988). In terms of the control of spawning migration, projections of gonadotropin-releasing hormone (GnRH) neurons and vasotocin neurons possess the most favorable features (Figure 15.2). They send their axons to many brain loci including the optic tectum, which is crucial for the control of orientation behavior in all vertebrate species, and to the neurohypophysis where GnRH and vasotocin are released into hypophysial vasculatures. Our recent result showed interaction of GnRH neurons and classical neurosecretory neurons (Saito *et al.*, 2003). However, since the aim of present article is an overview of molecular aspects of reproductive neuroendocrinology in salmon, we focus on the GnRH-GTH system and spawning migration.

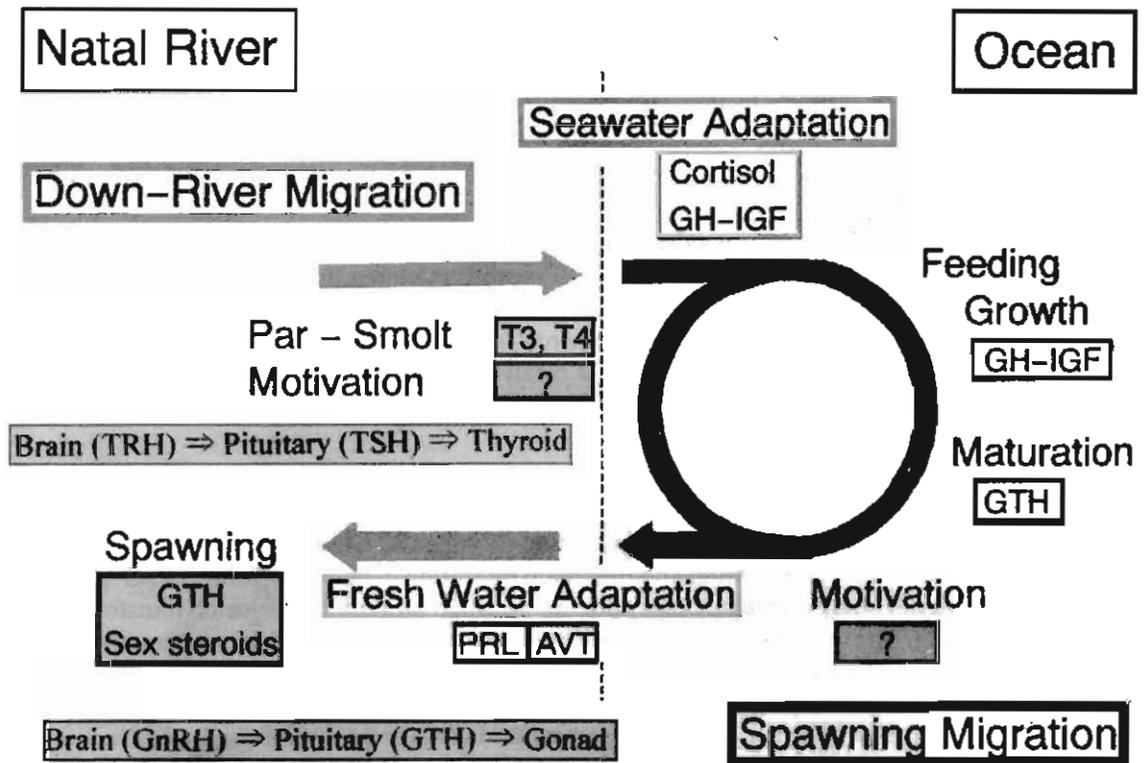


Figure 15.1: The Neuroendocrine Systems Involved in the Control of Migration in Salmon. See the text for explanation. (modified from Urano *et al.*, 1999)

Experimental Fishes: Model Fish and Wild Fish

So-called pacific salmon that belong to genus *Oncorhynchus* include seven species. Their life histories, in particular the duration of oceanic life, differ among species (Groot and Margolis, 1991). Recently diverged species like chum salmon (*O. keta*) spend several years in the North Pacific Ocean and the Bering Sea until they initiate spawning migration, whereas ancestral species like masu salmon (*O. masou*) return their natal river after one year of oceanic life. Salmonids in the former group can not sexually mature when they are aquacultured in fresh water, while species in the latter group can mature even in a freshwater tank. Nonetheless, neuroendocrine features of sexual maturation seemed to be almost the same among different species. We therefore selected masu salmon as a model salmon species to clarify molecular events during sexual maturation, and chum salmon as a representative wild salmon to study molecular mechanisms of spawning migration on the basis of experimental facts shown in the model system.

In our research, we used masu salmon of Mori hatchery strain, which was established for propagation of high return population in the Mori branch of Hokkaido Fish Hatchery. Fish of this strain retain ability of homing migration, nonetheless can grow, mature and spawn in a FW environment. Meanwhile, as wild fish, pre-spawning chum salmon of Ishikari stock, which were obtained from the

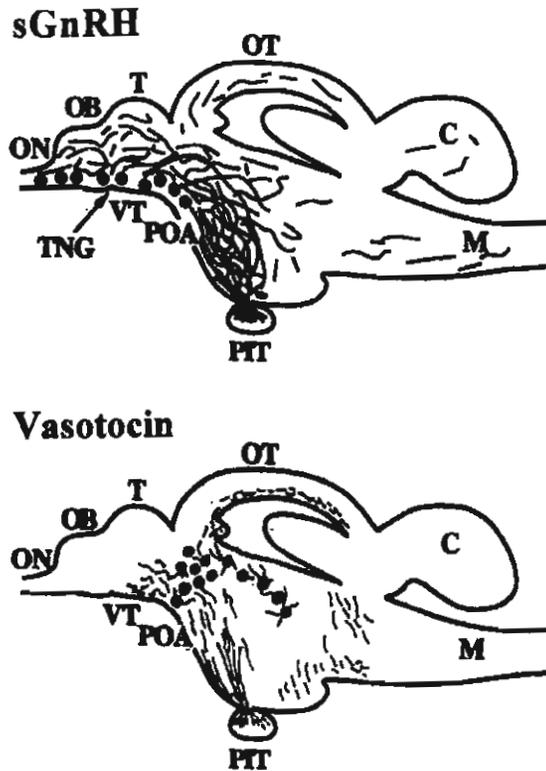


Figure 15.2: Schematic Diagrams Showing Projections of sGnRH Neurons (A) and vasotocin neurons (B).

Note that both sGnRH neurons and vasotocin neurons send their axons to various brain loci and the pituitary (PIT), favorable features for dual controls of the central and neuroendocrine systems. C, cerebellum; M, medulla oblongata; OB, olfactory bulb; ON, olfactory nerve; OT, optic tectum; POA, preoptic area; TNG, terminal nerve ganglion.

Ishikari River-Ishikari bay water system in Hokkaido, Japan, were mainly used in our study. In this vast water system, about 30 million juveniles were released every year from the Chitose Salmon Hatchery, which is located at the riverside of the Chitose River, a tributary of the Ishikari River, so that almost all fish used in the present study were regarded as returnees to the Chitose Salmon Hatchery. The distance from the mouth of the Ishikari River to the Chitose Salmon Hatchery is about 70 km, and chum salmon usually required two to three weeks to reach the hatchery from the mouth of the Ishikari River.

Quantitative Analysis of mRNA Amounts for Estimation of Hormonal Gene Expression

Description of molecular events in the neuroendocrine system in relation to reproductive function, in particular in seasonal breeders, requires information on changes of the absolute amounts of particular mRNAs for hormone precursors. We therefore developed an assay method to determine magnitudes of

changes in the amount of particular mRNA, as indices of expression of hormone genes in connection with salmon reproduction. First, we developed a quantitative dot blot analysis in which single-stranded sense cDNA was used as the standard for particular mRNA encoding a precursor of certain pituitary hormone. Validation tests showed that cross hybridization of the probe to mRNAs for other pituitary hormone precursors was less than 1 per cent, and intra- and inter-assay coefficients of variations were comparable to or less than those of radio immunoassays. The sensitivity was between 1 to 10 amol. However, this value is not sufficient to determine the levels of neurohormone mRNAs in the discrete brain loci. Hence, we incorporated a quantitative real-time PCR method, and established a reliable protocol which yields the sensitivity of about 100 copies of particular mRNA with satisfactory ranges of variations (Urano and Ando, 2002).

GnRH and GnRH Receptor Genes

Single vertebrate species usually have two or three GnRH forms. One form of GnRH, so-called hypothalamic GnRH, regulates gonadal maturation through stimulation of pituitary gonadotropes. The second form of GnRH is chicken GnRH-II (cGnRH-II), which is highly conserved from fish through mammals. cGnRH-II neurons are localized in the midbrain tegmentum and send their axons widely throughout the central nervous system, and modulates sexual behavior in some vertebrate species (Millar, 2003). The brain of salmonid fish contains two GnRH forms, salmon (s) GnRH and cGnRH-II, whereas the pituitary was shown to have only sGnRH. Many studies conducted in salmonids showed that sGnRH has a central role in the regulation of synthesis and release of GTHs, and thus promotes the gonadal maturation (Amano *et al.*, 1997).

Because of tetraploidy, salmon have two genes (sGnRH-I and -II) encoding sGnRH precursors (Higa *et al.*, 1997). These two genes are co-expressed in almost all sGnRH neurons in the forebrain of sockeye salmon (Amano *et al.*, 1998). However, the expression level of sGnRH-II gene is much higher than that of sGnRH-I gene in masu salmon and chum salmon (Ando *et al.*, 2001; Kitahashi *et al.*, 2004; Onuma *et al.*, 2005).

Multiple genes which encode GnRH receptors (GnRH-Rs) are present in single species (Lethimonier *et al.*, 2004; Millar *et al.*, 2004). Several GnRH-R subtypes with different structural characteristics are expressed in the brain, pituitary and various peripheral organs. We recently showed that, in masu salmon, five different GnRH-R genes, termed as msGnRH-R1, R2, R3, R4, and R5, are expressed in the brain, the pituitary and other peripheral tissues (Jodo *et al.*, 2003). Binding of GnRH to its receptors may activate multiple signal transduction pathways in target cells, and thus express multiple functions of GnRH in reproduction.

Seasonal Changes in Expression of Genes for sGnRH and GTHs in Masu Salmon

In the forebrains of masu salmon of both sexes, the amounts of sGnRH mRNAs were high during winter through spring in the prepubertal stage, declined toward summer, and then increased again in the spawning period (Figure 15.3) (Ando *et al.*, 2001; Kitahashi *et al.*, 2004). These changes correspond well with the changes in the content of sGnRH in the forebrain, whereas the amount of sGnRH in the pituitary gradually increased along with sexual maturation and reached its maximum in the spawning period (Amano *et al.*, 1992, 1993). Such difference in the contents of GnRH between the forebrain and the pituitary may indicate that the central roles of GnRH are independent of endocrine roles. The prepubertal elevation of sGnRH gene expression in the forebrain suggests a neuromodulatory action of sGnRH that is involved in homing migration, because masu salmon initiate homing migration at this stage.

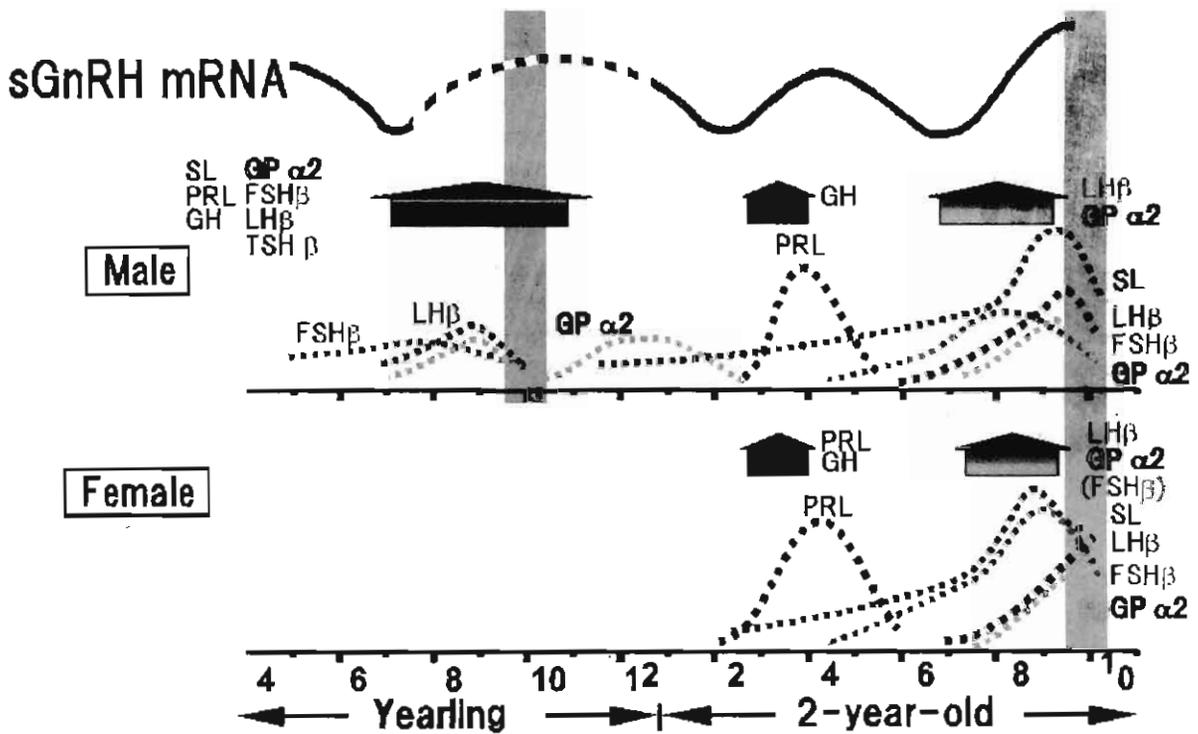


Figure 15.3: Seasonal Changes of Gene Expression for Precursors of sGnRH and Pituitary Hormones in Growing and Maturing Masu salmon in Aquaculture as the Model System. Yellow vertical bars show the breeding season. One-year old males, but not females, showed precocious maturation. See the text for further explanation. (from Kitahashi *et al.*, 2004).

The amounts of mRNAs encoding follicle-stimulating hormone (FSH) β , which was previously called as GTH I, and LH β increased along with sexual maturation, and reached to considerably high magnitudes in the spawning period. Nevertheless, the increase in the content of FSH β mRNA was initiated at early stages of gametogenesis, and the elevation of that of LH β mRNA was initiated at late stages of gametogenesis (Gomez *et al.*, 1999; Kitahashi *et al.*, 2004). In the primary pituitary cells of masu salmon, the amounts of $\alpha 2$, FSH β , and LH β mRNAs increased from March through May and reached their maximum at the pre-spawning stage in July (Ando *et al.*, 2004). The amounts of $\alpha 2$ and FSH β mRNAs then declined in September, while that of LH β mRNA remained at high levels.

We recently examined GTH release activity of masu salmon primary pituitary cell cultures at four reproductive stages in March (initiation of sexual maturation), May (early maturation), July (pre-spawning), and September (spawning period) (Ando *et al.*, 2004). FSH levels in the culture medium increased with sexual maturation and peaked in September, whereas LH release remained low until July and considerably increased in September. These results indicate that the differential regulation of releases of two GTHs can be achieved in part *in vitro* condition without hypophysiotropic stimuli and neuronal inputs such as dopaminergic and γ -aminobutyric acid (GABA)-ergic (Yaron *et al.*, 2003). It

thus appears that two different types of gonadotropes (FSH and LH cells) release GTH autonomously in different manners.

The different effects of GnRH on synthesis and release of two GTHs may be attributed in part to changes in expression of GnRH-Rs in FSH and LH cells. We therefore determined by use of real-time PCR seasonal variations in expression of the five msGnRH-R genes in the pituitary of masu salmon during the reproductive cycle (Figure 15.4). All five subtypes of msGnRH-R genes were expressed in the pituitary, although R4 mRNA was dominant. Interestingly, the expression patterns of five msGnRH-R genes differed among different subtypes. Among them, R4 mRNA increased only in the pre-spawning period, when expression of LH subunit genes was stimulated by GnRH_a, suggesting that the R4 subtype is involved in the GnRH-induced LH synthesis (Jodo *et al.*, 2004). Other subtype mRNAs increased in different periods, so that they can be involved in other action of GnRH in the pituitary, such as GnRH-induced PRL gene expression. Our recent unpublished results indicate that, in masu salmon primary pituitary cell cultures, sGnRH and sex steroids upregulate expression of msGnRH-R genes in gender and reproductive cycle dependent manners.

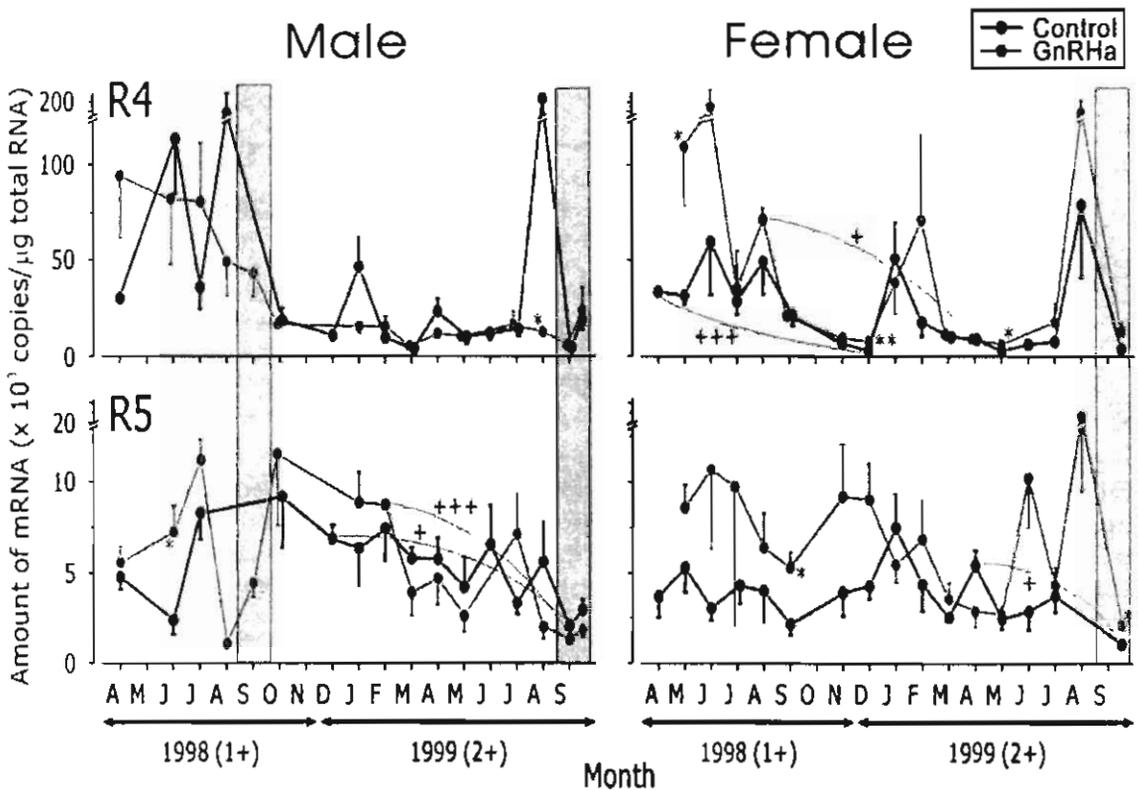


Figure 15.4: Seasonal Changes in Expression of GnRH-R Genes in the Pituitaries of Growing and Maturing Masu Salmon whose Gene Expression for sGnRH and Pituitary Hormones is Shown in Figure 15.3. See the text for explanation. (from Jodo *et al.*, 2004).

Gene Expression for sGnRH and GTHs in Homing Chum Salmon

Our previous studies suggested that sGnRH neurons regulate both final maturation and migratory behavior in homing salmonids (Urano *et al.*, 1999, Kitahashi *et al.*, 2004). As was seen in the model system of masu salmon, sGnRH neurons in the forebrain of chum salmon are considered to be active during pre-spawning to spawning periods when they show upstream migration. We therefore examined expression of two genes encoding precursors of sGnRH in discrete forebrain loci of wild pre-spawning chum salmon, which were captured along their migratory pathway in the Ishikari River-Ishikari bay water system. As was mentioned previously, salmon has two genes for sGnRH precursors, which are referred to as sGnRH-I and sGnRH-II, so that we determined the amounts of two mRNAs. Since the many previous reports showed that the salmon pituitary does not contain cGnRH II (see Ando and Urano, 2005), we did not determine amounts of cGnRH II mRNA.

Patterns of changes in activity of the GnRH-GTH system in wild homing chum salmon are subjects of year to year variations, since we found that there were year-to-year differences in plasma

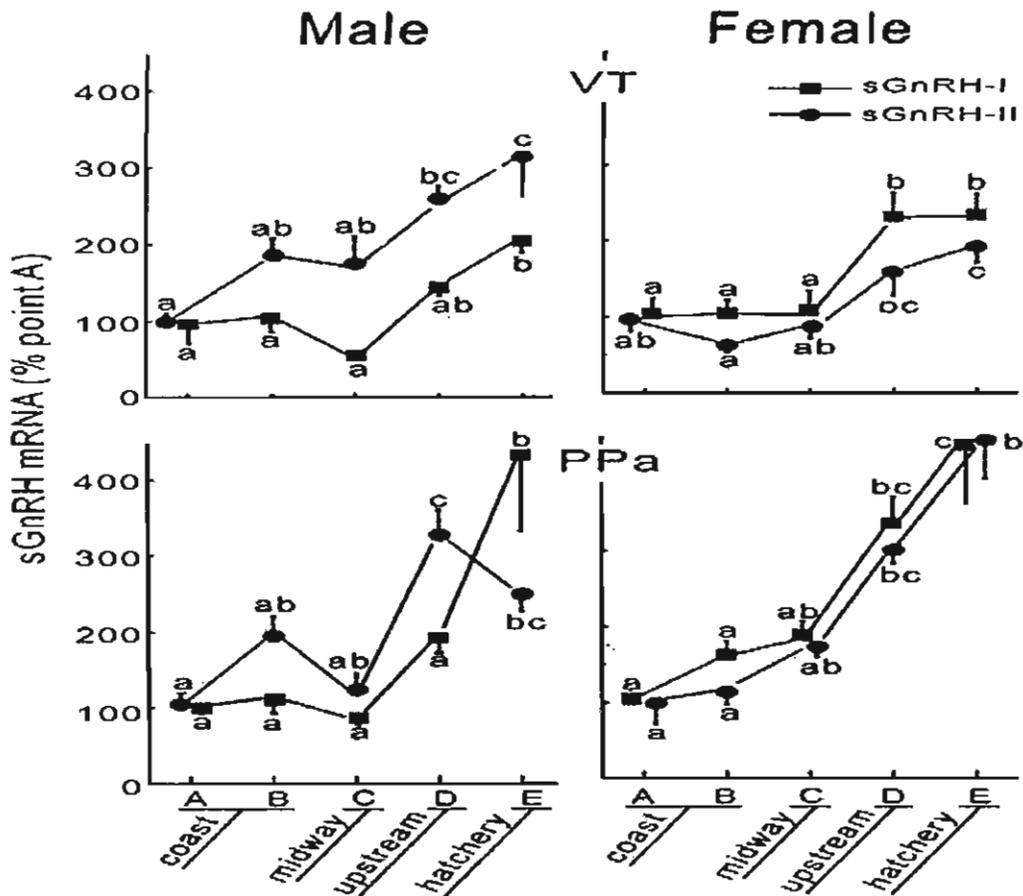


Figure 15.5: Elevation of sGnRH Gene Expression In the Ventral Telencephalon (VT) and the Nucleus Preopticus Parvocellularis Anterioris (PPa) of Homing Chum Salmon During Upstream Migration in 1999. Fish were captured at several points along their homing pathway in the Ishikari water system. (based on Onuma *et al.*, 2005)

levels of steroid hormones and sexual maturation during our six-year survey (Onuma *et al.*, 2003). By this reason, we used fish that were captured from 1997 through 1999 in our research in which changes in gene expression for sGnRH and GTHs were determined in homing chum salmon (Onuma *et al.*, 2005). They were fished along their homing pathway: coastal areas, a midway of the river, 4 km downstream of the natal hatchery, and the hatchery, and the brains and pituitaries were taken out to be frozen in liquid nitrogen. Amounts of sGnRH mRNAs were determined by real-time PCR in fresh frozen sections that were grouped into ten particular brain loci: the olfactory bulb (OB), terminal nerve (TN), ventral telencephalon (VT), nucleus preopticus parvocellularis anterioris (PPa) and nucleus preopticus magnocellularis (PM). The amount of sGnRH-II mRNA was higher than that of sGnRH-I mRNA, while they showed similar changes during upstream migration. In the OB and TN, the amounts of sGnRH mRNAs elevated from the coast to the natal hatchery. In the VT and PPa, they elevated along with the progress of final maturation (Figure 15.5). Such elevation was also observed in the rostroventral, middle and dorsocaudal parts of the PM. The amounts of gonadotropin II β (Figure 15.6) and somatolactin mRNAs in the pituitary also increased in consistent with the elevation of gene expression for sGnRH. These results in combination with lines of previous evidence (see Ando and Urano, 2005) indicate that sGnRH neurons are activated in almost all the forebrain loci during the last phases of spawning migration, resulting in coordination of final gonadal maturation and migratory behavior to the spawning ground.

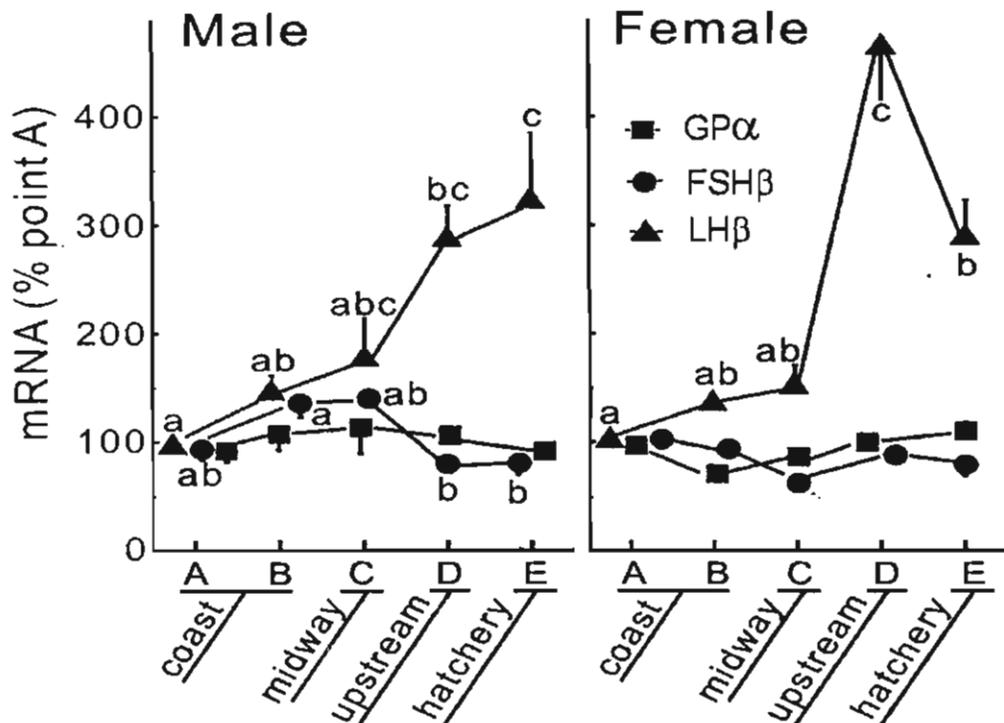


Figure 15.6: Changes in Expression of Genes for Precursors of GTH Subunits in the Pituitaries of the Same Homing Chum Salmon Shown in Figure 15.5.

Note that expression of LH β gene was highly elevated during upstream migration. This elevation is coincident with that of GnRH gene expression. (based on Onuma *et al.*, 2005)

Conclusion

In masu salmon as the model system and chum salmon as wild fish, seasonal variation of gene expression for sGnRH and GnRH-R were estimated through determination of the absolute amounts of mRNAs by the real-time PCR in the brains and the pituitaries of growing and maturing fish, in association with changes in expression of pituitary hormone genes. The expression of two sGnRH genes in the ventral telencephalon and the preoptic area of masu salmon elevated twice in late spring to early summer and in breeding season in autumn, whereas patterns of changes in expression of GnRH-R genes in the pituitaries differed among different types. Nonetheless, in masu salmon, elevation of gene expression for LH subunits in the pre-spawning season coincided well with that for two sGnRH precursors and some types of GnRH-R. Homing chum salmon of the Ishikari stock showed similar patterns of changes in expression of genes for sGnRH precursors and LH subunits during upstream migration. These coincident molecular events may be important at least for control of final maturation accompanied by upstream migration. Further, a similarity between masu salmon and chum salmon seen in pre-spawning and spawning periods promises that a use of masu salmon as the model system should yield fruitful results to clarify cellular and molecular bases of neuroendocrine mechanisms in reproduction of salmon and also other teleost fishes.

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