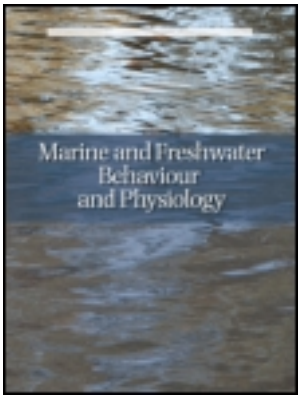

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Effect of hypo-osmotic environmental changes on the expression of gonadotropin-releasing hormone, its receptor, and gonadotropin hormone subunit mRNA in adult chum salmon (Oncorhynchus keta)

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Effect of hypo-osmotic environmental changes on the expression of gonadotropin-releasing hormone, its receptor, and gonadotropin hormone subunit mRNA in adult chum salmon (*Oncorhynchus keta*)

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Migrating fish such as salmonids are affected by external environmental factors and salinity changes are particularly important, influencing spawning migration. The aim of this study was to test whether changes in salinity would affect the expression of the hypothalamic-pituitary-gonadal (HPG) axis hormones (gonadotropin-releasing hormones (GnRHs) [salmon GnRH and chicken GnRH-II], GnRH receptors [GnRHR1 and GnRHR5], and mRNA of the gonadotropin hormone [GTH] subunits [GTHα, follicle stimulating hormone β, and luteinizing hormone β]) in chum salmon (*Oncorhynchus keta*). Fish were progressively transferred from seawater (SW) through 50% SW to freshwater (FW), and the relationship between the osmoregulatory hormone prolactin (PRL) and sexual maturation was determined. The expression and activity of HPG hormones and their receptors, and levels of estradiol-17β and PRL increased after fish were transferred to FW, demonstrating that changes in salinity stimulate the HPG axis and PRL production in migrating chum salmon. These findings reveal details about the role of the endocrine system in maintaining homeostasis and stimulating sexual maturation and reproduction in response to salinity changes in this species.

Keywords: chum salmon; *Oncorhynchus keta*; GnRH; hypothalamic-pituitary-gonadal axis; salinity change

Introduction

Chum salmon (*Oncorhynchus keta*) is an anadromous fish that spawns in rivers; the fry then migrate downstream to the sea to complete their early life stages, following which they return to their natal rivers to reproduce (Ueda 2011). Anadromous salmon of the genus *Oncorhynchus* (e.g. pink salmon [*O. gorbuscha*], coho salmon [*O. kisutch*], and masu salmon [*O. masou*]) migrate upstream and spawn in their natal rivers in fall (Machidori & Katou 1984; Quin 2005). Maturing salmon are regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which controls not only this migration, but also the associated sexual maturation. It has been found that the exogenous application of gonadotropin-releasing hormones (GnRHs) can activate the HPG axis, accelerating

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maturation and the timing of freshwater (FW) entry and upstream movement in many species of salmonids (Mylonas & Zohar 2001). In fish and other vertebrates, the control of reproduction via GnRHs is a complex process that involves the interaction of a number of factors, including the gonadal steroid hormones gonadotropin hormones (GTHs) (Urano et al. 1999; Mylonas & Zohar 2001).

GnRHs, which are produced by the hypothalamus and regulate maturation and gonadal development in fish, are key regulators of this system. Furthermore, they also stimulate the synthesis and release of pituitary hormones, most notably, follicle stimulating hormone (FSH) and luteinizing hormone (LH), which, in turn, stimulate gonadal gametogenesis and steroidogenesis (Ando & Urano 2005). In addition, GTHs stimulate the release of the maturation-inducing steroid in the gonad, which induces gonadal maturation and ovulation in salmon (Nagahama et al. 1983; Nagahama & Adachi 1985).

Two types of GnRH isoforms are conserved in salmonids: salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II) (Amano et al. 1992). In some teleosts, sGnRH facilitates the electrical activity of the central neurosecretory cells and controls GTH secretion, as well as being implicated in the regulation of spawning behavior (Saito et al. 2003). It has also been shown that sGnRH neurons regulate both final maturation and migratory behavior in homing salmonids via a GnRH analog (GnRHa), which increases the amount of GTH mRNAs in the pituitary and induces further acceleration of resumption of meiosis in the oocytes, which takes place just before ovulation (Kitahashi et al. 1998). cGnRH-II neurons are localized in the midbrain tegmentum and project their axons widely throughout the central nervous system to modulate the sexual and feeding behavior of salmonids (Onuma et al. 2010).

The timing of sexual maturation and spawning in fish may also be affected by environmental parameters, such as photoperiod, salinity, and water temperature (i.e. FW and/or seawater [SW] adaptation) (Takashima & Yamada 1984; Hirano et al. 1990; Aida 1991; Pankhurst & Thomas 1998; Duncan et al. 2000). Furthermore, the behavior and physiology of migrating fish such as salmonids are also affected by external environmental factors, such as photoperiod and water temperature (Duncan et al. 2000). Salinity change is one of the most important direct environmental factors that influences spawning migration. However, little information is available about how changes in environmental salinity affect sex steroid hormones and maturation. Previous studies have shown that the maturation that occurs as fish move from SW to FW environments can be regulated by GnRHa treatment (Cooperman et al. 2010), the pituitary-gonadal (PG) axis, and prolactin (PRL), all of which act as FW-adaptation hormones during spawning migration (Hirano et al. 1987; Onuma et al. 2005). The sGnRH may also regulate PRL cells directly or indirectly in combination with the PG axis, e.g. via the production of sex steroid hormones (Onuma et al. 2005).

The aim of the study was to investigate how a change in salinity stimulates the HPG axis hormones (GnRHs [sGnRH and cGnRH-II], GnRH receptors [GnRHR1 and GnRHR5], and mRNA of the GTH subunits [GTHα, FSHβ, and LHβ]) in chum salmon as they travel from coastal SW to hatchery FW environments. The effect of salinity on the osmoregulatory hormone PRL was also examined by observing changes in hormone-related maturation as fish were transferred into a FW environment. Fish were progressively transferred from 100% SW through 50% SW to FW to record changes in the expression of GnRHs, GnRH receptors, and GTH subunits through time. Adult chum salmon were also transferred from coastal SW to an artificially hypo-osmotic environment (SW→50% SW→FW) to examine the response of plasma GnRH, FSH, LH, and estradiol-17β (E_2) levels and PRL to salinity changes.

Materials and methods

Experimental fish

Mature female chum salmon ($n = 4$–5; length = 62.4 ± 7.4 cm, weight = 2.62 ± 0.57 kg, gondosomatic index [gonad weight/body weight] = 18.3 ± 3.8) were collected from Ishikari Bay, Hokkaido, Japan (coastal sea) on 15 October 2011 and transported to Hanazono, Hokkaido, Japan. Fish were maintained in four 40 L tanks for the duration of the experiment (3 days).

The transfer of chum salmon from SW (35 psu) to FW (0 psu) followed a specific procedure. The salmon were acclimated in a square tank filled with SW, following which ground water was poured into the tank to give a concentration of 50% SW (17.5 psu); fish were maintained in this water for 24 h, after which more ground water was added to completely dilute the tank water to FW, in which the fish were held for a further 24 h. The water temperature was maintained at 20 ± 0.5 °C. No fish died during the experimental period.

Sampling

The brain and pituitary were collected from 5 randomly selected fish from each salinity group (SW, 50% SW, and FW) 24 h after transfer. The organs were immediately frozen in liquid nitrogen and stored at −80 °C until total RNA extraction was performed. In addition, blood was collected from the caudal vasculature using a 3 mL heparinized syringe and centrifuged (10,000 × g, 4 °C, 5 min), following which the plasma was stored at −80 °C until analysis.

Quantitative real-time PCR (QPCR)

QPCR was conducted on the total RNA extracted from the brain and pituitary of chum salmon to determine the relative expression of 2 GnRH types (sGnRH and cGnRH-II), GnRH receptors (GnRHR1 and GnRHR5), mRNA of the GTH subunits (GTHα, FSHβ, and LHβ), and PRL using a TRIzol kit (Gibco/BRL). Primers for QPCR were designed with reference to the known sequences of chum salmon, which are shown in Table 1. QPCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following conditions: 0.5 μL cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, SYBR green, and Taq polymerase in buffer (10 mM Tris–HCl [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, and 20 nM fluorescein), which were made up to a total volume of 25 μL. QPCR was performed as follows: 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, and annealing at 55 °C for 20 s. For each experimental group, QPCR was run in triplicate to confirm consistency and, as an internal control, experiments were repeated using β-actin, which gave a reaction efficiency of 95.7%. All data were expressed as change with respect to the corresponding β-actin-calculated cycle threshold (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and the internal control (β-actin) was calculated as $\Delta\Delta Ct = 2\text{^} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$. In addition, to ensure that the primers amplified a specific product, we performed a melt curve analysis, which showed that the products of each primer pair had a single melting point.

Western blot analysis

Total protein was extracted from the brain and pituitary of female chum salmon using a protein extraction buffer (5.6 mM Tris, 0.55 mM ethylenediaminetetraacetic acid

Table 1. Primers used for QPCR amplification.

Genes	Primer	DNA sequences
sGnRH (JX183101)	Forward	5′-CTT AGC AAC AGA ACG GTC G-3′
	Reverse	5′-CCA GGT AGC CAG CCA TAC-3′
cGnRH-II (AB365004)	Forward	5′-TGT GTC TGG GAG CCC AGC T-3′
	Reverse	5′-CAG AGG TGG TAA ATG AGT CCA-3′
GnRHR1 (AB107910)	Forward	5′-CAC CTT AAT CCT CTT CCT ATT TGC C-3′
	Reverse	5′-GCT CAT GAT GAG TGG TCG CAG-3′
GnRHR5 (JX183102)	Forward	5′-AAT GTT CCC GCC AGA GAA-3′
	Reverse	5′-AGA GGT TGG AGA AGG CAG-3′
GTHα (M27152)	Forward	5′-CTC ATC CTG TCC GCA CTT-3′
	Reverse	5′-GAG AAG CAG CAG CCT GTA-3′
FSHβ (M27153)	Forward	5′-ACC ATC ATC GTG GAG AGA G-3′
	Reverse	5′-GAT AGT TCA GGT CCG TTG TTT C-3′
LHβ (M27154)	Forward	5′-TTT CAA GAG CCC ATT TTC CA-3′
	Reverse	5′-CCA CAG GGT AGG TGA CAT-3′
PRL (D00249)	Forward	5′-CTT CAC TCA CTC AGC ACT TC-3′
	Reverse	5′-CCT TGT CCT TGG GTG TCT-3′
β-actin (JX183093)	Forward	5′-ATT TGG CAT CAC ACC TTC T-3′
	Reverse	5′-TTC TCC CTG TTG GCT TTG-3′

(EDTA), 0.55 mM ethyleneglycotetraacetic acid (EGTA), 0.1% sodium dodecyl sulfate (SDS), 0.15 mg mL^{-1} phenylmethylsulfonyl fluoride, and 0.15 mg mL^{-1} leupeptin). It was then sonicated and quantified using the Bradford method (Bio-Rad). Total protein (30 µg per lane) was loaded onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel, and a protein ladder (Fermentas, Hanover MD, USA) was used for reference. Samples were electrophoresed at 80 V through the stacking gel and at 150 V through the resolving gel until the bromophenol blue dye front had run off of the gel. The gels were then immediately transferred to a 0.2 µm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 1.5 h at 4 °C. Thereafter, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min, following which they were washed in TBS. The membranes were incubated with GnRH antibodies (LRH13; a monoclonal mouse antiserum that recognizes most vertebrate GnRH forms; dilution, 1:5000; courtesy of M.K. Park (Park & Wakabayashi 1986)), followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (dilution, 1:5000; Bio-Rad) for 60 min. In addition, the membranes were incubated with GTHα antibodies (anti-goldfish GTHα; a polyclonal rabbit antibody; dilution, 1:2000; courtesy of Kobayashi [Kobayashi et al. 2006]), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:5000; Bio-Rad, USA) for 60 min. The internal control was β-tubulin (dilution, 1:5000; ab6046, Abcam, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:5000; Bio-Rad) for 60 min. Bands were detected using the sensitive electrochemiluminescence (ECL) systems (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposed for 2 min using a Molecular Imager® ChemiDoc™ XRS + Systems (Bio-Rad).

Plasma parameter analysis

Plasma osmolality was examined using a Vapor Pressure Osmometer (Vapro 5600; Wescor Co., Logan, UT). Plasma GnRH, FSH, and LH levels were analyzed using the immunoassay technique with the ELISA kits E0843f, E0462f, and E0441f, respectively

(EIAab Science, Wuhan, China), and plasma E_2 and PRL levels were analyzed using the immunoassay technique with the ELISA kits E13017Fh and E12695Fh, respectively (Cusabio Biotech, Hubei, China).

An anti-antibody that was specific to the antibody of the hormones (GnRH, FSH, LH, E_2, and PRL) was pre-coated onto a microplate, following which 50 µL of plasma, 50 µL of HRP-conjugate, and 50 µL of the antibody were added to each well. These were mixed well and then incubated for 2 h at 37 °C. Following the last wash, any remaining Wash Buffer was aspirated or decanted off, and 50 µL each of Substrates A and B were added to each well. These substrate solutions were then incubated for 15 min at 37 °C in the dark, during which they changed from colorless or light blue to darker shades of blue. Following incubation, 50 µL of stop solution was added to each well, resulting in the color changing from blue to yellow. The optical density of the solution in each well was then determined within 10 min, using a microplate reader set to 450 nm. The following standard curve concentrations were used for the ELISA: GnRH – 500, 250, 125, 62.5, 3.12, 15.6, and 7.8 pg mL^{-1}; GTHs (FSH and LH) – 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mIU mL^{-1}; E_2 – 1000, 500, 200, 60, and 20 pg mL^{-1}; and PRL – 4000, 1200, 200, 40, and 16 µIU mL^{-1}.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way analysis of variance followed by Tukey's *post hoc* test was used to test for significant differences in the data ($p < 0.05$). Values are expressed as mean ± standard error (SE).

Results

Effect of changing salinity on the expression of sGnRH and cGnRH-II mRNA

Western blot analysis detected LRH13 protein of a size that corresponded to the predicted size for chum salmon (approximately 52 kDa), which exhibited similar mRNA expression across the 3 salinities (Figure 1(a)). By contrast, the expression of both sGnRH (Figure 1(b)), and cGnRH-II (Figure 1(c)) mRNA in the brain was significantly higher following transfer to FW (approximately 5.4- and 1.9-fold higher, respectively) than in 50% SW and SW.

Effect of changing salinity on the expression of GnRHR1 and GnRHR5 mRNA

The expression of GnRHR1 and GnRHR5 mRNA in the pituitary is shown in Figure 2. It was found that both GnRHR1 (Figure 2(a)) and GnRHR5 (Figure 2(b)) mRNA were significantly higher following transfer to FW (approximately 23.2- and 9.8-fold higher, respectively) than in 50% SW and SW.

Effect of changing salinity on the expression of GTH subunit mRNA

The expression of mRNA of the GTH subunits (GTHα, FSHβ, and LHβ) in the pituitary is shown in Figure 3. It was found that the mRNA of all 3 subunits was significantly higher following transfer to FW (approximately 15.1-, 5.9-, and 13.5-fold higher, respectively) than in 50% SW and SW. In addition, western blot analysis

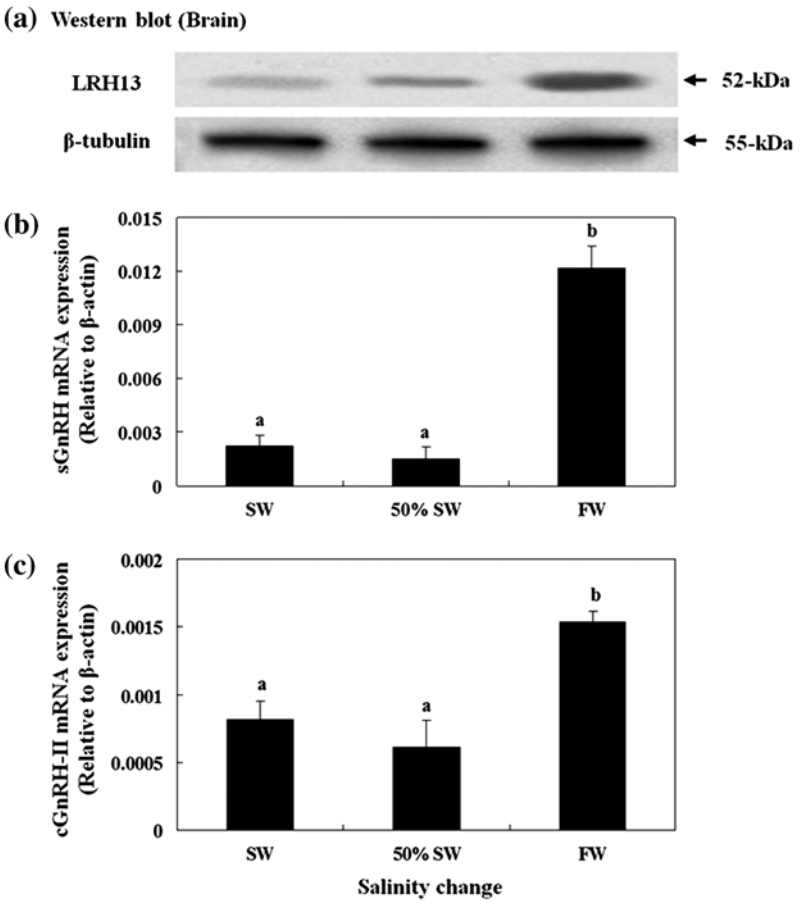

Figure 1. sGnRH and cGnRH-II mRNA expression levels in the brain of chum salmon after a change in salinity by transferring them from SW (35 psu) to FW (0 psu). (a) Western blot of LRH13 (a monoclonal mouse antiserum that recognizes most vertebrate GnRH forms; dilution, 1:5000; 52 kDa) protein expression in the brain of chum salmon during the salinity change. β-tubulin (55 kDa) was used as the internal control. Expression of sGnRH mRNA level (b) and cGnRH-II mRNA level (c) in the brain of chum salmon female during salinity change by using QPCR. We reverse-transcribed 3 μg of total RNA prepared from the brain and amplified the sample using gene-specific primers. The results are expressed as a normalized fold expression (relative to the control) with respect to β-actin levels for the same sample. Values are mean ± SE ($n = 4$–5). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time period after salinity change ($p < 0.05$).

detected a GTHα protein of a size that corresponded to the predicted size for chum salmon (approximately 35 kDa), which exhibited similar mRNA expression across all 3 salinities (Figure 3(a)).

Plasma GnRH, FSH, and LH assays

Plasma GnRH, FSH, and LH concentrations were $13.6 \pm 2.3\ \text{pg mL}^{-1}$, $18.9 \pm 4.2\ \text{mIU mL}^{-1}$, and $18.8 \pm 4.2\ \text{mIU mL}^{-1}$, respectively, in SW. Following the transfer to FW, plasma GnRH levels gradually increased to reach levels that were approximately

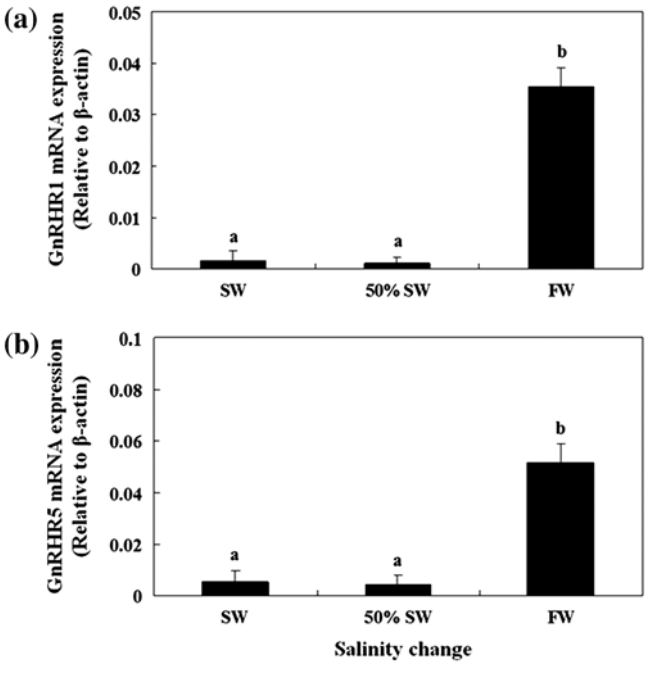

Figure 2. GnRHR subunits of mRNA expression levels in the pituitary of chum salmon after salinity transfer from SW (35 psu) to FW (0 psu). Expression of GnRHR1 mRNA level (a) and GnRHR5 mRNA level (b) in the pituitary of chum salmon females during salinity change. We reverse-transcribed 3 μg of total RNA prepared from the pituitary and amplified the sample using gene-specific primers. The results are expressed as a normalized fold expression (relative to the control) with respect to β-actin levels for the same sample. Values are mean ± SE ($n = 4$–5). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time period after salinity change ($p < 0.05$).

5.2-fold higher, and plasma FSH and LH levels were also significantly higher (4- and 15.9-fold, respectively) than in 50% SW and SW (Figure 4).

Plasma E_2 assay

Plasma E_2 levels were $306.2 \pm 50.0\ \text{pg mL}^{-1}$ in SW. Following the transfer to FW, these levels significantly increased to $2659.9 \pm 300.2\ \text{pg mL}^{-1}$ (Figure 5).

Plasma osmolality assay

Plasma osmolality levels were $400.8 \pm 27.8\ \text{mOsm kg}^{-1}$ at the start of the experiment. At 24 h after transfer to 50% SW and FW, these levels significantly declined to 339.0 $\pm 17.0\ \text{mOsm kg}^{-1}$ and $304.4 \pm 4.4\ \text{mOsm kg}^{-1}$, respectively (Table 2).

Effect of changing salinity on the expression and activity of PRL

The expression of PRL mRNA in the pituitary is shown in Figure 6. PRL mRNA was significantly higher following transfer to FW (approximately 35.7-fold higher) than in

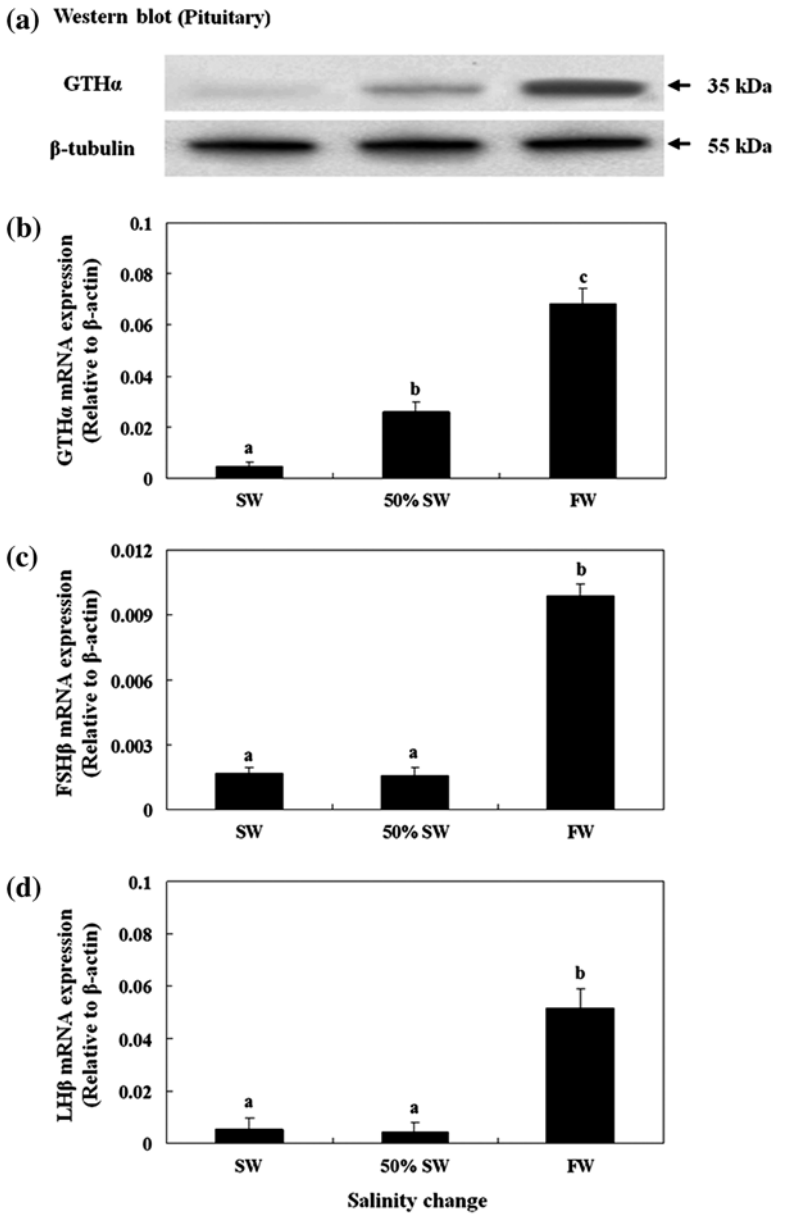

Figure 3. GTH subunits of mRNA expression levels in the pituitary of chum salmon after salinity transfer from SW (35 psu) to FW (0 psu). (a) Western blot of GTHα (a monoclonal rabbit antiserum; dilution, 1:5000; 35 kDa) protein expression in the pituitary of chum salmon during the salinity change. β-tubulin (55 kDa) was used as the internal control. The expression of GTHα mRNA level (b), FSHβ mRNA level (c), and LHβ mRNA level (d) in the pituitary of chum salmon females during salinity change by using QPCR. We reverse-transcribed 3 μg of total RNA prepared from the pituitary and amplified the sample using gene-specific primers. The results are expressed as a normalized fold expression (relative to the control) with respect to β-actin levels for the same sample. Values are mean ± SE ($n = 4$–5). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time period after salinity change ($p < 0.05$).

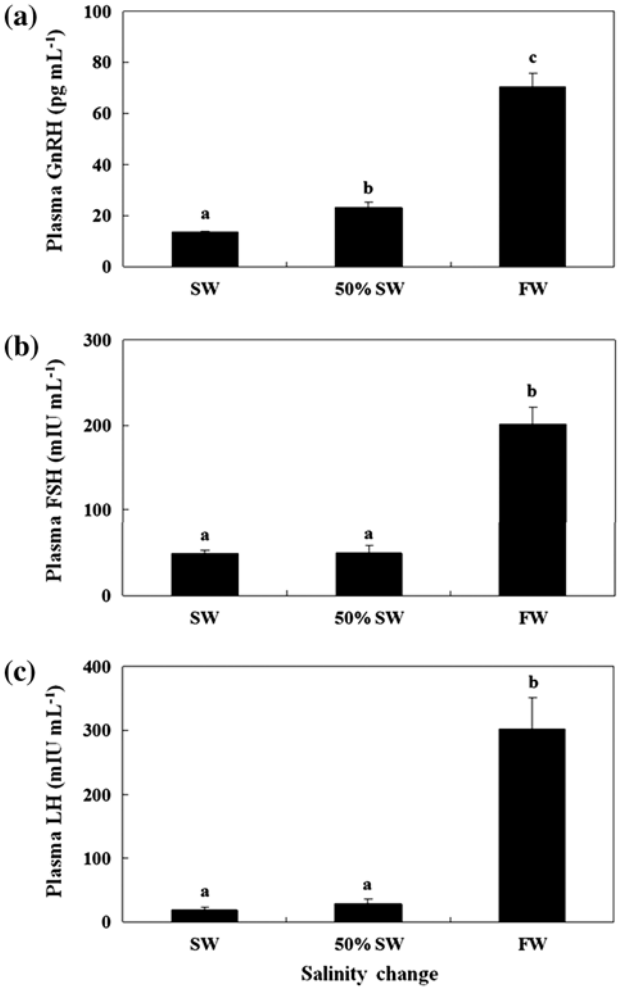

Figure 4. Plasma GnRH (a), FSHβ (b), and LHβ (c) levels during salinity change in chum salmon females. Values are mean ± SE ($n = 4$–5). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time period after salinity change ($p < 0.05$).

50% SW and SW. Plasma PRL levels also significantly increased following the transfer to FW, from an initial level of $10.5 \pm 3.3\ \mu IU\ mL^{-1}$ in SW to $40.2 \pm 5.2\ \mu IU\ mL^{-1}$ in FW (Figure 6).

Discussion

The expression and concentration of GnRHs (sGnRH, cGnRH-II), GnRHRs (GnRHR1 and GnRHR5), and GTH subunits mRNA were compared following the transfer of migrating adult female chum salmon from SW to an artificially hypo-osmotic environment (SW→50% SW→FW). In addition, changes in salmon plasma E_2 levels in response to these salinity changes were examined. Our findings suggest that the HPG axis is affected by salinity changes during the migratory maturation process in chum salmon.

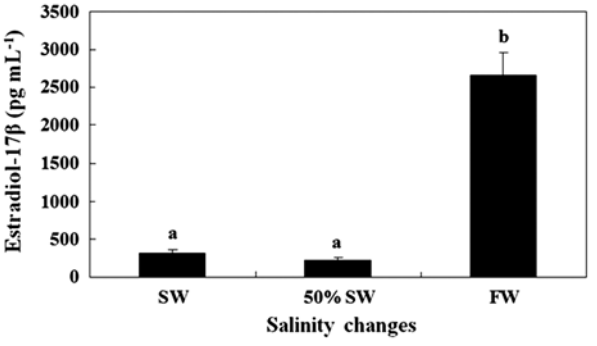

Figure 5. The plasma estradiol-17β (E_2) levels during salinity change in chum salmon females. Values are mean ± SE ($n = 4$–5). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time period after salinity change ($p < 0.05$).

Table 2. Plasma osmolality levels during salinity changes in chum salmon.

Ambient	Osmolality (mOsm kg^{-1})
SW (35.0 psu)	400.8 ± 27.8^{c}
50% SW (17.5 psu)	339.0 ± 17.0^{b}
FW (0 psu)	304.4 ± 4.4^{a}

Note: Different letters indicate significant differences ($p < 0.05$). All values are mean ± SE ($n = 4$–5).

The level of osmolality decreased when anadromous adult chum salmon were transferred to an artificially hypo-osmotic environment (Table 2), supporting findings from other studies that have investigated the effect of exposure to hypo-osmotic conditions on various fish species, including chum salmon (Hirano et al. 1987), black porgy (*Acanthopagrus schlegeli*; An et al. 2008), and cinnamon clownfish (*Amphiprion melanopus*; Park et al. 2011). This observed decline in osmolality may result from the osmoregulation of water inflow and ion outflow from the body (An et al. 2008; Park et al. 2011).

The expression of GnRHs (sGnRH and cGnRH-II) mRNA in the brain increased in FW (Figure 1), supporting the findings of previous studies on chum salmon (Onuma et al. 2005, 2010) and sockeye salmon (*O. nerka*; Cooperman et al. 2010), which showed that the expression of sGnRH mRNA increased after fish shifted from a SW to FW environment during upstream migration, as occurs in spawning salmonids. Onuma et al. (2010) reported that the expression of sGnRH mRNA increased during upstream migration, with sGnRH neurons contributing to the control of the PG axis and stimulation of the sex steroid hormone; they also found that the HPG axis was activated in fish when they moved into a FW environment. This may have been due to the elevated sGnRH levels regulating the PG axis, as HPG axis activity in salmonids is regulated by sGnRH, levels of which gradually increase in the brain with gonadal maturation during spawning (Amano et al. 1992). It is thus conceivable that the PG axis is activated by sGnRH neurons when fish migrate during the last stage of the spawning migration season, which subsequently induces upstream movement. Previous research has indicated that sGnRH is involved in the neuroendocrine control of FW adaptation in chum salmon, and that elevated activity of the neurohypophysial hormone produces neurons that are involved in the control of osmoregulation and reproductive behavior (Urano et al. 1994).

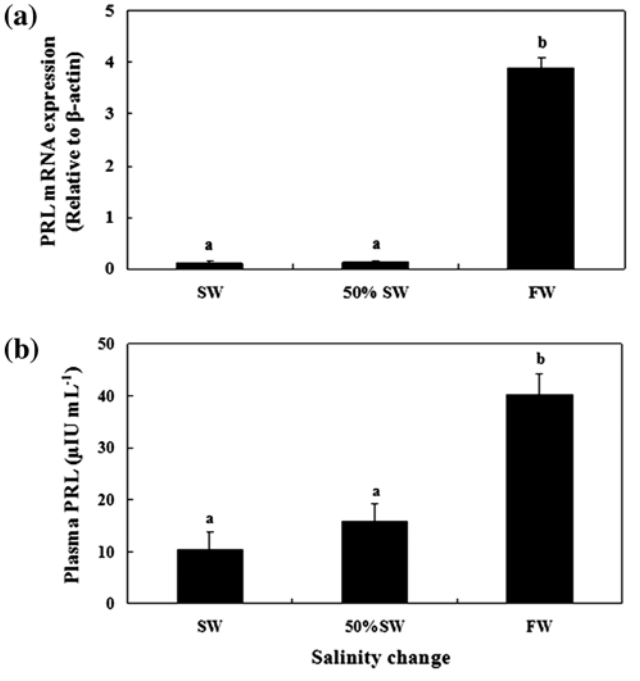

Figure 6. PRL mRNA expression levels and plasma PRL levels of chum salmon after salinity transfer from SW (35 psu) to FW (0 psu). (a) The expression of PRL mRNA level in the pituitary of chum salmon females during salinity change by using QPCR. We reverse-transcribed 3 μg of total RNA prepared from the pituitary and amplified the sample using gene-specific primers. The results are expressed as a normalized fold expression (relative to the control) with respect to β-actin levels for the same sample. (b) The plasma PRL levels during salinity change in chum salmon females. Values are mean ± SE ($n = 4$–5). Values with letters indicate significant differences between SW, 50% SW, and FW within the same time period after salinity change ($p < 0.05$).

The *in vitro* application of sGnRH to pituitary cells obtained from pre-spawning masu salmon (*O. masou*) has been shown to lead to an increase in PRL, which is a FW-adaptation hormone (Hirano et al. 1987; Onuma et al. 2005). It has also been shown that sGnRH stimulates the secretion and composition of PRL cells (Weber et al. 1997), and modulates salt and water homeostasis activity. In the present study, it was found that the expression of PRL mRNA in the pituitary and plasma PRL both increased in FW (Figure 6). It has previously been suggested that sGnRH is mediated by protein kinase A, because cyclic adenosine monophosphate (cAMP) has been shown to modulate the PRL content in the pituitary of rainbow trout (*Salmo gairdneri*; Johnston and Wigham 1988) and tilapia (*Oreochromis mossambicus*: Grau et al. 1982). Therefore, it seems that a FW environment enhances the production of PRL, sGnRH, and sex steroid hormone.

cGnRH-II has a direct effect on neuronal activity in the salmonid brain (Onuma et al. 2010). In rainbow trout, cGnRH-II increases the electrical activity of magnocellular neurosecretory neurons in a GnRHR-dependent manner (Saito et al. 2003); and Volkoff and Peter (1999) reported that intraventricular injections of cGnRH-II induced spawning behavior. Furthermore, Urano et al. (1999) hypothesized that the reproductive phenomena underlying spawning migration are coordinated by neuroendocrine cells such as GnRH neurons, which appropriate anatomical features to coordinate the functioning of the HPG axis and the central nervous system. Hence, it seems likely that

cGnRH-II neurons are involved in regulating the upstream migration, sexual maturation and spawning in chum salmon.

It was found that levels of GnRHR1 and GnRHR5 mRNA increased when fish were transferred to a FW environment (Figure 2). In masu salmon, 5 different GnRHR genes (GnRHR1, GnRHR2, GnRHR3, GnRHR4, and GnRHR5) are expressed in the brain, pituitary, and other peripheral tissues (Jodo et al. 2003). Jodo et al. (2005) reported that GnRHR1 is able to generate truncated GnRHR, the levels of which increase during growth and sexual maturation; and GnRHR5 is highly paralogous to GnRHR4, and is involved in stimulating the synthesis and release of GTH by GnRH during the pre-spawning period. The expression of all 5 GnRHR gene subtypes has been shown to increase during growth and sexual maturation of masu salmon in relation to season and sex. Therefore, it is possible that increased GnRH may regulate GnRHR genes when fish move from a SW to FW environment.

In the present study, the expression and levels of HPG axis hormones (GnRH, GTHα, FSHβ, LHβ mRNA, and plasma GTHα, FSHβ, LHβ, and E_2) increased following transfer to FW; indeed, PG axis activity of adult fish increased to the levels recorded during spawning. Previous studies have shown that gonadal maturation in chum salmon is primarily regulated by elevated GTH, FSH, LH, and sex steroid hormones such as E_2, testosterone (T) and 11-ketotestosterone (11-KT) during spawning migration (Swanson et al. 2003; Onuma et al. 2009). GTH plasma levels also increase during upstream migration in pre-spawning chum salmon (Ueda et al. 1984; Kitahashi et al. 1998), pink salmon (Dye et al. 1986), and sockeye salmon (Truscott et al. 1986), although it should be noted that these studies mainly detected LH because antiserum that distinguishes FSH from LH was not used. Makino et al. (2007) reported that elevated levels of sex steroid hormones, such as E_2, are associated with FW adaptation in pre-spawning chum salmon, and the application of sex steroid hormones has been shown to directly modulate the PRL gene during FW acclimation in adult chum salmon (Hirano et al. 1987; Onuma et al. 2005). Therefore, it is likely that the endocrine systems that govern salt and water homeostasis metabolism in homing salmon are regulated by PG axis activity.

This study showed that the expression and activity of GnRHs in chum salmon increases following artificial transfer from SW to FW, which, in turn, increased reproduction hormone activity. The salinity change also led to changes in sGnRH, which stimulated GTH subunits and plasma E_2 by activating the HPG axis. These findings suggest that an artificial hypo-osmotic environmental change activated the HPG axis and induced reproduction in chum salmon. Therefore, it can be concluded that a change in salinity stimulates the HPG axis of migrating chum salmon, which, in turn, affects the endocrine systems, e.g. by maintaining homeostasis (chemical and water balance in body), allowing chum salmon to reach sexual maturation and reproduce.

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