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Sexually Different Expression of Neurohypophysial Hormone Genes in the Preoptic Nucleus of Pre-Spawning Chum Salmon

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ABSTRACT—Vasotocin (VT) and isotocin (IT) are teleost neurohypophysial hormones produced by neurosecretory neurons in the magnocellular part of preoptic nucleus (PM) of the hypothalamus. Several previous studies indicated that neurohypophysial hormones are involved in teleost reproductive behavior. The changes in the expression of VT and IT genes were thus studied by an *in situ* hybridization technique and an immunohistochemical avidin-biotin-complex method in pre-spawning chum salmon (*Oncorhynchus keta*). Male and female fish were caught at Atsuta, the mouth of the Ishikari Bay, and at Chitose, upstream to the Ishikari River in October, 1994. The former and the latter are referred to as bay fish and river fish, respectively. The intensity of autoradiographic hybridization signals were determined in individual neurosecretory neurons in the rostroventral, middle, and dorsocaudal parts of the PM. In the females, the levels of VT and IT mRNAs in the river fish were significantly lower than those in the bay fish in all the three loci in the PM, whereas VT and IT immunoreactivities in the river fish were higher than those in the bay fish. These results suggest that both the rates of transcription and release of VT and IT were decreased in pre-spawning female chum salmon. Contrary, in the males, the levels of IT mRNA and IT immunoreactivity in the river fish were greater than those in the bay fish particularly in the rostroventral part of the PM, whereas conspicuous changes were not seen in the levels of VT mRNA and VT immunoreactivity. The present results thus revealed sexually different expression of neurohypophysial hormone genes in the preoptic nucleus of pre-spawning chum salmon when compared between bay and river fish. The regulation of neurohypophysial hormone gene expression may be different between the male and the female during the last stages of spawning migration.

INTRODUCTION

In teleosts, vasotocin (VT) and isotocin (IT) are neurohypophysial hormones produced by hypothalamic neurosecretory neurons in the preoptic nucleus (see Acher, 1985). Several studies indicated that neurohypophysial hormones are involved in reproductive behavior in teleosts (see Urano *et al.*, 1994) as well as in other vertebrates (see Moore, 1992). Since the spawning migration of salmon is considered to be a kind of reproductive behavior, it is probable that VT and IT are involved in control of salmon spawning migration.

The nucleotide sequences of cDNAs for VT and IT precursors have been analyzed in three salmonid fishes, chum salmon (*Oncorhynchus keta*) (Heierhorst *et al.*, 1990; Hyodo *et al.*, 1991), masu salmon (*O. masou*) (Suzuki *et al.*, 1992), and sockeye salmon (*O. nerka*) (Hiraoka *et al.*, 1993). The level of VT mRNA decreased after transfer of fish from freshwater (FW) to 80% sea water (SW), and increased after transfer back to FW (Hyodo and Urano, 1991a), suggesting that neurohypophysial hormones are involved in osmoregulation in salmonid fish. Since salmon need to adapt to FW environments when they enter their natal river, an involvement of neurohypophysial hormones in salmon spawning migration in respect to osmoregulation is also interesting.

Preliminary experiments by our group in 1992 and 1993 appeared to show that the levels of VT and IT mRNAs differed between SW and FW chum salmon, although the results were not conclusive (Hyodo *et al.*, 1994). Hence, in the autumn of 1994, we conducted extensive survey to obtain conclusive results by use of both quantitative Northern blot analysis (Hiraoka *et al.*, 1995a) and semiquantitative molecular- and immuno-histochemistry results of which are reported in this

paper.

In the present study, we analyzed changes in expression of VT and IT genes in individual neurosecretory neurons in the male and female pre-spawning chum salmon which were caught in the Ishikari Bay, and in the Chitose River. Both of VT and IT neurons are localized mainly in the magnocellular part of preoptic nucleus (PM). The PM actually can be divided into three loci, pars parvocellularis (PMp), pars magnocellularis (PMm) and pars gigantocellularis (PMg) (Braford and Northcutt, 1983). The PMp consists of relatively small cells in the rostroventral part of the PM, the PMm consists of large neurons in the middle part of the PM, and the PMg is composed of larger cells which are localized caudal to the PMm. Neurosecretory neurons in these discrete loci may have different characteristics among each other, such as functions and afferent innervations, as have been shown in the magnocellular and parvocellular neurons in the mammalian paraventricular nucleus (see Hyodo and Urano, 1991b; Swanson and Sawchenko, 1983). An *in situ* hybridization technique and an immunohistochemical avidin-biotin-complex (ABC) method were therefore applied to detect changes in expression of VT and IT genes in neurosecretory neurons in the three loci in the PM of pre-spawning chum salmon.

MATERIALS AND METHODS

Fish

Sexually matured chum salmon of both sexes (n=5, each) were caught at Atsuta, a fisherman village faced to the Ishikari bay, and at Chitose, a town through which a branch of the Ishikari river runs, in October, 1994. The fish caught at Atsuta and Chitose are referred to as bay fish and river fish, respectively. After the fish were weighed and measured of body length, blood was collected from the caudal vasculature. Blood samples kept in ice were later centrifuged at 3000 rpm for 15 min to obtain plasma. Plasma osmolality, and sodium and chloride concentrations were measured by a vapor pressure osmometer (Wescor 5500), an ion analyzer (AVL 984-S, Graz) and a chloridometer (Buchler), respectively.

In the bay fish, the nuptial color was not yet developed, whereas silver color was still apparent. Most of the eggs were not yet ovulated in the female bay fish. The river fish showed typical nuptial color, and further, the females had completed ovulation, and the males excreted milt when pressed the abdomen. The GSI (gonad weight × 100/body weight) values were increased in the females and decreased in the males during the last stages of spawning migration from Atsuta to Chitose (Table 1), indicating that the final maturation occurred during this period.

The plasma osmolality, and the sodium and chloride concentrations were significantly lower in the river fish than in the bay fish in both sexes (Table 1), indicating that the fish captured at Chitose were well adapted to FW.

Tissue preparation

Immediately after the collection of blood samples, animals were decapitated and tissue blocks from the forebrains which mainly included the hypothalamus and the pituitary were taken out. They were then immersed in 4% paraformaldehyde in 0.05 M phosphate buffer (pH 7.3) at 4°C for 2 days. After fixation, the tissues were washed in cold 70% ethanol overnight, dehydrated through graded ethanols, and were embedded in paraplast. Serial transverse sections were cut at 8 μm, separated into several groups, and were mounted on gelatinized slides.

In situ *hybridization (ISH)*

The procedure for *in situ* hybridization histochemistry and quantification of hybridization signals followed those previously described by Hyodo *et al.* (1988) and Hyodo and Urano (1991a). For *in situ* hybridization probes, 46mer synthetic oligonucleotides were also used as was described in the previous paper. The nucleotide sequences of probes correspond to the regions in the chum salmon mRNAs encoding proVT (-5 to 11) and proIT (-5 to 11). The VT probe was designed to hybridize with salmonid VT-I and -II mRNAs, whereas the IT probe recognizes salmonid IT-I and -II mRNAs. The probes were labeled at the 3' ends with [α-^{35}S] dATP (DuPont/NEN Products) by 3'-end labeling system (Amersham), and purified with NENSORB purification cartridges (DuPont/NEN Products). Final specific activity of the probes were 0.9-1.2 × 10^8 cpm/μg. The radiolabeled probes were diluted in a hybridization buffer (0.9 M NaCl, 0.6 mM EDTA, 0.02% bovine serum albumin (BSA), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10 mM dithiothreitol, 100 μg/ml denatured calf thymus DNA, and 10% dextran sulfate in 20 mM Tris buffer, pH 7.5) so as to apply 7.5 ng/90 μl of the probe to each slide glass. Hybridization with the labeled probe was performed at 46°C for 16 hr. The sections were then washed in 1× SSC at room temperature for 10 min, twice in 1× SSC at 45°C for 30 min, and finally in 1× SSC at room temperature for 10 min. Specificity of hybridization signals was previously confirmed by several tests (Hyodo and Urano, 1991a).

For quantification of hybridization signals, the numbers of autoradiographic silver grains were counted in individual neurons in the PMp, PMm and PMg. Since the background staining was quite low in the present study, the numbers of background silver grains were not counted to calibrate the measured values. The sizes of individual neurons which contained hybridization signals were determined by measuring the weights of cell-shaped papers cut out from highly enlarged drawings, and the numbers of grains per unit area (100 μm^2) were calculated to compare transcriptional activity among neurons of big size deference using normalized values.

Immunohistochemistry

Tissue sections adjacent to those used for the ISH were immunohistochemically stained by the avidin-biotin-complex method. In this study, the primary antisera (gifts from Dr. Kawashima) were used as follows: rabbit anti-vasopressin preabsorbed with IT was

Table 1. Changes in gonadosomatic index (GSI), plasma osmolality, sodium and chloride concentrations between bay fish (Atsuta) and river fish (Chitose). Values are mean ± SEM.

Sampling area	Sex	Body color*	N	BW (kg)	GW (g)	GSI (%)	Plasma osmolality (mOsm/kg)	Na^+ (mM)	Cl^- (mEq/1)
Atsuta	♂	SN	5	2.3 ± 0.2	112 ± 9.7	5.0 ± 0.5	411.4 ± 6.8	204.4 ± 23.9	168.4 ± 2.2
Chitose	♂	N	5	3.2 ± 0.1	146 ± 23.2	4.5 ± 0.6	327.0 ± 2.3	182.2 ± 1.6	142.6 ± 4.4
Atsuta	♀	SN	5	2.9 ± 0.4	472 ± 35.0	16.8 ± 1.3	393.4 ± 5.1	237.9 ± 22.5	174.0 ± 8.3
Chitose	♀	N	5	3.5 ± 0.1	768 ± 27.3	21.8 ± 0.2	308.2 ± 9.6	160.7 ± 12.7	126.4 ± 8.4

*SN, intermediate between smolt and nuptial color; N, nuptial color

diluted 1:10000 with phosphate buffered saline containing 0.5% BSA (PBS-BSA, pH7.6) and rabbit anti-oxytocin preabsorbed with VT was diluted 1:20000 with PBS-BSA. These values for dilution of the antisera, which were determined by serial dilution experiments, stained magnocellular neurosecretory cells sub-maximally, so that changes upward or downward could be detected. Specificity of immunohistochemical staining was confirmed by pre-absorption tests in which the primary antisera were pretreated with antigen-conjugated CNBr-activated Sepharose 4B (Pharmacia). The magnitudes of immunoreactive stainability were determined by measuring the brightness of unit area in each of stained cells by use of a illuminometric function of multipurpose research microscope (Nikon Microphoto FXA).

Statistics

Differences in the number of silver grains and immunoreactivity between the bay fish and the river fish were tested by Student's *t*-test.

RESULTS

VT and IT-immunoreactive (-ir) neurons, which are densely localized over the PMp, PMm and PMg in the preoptic nucleus, showed somewhat different distributional patterns (Fig. 1). In addition, IT-ir neurons are distributed in the ventrocaudal portion of the nucleus preopticus parvocellularis anterioris (PPa), locating rostral to the PM. IT-ir neurons in the PMg are smaller than VT-ir neurons, and localized rather in the ventral portion. The VT-ir and IT-ir neurons in the PMp, PMm and PMg have irregular oval shape, and their long axis are approximately 30-50 μm, 50-70 μm and 70-90 μm, respectively. The localization of neurons which include hybridization signals for VT and IT mRNA generally coincide with the distribution of VT and IT-ir neurons. Comparison of the localization of hybridization signals in the alternate sections showed that VT and IT mRNA signals were localized in discrete magnocellular neurons (Fig. 2).

Hybridization signals for VT mRNA in individual neurons

In the females, the number of silver grains for VT mRNA in individual neurons were apparently fewer in the river fish than in the bay fish (Fig. 3a, b). Quantitative analysis confirmed that such differences were statistically significant in all the three PM loci (Fig. 4). Contrary, in the males, no significant changes in the number of grains were observed between the bay and the river fish. Reflecting these sexually different changes, the level of VT mRNA in the males was higher than those in the females when compared within the river fish. Although such changes in the level of VT mRNA were seen, the sizes of hybridization positive cells were not remarkably changed during the last stage of spawning migration (Fig. 4).

Hybridization signals for IT mRNA in individual neurons

In the males, the level of IT mRNA in the PMp and the PMm was significantly higher in the river fish than in the bay fish, although not statistically significant in the PMg (Fig. 5). Contrary, in the females, the level of IT mRNA was significantly lower in the river fish than in the bay fish, except for the PMg which showed a similar but not significant change. As well as the level of VT mRNA, the level of IT mRNA in the male river fish was higher than that in the females in the PMp and the PMm. The sizes of IT-hybridization positive cells were not remarkably changed during the last stages of spawning migration.

Hybridization signals in the unit area

The pattern of changes in the number of grains per unit area between the bay and the river fish generally coincided with that in the number of grains per cell in both VT and IT hybridization positive cells (Fig. 6). In contrast to the difference in the number of grains among the individual PMp, PMm and PMg neurons, the number of grains per unit area in the three loci were almost at the same level. The differences in the number of grains per cell among the three loci (Figs. 4 and 5) may only reflect the difference of cell sizes.

Immunoreactivity

In the females, VT and IT immunoreactivities in the river fish were higher than those in the bay fish in the three PM loci (Figs. 3a, b and 7). Whereas, in the males, IT immunoreactivity was higher in the river fish than in the bay fish only in the PMp, although VT immunoreactivity did not show any significant changes. In the neurohypophysis, not so remarkable but significant decrease was observed only in the IT immunoreactivity in the female river fish when compared with the female bay fish.

DISCUSSION

In the present study, we found that the expression of neurohypophysial hormone genes in preoptic magnocellular neurons were sexually different in pre-spawning chum salmon. When the magnitudes of hybridization signals for VT mRNA were compared between the bay and the river fish of both sexes, the level of VT mRNA was decreased in the females, but not changed in the males, coincidentally in all the three PM loci, PMp, PMm and PMg. The level of IT mRNA in the river fish was lower in the females and higher in the males than those in the bay fish. These changes in the IT mRNA level were predominant in the PMp and the PMm. The changes in immunohistochemical stainability did not necessarily coincide with those in the intensities of hybridization signals. In the females, when compared the river fish with the bay fish, VT and IT immunoreactivities were increased in contrast to the decrease in the VT and IT mRNA levels just mentioned above. In the males, VT immunoreactivity was not changed, but IT immunoreactivity was increased in the PMp of the river fish.

Hiraoka *et al.* (1995a) determined the changes in the VT and IT mRNA levels in the forebrain which included the entire PM by quantitative Northern blot analysis using chum salmon of the same population, which were collected concomitantly with the animals used in the present study. The patterns of changes in the VT and IT mRNAs in the forebrain coincided well with the present morphometric results in individual VT

and IT neurons. Further, the pattern of changes in VT gene expression were consistent in animals captured in both 1993 and 1994, however, those in IT gene expression were not consistent between 1993 and 1994 samples. We therefore consider that the decrease in the VT mRNA level in individual PM neurons in the females is consistent and also biologically

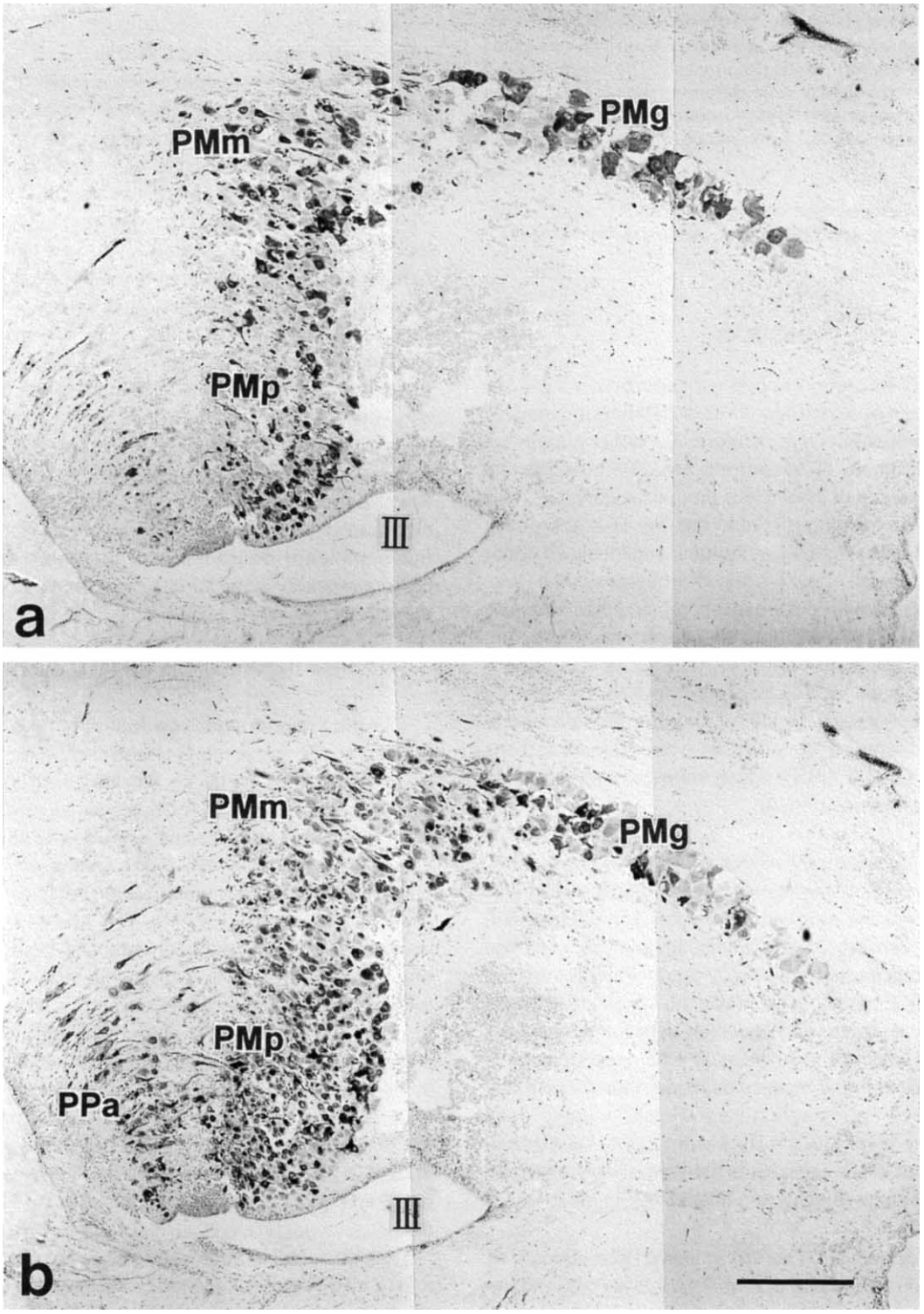

Fig. 1. Distribution of immunoreactive VT (a) and IT (b) neurons in adjacent sagittal sections of the preoptic nucleus of the female river fish. Most of immunoreactive neurons are darkly stained, whereas neighboring non-immunoreactive neurons are lightly counterstained with cresylviolet. The VT immunoreactive (-ir) cells and IT-ir cells were localized in three PM loci, pars parvocellularis (PMp), pars magnocellularis (PMm) and pars gigantocellularis (PMg). IT-ir cells were also localized in the ventrocaudal portion of the nucleus preopticus parvocellularis anterioris (PPa). III, third ventricle. Scale bar, 0.5 mm.

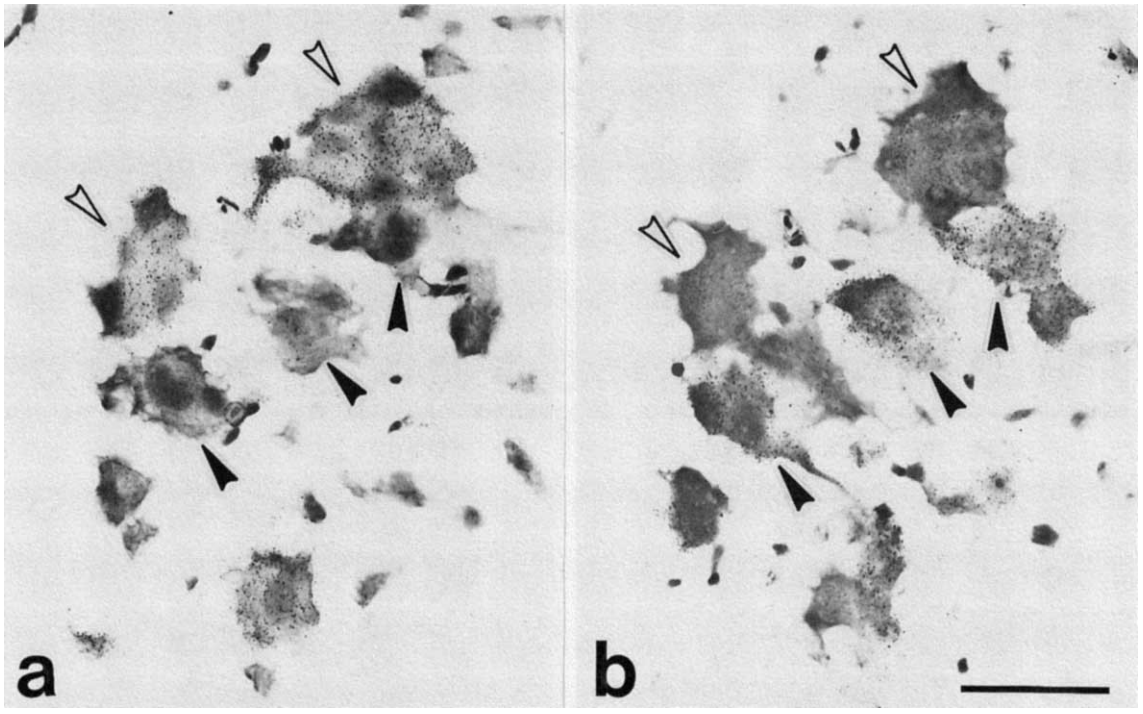

Fig. 2. Comparison of localization of VT mRNA hybridization signals (a) with that of IT mRNA hybridization signals (b) in alternate sections of the female river fish. Open arrowheads show neurons which contain hybridization signals for VT mRNA, and filled arrowheads show neurons which contain hybridization signals for IT mRNA. VT and IT mRNAs are expressed in separate magnocellular neurons. Sections are counterstained with cresylviolet. Scale bar, 50 μm.

meaningful phenomenon in pre-spawning salmon during the last stages of spawning migration.

The decrease in the VT mRNA level in the female river fish suggests that the expression of VT gene was repressed in the pre-spawning females. The present result that the VT immunoreactivity in individual somata was increased in the female river fish probably indicates reduction in the rates of transport and release of VT. In the rat, osmotic stimulation increased the size of neurosecretory neurons in the supraoptic and paraventricular nuclei and also the proportion of cells which had dilated endoplasmic reticulum (Morris and Dyball, 1974). The size of cells thus can be considered to reflect the synthetic activity of hormones. The present result that the cell size remained rather unchanged may reflect that translational activity was not so much affected in magnocellular neurons. It is probable that secretory activity of VT neurons is suppressed in the female river fish during the last stages of spawning migration.

Despite different sizes and location of VT-ir neurons, the decrease in VT gene expression in the female river fish coincided in all the three PM loci. This result suggests that, in the pre-spawning chum salmon, the expression of VT gene in the PM may be regulated by a common input signal, such as a humoral factor. In mammals, sex steroid hormones had regulatory influences on vasopressin and oxytocin gene expression (Adan and Burbach, 1992), and vasopressin gene expression was repressed by glucocorticoid in the paraventricular nucleus (Davis *et al.*, 1986). In rainbow trout, estrogen receptor-immunoreactive cells and cells which produce estrogen receptor mRNA are located in three brain regions, the ventral telencephalon, the anterior ventral preoptic region, and the mediobasal hypothalamus (Anglade *et al.*, 1994; Salbert *et al.*, 1991). Ueda *et al.* (1984) determined changes in the plasma steroid hormone levels in pre-spawning chum salmon, and found that the concentration of estradiol-17β was high in the earlier phase of up-river migration, but decreased in the spawning period in females. Such changes in plasma sex steroid hormone may induce changes in expression of VT gene in female pre-spawning chum salmon. In addition, Hirano *et al.* (1990) reported that, in pre-spawning chum salmon caught at the Otsuchi bay, the plasma cortisol level was higher in females than in males. Particularly in the females, the cortisol levels were higher in the bay and the river fish than those in the ocean fish. Such changes in the plasma cortisol levels seem to be opposite to those in the level of VT mRNA in the chum salmon also caught at the Otsuchi bay (Hiraoka *et al.*, 1995b). However, since the fish in the Otsuchi bay completed gonadal maturation before entering the river, the maturity of the bay fish in this study seemed to correspond to that of the ocean fish in the Otsuchi population. It is therefore probable that expression of VT gene was repressed by cortisol in the pre-spawning female. In fact, three estrogen responsive elements and four corticoid responsive elements are present in the 5'-upstream region of the VT-I gene in chum salmon (Satomi *et al.*, 1994).

Several studies have indicated that neurohypophysial hormones are involved in osmoregulation in teleosts (see Urano *et al.*, 1994). In immature rainbow trout, the level of VT

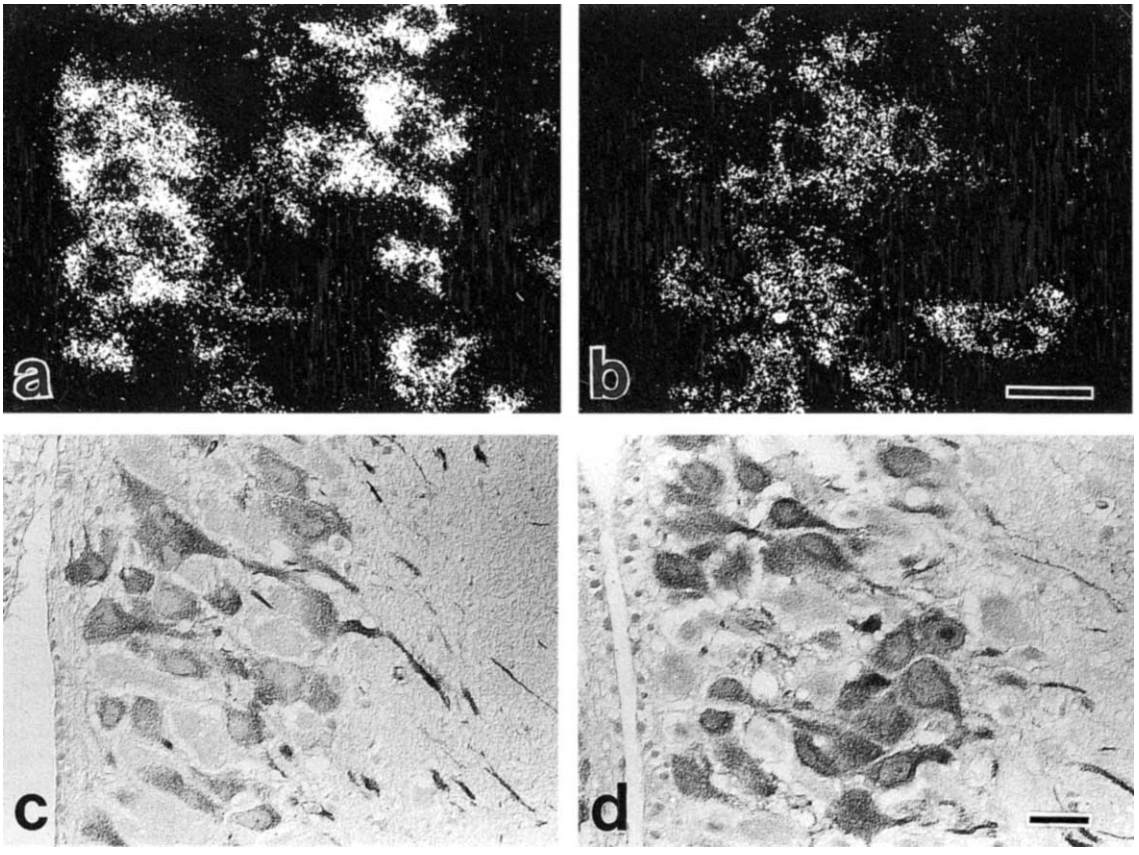

Fig. 3. Difference in VT mRNA level and VT immunoreactivity between the bay fish and the river fish in the females. The number of silver grains in magnocellular neurons of the bay fish (a) are greater than that of the river fish (b), whereas VT immunoreactivity in the bay fish (c) are lower than that of the bay fish (d). Scale bar, 50 μm.

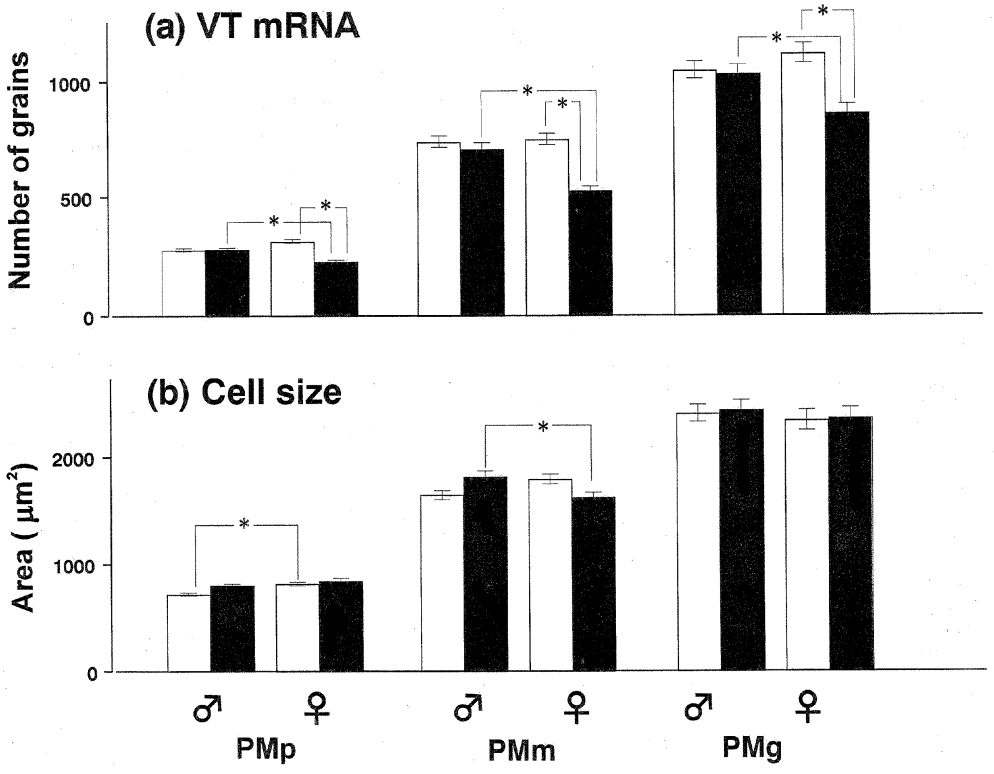

Fig. 4. The number of grains per cell (a) and cell size (b) of neurons labeled for VT mRNA in PMp (n=150), PMm (n=74-91) and PMg (n=11-51). Mean ± SEM. □ Bay fish; ■ River fish. *P<0.01.

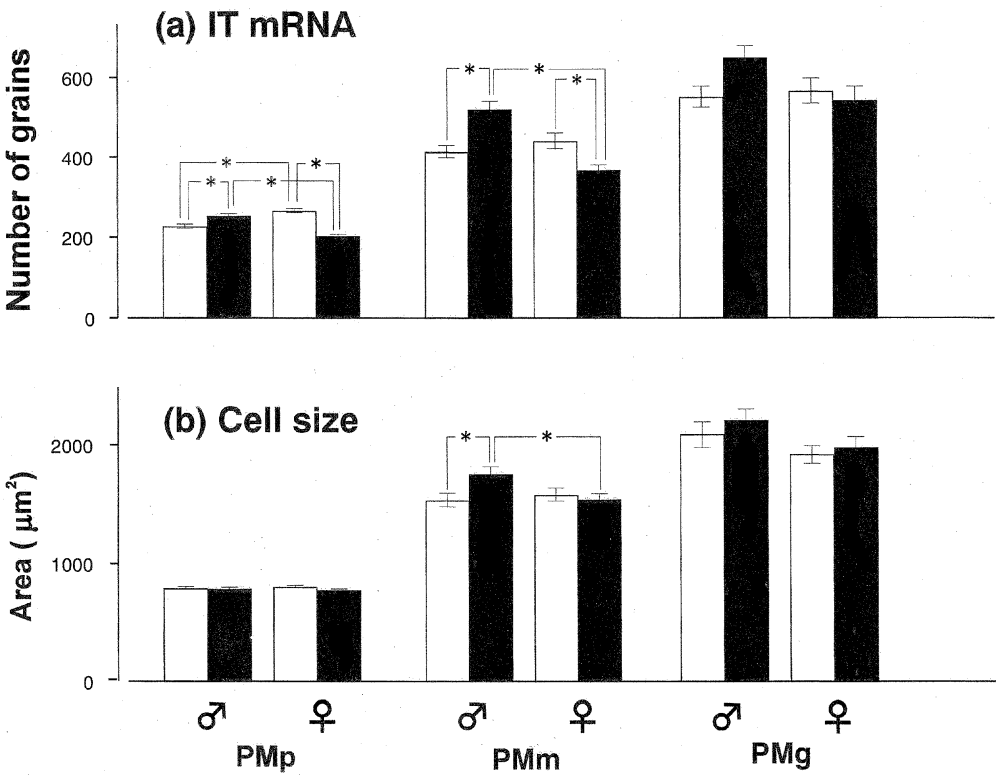

Fig. 5. The number of grains per cell (a) and cell size (b) of neurons labeled for IT mRNA in PMp (n=150), PMm (n=65-75) and PMg (n=36-50). Mean ± SEM. □ Bay fish; ■ River fish. *P<0.01.

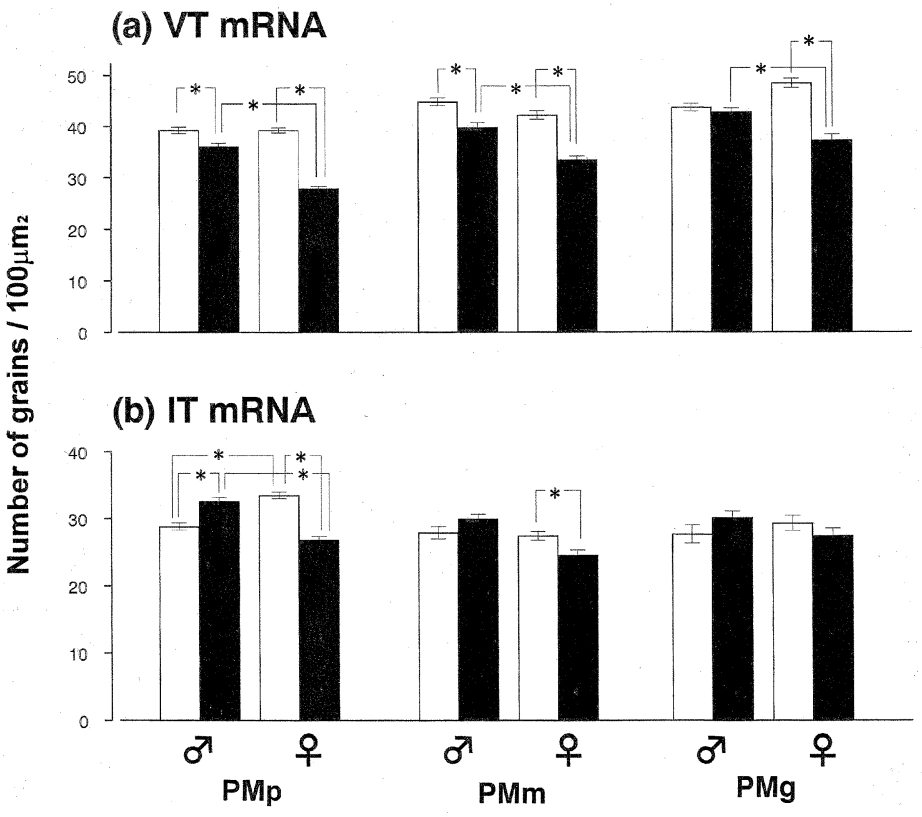

Fig. 6. The number of grains per unit area in VT neuron (a) and IT neuron (b) which were calculated from the number of grains in individual neurons and their area shown in Fig. 4 and Fig. 5. Mean ± SEM. □ Bay fish; ■ River fish. *P<0.01.

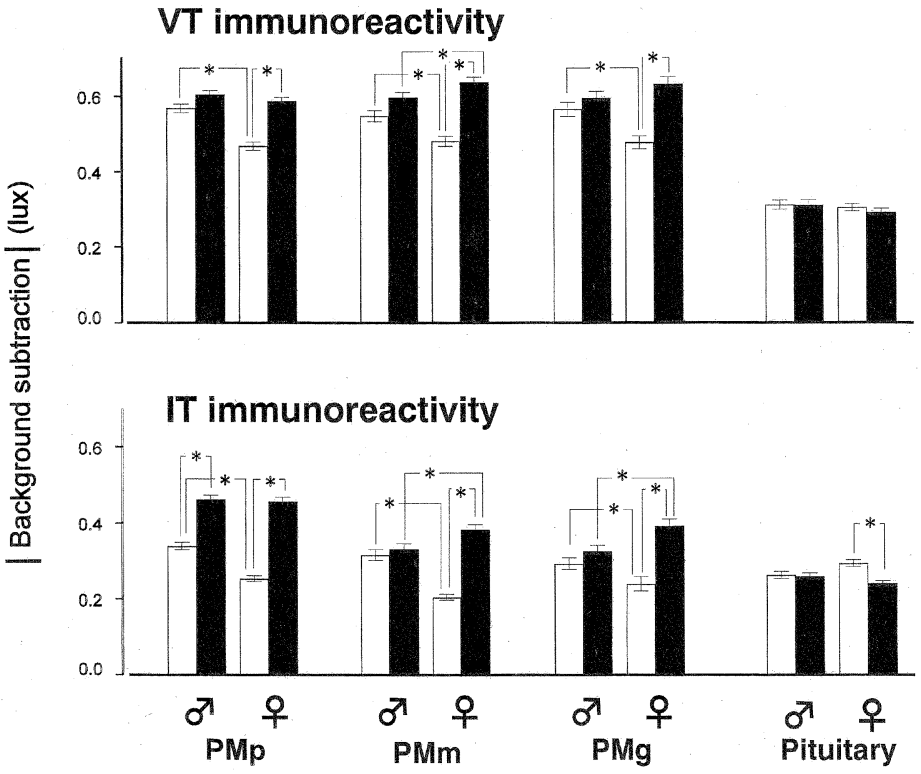

Fig. 7. VT and IT immunoreactivities of neurons in PMp (n=150), PMm (n=78-100) , PMg (n=51-75) and neurohypophysis (n=100). Immunostainabilities are expressed as the |background subtraction|. Mean ± SEM. □ Bay fish; ■ River fish. *P<0.01.

mRNA in the magnocellular neurosecretory neurons was decreased by transfer from FW to 80% SW, and was increased by transfer back to FW from 80% SW, suggesting that VT is involved in FW adaptation (Hyodo and Urano, 1991a). However, the present results in the females showed that the expression of VT gene was decreased in the animals apparently well adapted to FW (Table 1). The plasma osmolality and the sodium concentration were also not different between the males and the females. Thus, the sexually different expression of VT gene in pre-spawning chum salmon can not be explained by the changes in environmental osmolality.

It has been indicated that VT is involved in spawning behavior in teleosts (see Liley and Stacey, 1983). Further, VT has regulatory effects in reproductive behavior in several vertebrate species (see Moore, 1992). In the males, mating behavior was enhanced by VT injection in the pigeon, domestic chicken and rough-skinned newt. In the male prairie voles (*Microtus ochrogaster*), cohabitation with a female increased the level of vasopressin mRNA in the bed nucleus of the stria terminalis which is the presumed source of vasopressin-immunoreactive fibers in the lateral septum and the lateral habenular nucleus, suggesting that vasopressinergic projection may play an important role in mating-induced changes in social behavior (Wang *et al.*, 1994). The present result that VT gene expression was maintained in the males but decreased in the females may indicate an involvement of VT in the control of male mating behavior rather than osmoregulation.

In conclusion, the changes in the expression of VT and IT genes were sexually different during the last stages of spawning migration. The expression of VT gene in pre-spawning females was significantly decreased in all the three PM loci. These results suggest that the regulations of VT and IT gene expression are sexually different between the bay fish and the river fish.

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