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FULL PAPER

Effects of exogenous cortisol and seawater adaptation on thyroid hormone receptors in the smolt stage of the sockeye salmon, *Oncorhynchus nerka*

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Abstract The objective of this investigation was to quantify how thyroid hormone receptors of the sockeye salmon, *Oncorhynchus nerka*, respond to salinity changes from freshwater (FW) to seawater (SW) conditions. Thyroid hormone receptors (TRs) mRNA and protein expressions levels significantly increased when the fish were transferred from FW to SW, and the plasma T_3 and T_4 levels were significantly highest at 50 % SW and then maintained as control. Moreover, these parameters were significantly lower in the cortisol-injected groups than in the control. Hence, TRs, T_3, and T_4 may play a role in SW adaptation, when the fish migrate from FW to SW environments. We showed a negative correlation between cortisol and thyroid hormone levels, and a significant increase in plasma K^+ levels in the kidney when the fish were transferred to SW, with levels being significantly lower in the cortisol-injected group. Hence, cortisol appears to be a stress hormone, and the plasma Na^+ and Cl^- levels significantly increased when the fish were transferred to SW, with levels being significantly lower in the cortisol-injected group. These results indicate that cortisol modulates ion transportation in the plasma.

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Introduction

Salinity is an important environmental factor in fish. Changes in salinity cause physiological stress to fish and also affect growth, reproduction, metabolism, osmoregulation, and immunity (Ackerman et al. 2000). In sockeye salmon, juveniles stay in freshwater (FW) for 16–18 months to grow into smolts that have the ability to tolerate seawater (SW). They migrate downstream where some river or lake residents also exist. Adult salmon migrate upstream 4–5 months prior to final gonadal maturation (Groot and Margolis 1991). This tolerance facilitates the downstream migration of juveniles and the subsequent upstream migration of adults, prior to final gonadal maturation, in which a series of endocrine changes occur in response to environmental changes in salinity (McCormick and Saunders 1987). The development of salinity tolerance is obviously important to seaward migrants and has been the most widely studied physiological change that occurs during smolting. The mechanisms for increased hypoosmoregulatory ability include differentiation of the gill, gut, and kidney (Hoar 1988).

The parr–smolt transformation of salmonid teleosts involves a series of morphological, behavioral, and physiological changes that prepare an FW-dwelling parr for life as an SW-adapted smolt (Hoar1988), and salmonids are anadromous and migrate to the ocean after complex morphological, physiological, and behavioral changes; these changes are termed parr–smolt transformation, smoltification, or smolting (McCormick and Saunders 1987). It is

characterized by a series of endocrine changes (Barron 1986) including a dramatic increase in thyroid activity, originally shown histologically (Hoar 1939) and later through measurement of plasma thyroid hormone levels (Dickhoff and Darling 1983). Thus, smoltification is an excellent model for studying thyroid activity in teleost fish (Larsen et al. 2011), and salinity is one of the factors affecting smoltification of salmon. In general, teleosts exhibit various physiological mechanisms that protect against external salinity changes in the environment (Marshall and Grosell 2006).

Several hormones are related to various physiological mechanisms in fish. For example, thyroid hormones (THs) are pleiotropic factors involved in growth, development, and metabolism; they also contribute to the acclimation of fish to changes in environmental salinity (Brent 1996). Thyroid activity is regulated through the hypothalamic-pituitary–thyroid (HPT) axis. For instance, the hypothalamus stimulates the production and release of the thyroid stimulating hormone (TSH) by the pituitary (MacKenzie et al. 2009). In turn, TSH stimulates the secretion of thyroxine (T_4) by the thyroid, which is subsequently converted to the more biologically active triiodothyronine (T_3) (MacKenzie et al. 2009). The biological actions of THs are mediated through the thyroid hormone receptor (TR), which belongs to the nuclear receptor superfamily (Mangelsdorf et al. 1995). There are 2 principal TR isoforms (TRα and TRβ), which are the products of distinct genes (Yaoita and Brown 1990; Lazar 1993). TRα and TRβ are highly conserved in biochemical properties, with TRβ1 and TRβ2 isoforms being splice variants generated from the TRβ gene (Lazar 1993).

Cortisol is a hormone that contributes to the acclimation of fish to SW. It is pivotal in stress responses and in osmoregulatory processes of fish (Wendelaar Bonga 1997). Also, it is often referred to as a SW-adapting hormone, because it is heavily implicated in the ability of fish to maintain water and electrolyte balance when in SW environments (Mommsen et al. 1999) and increased cortisol levels contribute to the regulation of smoltification (Nagae et al. 1994; Prunet et al. 1994; Mizuno et al. 2001). Veillette et al. (2005) reported that cortisol levels regulate water absorption in the intestine during parr–smolt transformation.

During changes in salinity, fish adjust their plasma osmolality by detecting changes in Na^+ and Cl^- as water flows across specialized osmoregulatory organs, such as the gills (Evans 1993). Fish generally maintain a constant body homeostasis, despite changes in osmolality caused by environmental salinity change. Moreover, specific hormones and proteins regulate internal and external body ions and fluids; this allows fish to acclimate to salinity changes in osmoregulatory organs, such as the gills, kidneys, and intestines. Cortisol and TH have a synergistic effect on fish, influencing peripheral interactions of glucocorticoids with the thyroid axis, particularly with respect to osmoregulation (Arjona et al. 2008); however, these processes are not completely understood.

Therefore, we investigated the response of TR mRNA and protein expressions when experimental sockeye salmon, *Oncorhynchus nerka*, acclimated to FW were transferred to SW. We also examined the response of plasma T_3 and T_4; Na^+, Cl^-, and K^+ ions; and cortisol levels to salinity changes. Furthermore, we injected the SW acclimation hormone, cortisol, into fish to investigate its effect and then examined how TRs interact with cortisol when sockeye salmon acclimate to SW.

Materials and methods

Experimental fish. One-year-old sockeye salmon (*Oncorhynchus nerka*; $n = 150$, body length 9.5 ± 0.5 cm; body weight 8.4 ± 1.0 g) were reared at Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University, Japan, in outdoor FRP tanks supplied with a continuous flow of spring water, at an ambient temperature and under natural photoperiod conditions (Japan). The fish were collected for the experiments from 20 February 2012. We collected both baseline and experimental specimens. The former were the first 150 sockeye salmon landed each day, which were immediately killed for physiological biopsy. Physiological biopsy involved measuring all variables, plus an extensive sequence of additional sampling. Fish were maintained in four 40-L tanks, and the temperature was maintained at 12 ± 0.5 °C.

The transfer of sockeye salmon from FW (0 psu) to SW (35 psu) was performed by following a specific protocol. Briefly, underground water was poured into square 40-L tanks, and the fish were kept at 25 % SW, 50 % SW, and 75 % SW for subsequent 24-h periods, by adding natural SW to convert the water in the tanks into SW, and a day of conversion rate of water was 0.7–1.3 %. No mortalities were observed. The temperature was maintained at 12 ± 0.5 °C, and the photoperiod was maintained at a 12:12 h light–dark cycle.

Cortisol injection. The fish were injected with hydrocortisone-21-hemisuccinate (cortisol; H2882; Sigma, St. Louis, MO, USA) and allowed to adapt in 40-L tanks, and the temperature was maintained at 12 ± 0.5 °C. Cortisol was first dissolved in saline. The fish were then anesthetized with 0.005 % eugenol (4-ally-2-methoxyphenol), and then each fish was given an intraperitoneal injection of cortisol [10 and 50 μg/g body mass (BM)] using 1-mL syringe. The sham group of fish was injected with a dissolved equal volume of saline (10 μL/g BM). After the

intraperitoneal injection, the cortisol-injected fish were transferred from FW to SW in five 24-h stages of FW, 25 % SW, 50 % SW, 75 % SW, and 100 % SW, sequentially.

Sampling. Five fish in each group (FW, 25 % SW, 50 % SW, 75 % SW, 100 % SW, and a cortisol injection group) were randomly selected for blood and tissue sampling. Immediately after tissue collection, the fish were frozen in liquid nitrogen and stored at –80 °C until total RNA extraction was performed. In addition, blood was obtained from the caudal vasculature by using a 1-mL heparinized syringe. After centrifugation (4 °C, 10,000 × *g*, 5 min), the plasma was stored at –80 °C until analysis.

Quantitative real-time polymerase chain reaction. Quantitative real-time polymerase chain reaction (QPCR) was conducted to determine the relative expression of TRα (accession no. JX232610), TRβ1 (JX232611), and TRβ2 (JX235711) mRNA by using total RNA (treated by DNase for removing genomic DNA) extracted from the brain and gill of sockeye salmon. Primers for QPCR were designed with reference to known sequences of sockeye salmon. These sequences included TRα forward primer (5'-CAA GTT CCT GCC TGA GGA TAT AG -3'), TRα reverse primer (5'-TCT TGG TGA ACT CGC TGA AG -3'), TRβ1 forward primer (5'-TCA AGC GCC GGT CAT AAA T -3'), TRβ1 reverse primer (5'-AAA GTC CAC CAC TCT GGT AAT G -3'), TRβ2 forward primer (5'-GGA CGA GTT ATG TGT GGT ATG T-3'), TRβ2 reverse primer (5'-CGT TGG GTT CAG GTT CTT CT -3'), β-actin forward primer (5'-ATC TGG CAT CAC ACC TTC TA-3'), and β-actin reverse primer (5'-CTT CTC CCT GTT GGC TTT-3'). PCR amplification was conducted using a BIO-RAD iCycleriQ Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad, CA, USA), according to the manufacturer's instructions. QPCR was performed as follows: 95 °C for 5 min, followed by 35 cycles each of 95 °C for 20 s and 55 °C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data were expressed relative to the corresponding β-actin calculated threshold cycle (CT) levels. The calibrated ΔCt value ($\Delta\Delta Ct$) for each sample and internal controls was calculated as $\Delta\Delta Ct = 2^{-(\Delta Ct_{\text{sample}} - \Delta Ct_{\text{internalcontrol}})}$.

Western blot analysis. Total protein isolated from the brain and gill of sockeye salmon was extracted using a protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1 % SDS, 0.15 mg/mL phenylmethylsulfonyl fluoride, and 0.15 mg/mL leupeptin). It was then sonicated and quantified using the Bradford method (Bio-Rad). Total protein (30 μg) was loaded in separate lanes on 4 % acrylamide stacking gel and 12 % acrylamide resolving gel. For reference, a protein ladder (Fermentas, Vilnius, Lithuania) was used. Samples were electrophoresed at 80 V through the stacking gel and 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 and then washed in TBS. The membranes were incubated with TRα (dilution, 1:200; ab5621, Abcam, UK) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:2,000; Bio-Rad) 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 (1:5,000; Bio-Rad) for 60 min. Bands were detected using standard ECL and the more sensitive ECL systems (ECL Advance; GE Life Sciences, Sweden). They were then exposed to autoradiography sensitive film for 2 min.

Plasma parameter analysis. Plasma T_3 and T_4 levels were analyzed using enzyme immunoassay (EIA) with the T_3 and T_4 EIA Kit (Biosewoom, Korea). Plasma cortisol concentration was determined using the enzyme-linked immunosorbent assay (ELISA) kit (EIAab, Wuhan, China). Plasma Na^+, Cl^-, and K^+ were analyzed using the Biochemistry Autoanalyzer (FUJI DRI-CHEM 4000i; FujiFilm, Tokyo, Japan).

Statistical analysis. All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). Two-way ANOVA followed by Tukey's–Kramer test as post hoc test was used to assess statistically significant differences for the different levels of salinity and different cortisol injection concentrations. A value of $P < 0.05$ was considered statistically significant.

Results

Expression of TR isoforms mRNA in the brain and gill. The quantity of TRα, TRβ1, and TRβ2 mRNA significantly increased from FW to SW conditions. Moreover, TR mRNA isoform expression in the cortisol-injected group was significantly lower than that in the control group.

Western blot analysis revealed a protein with TRα-specific immunoreactivity, which had a mass that corresponded to the predicted mass for sockeye salmon TRα (47 kDa). The expression pattern of the protein resembled that of the TRα, TRβ1, and TRβ2 mRNA expressed in the sockeye salmon brain and gill (Figs. 1a, 2a).

Plasma T_3 and T_4 levels. The plasma T_3 (182.7 ± 17.2 pg/mL) and T_4 (2.3 ± 0.2 pg/mL) levels significantly increased at 50 % SW (T_3, 489 ± 30.5 pg/mL; T_4, 5.7 ± 0.3 pg/mL) and then decreased. Furthermore, plasma T_4

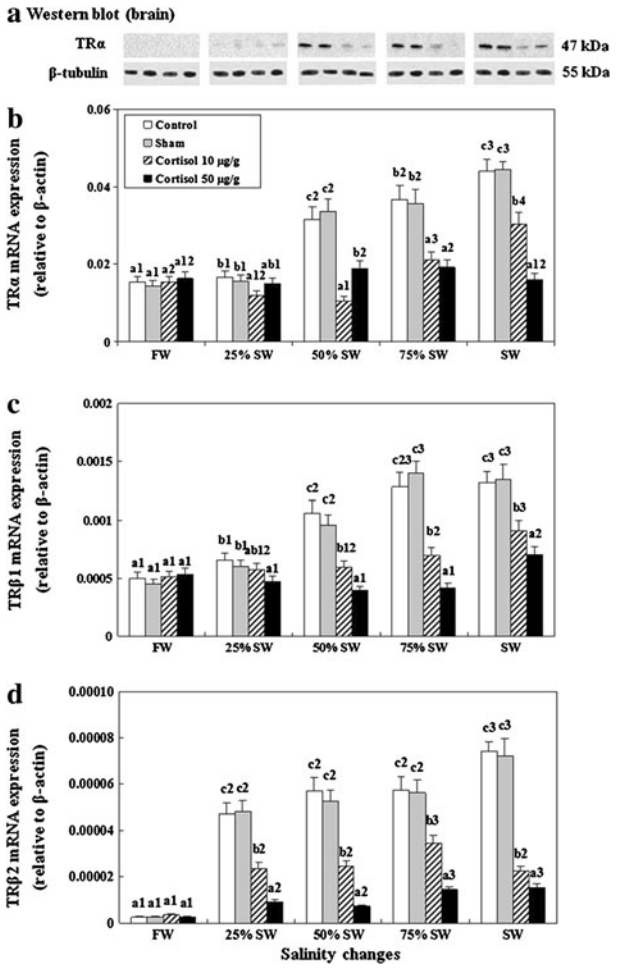

Fig. 1 TRα, TRβ1, and TRβ2 mRNA expression levels in the brain of 4 groups of sockeye salmon: without injection (*control*), injected with 10 and 50 μg/g cortisol, and injected with saline water (*sham*), which were transferred from freshwater (*FW*) to seawater (*SW*). **a** Western blot analysis using TRα (47 kDa) to examine protein expression in the brain of sockeye salmon; the internal control was 55 kDa β-tubulin. **b** TRα, **c** TRβ1, and **d** TRβ2 mRNA levels relative to β-actin mRNA levels in the brain of sockeye salmon and measured using quantitative real-time PCR. Values with letters that differ indicate significant differences between cortisol-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (*control*, *sham*, cortisol 10μg/g, and cortisol 50 μg/g) ($P < 0.05$). All values are mean ± SD ($n = 5$)

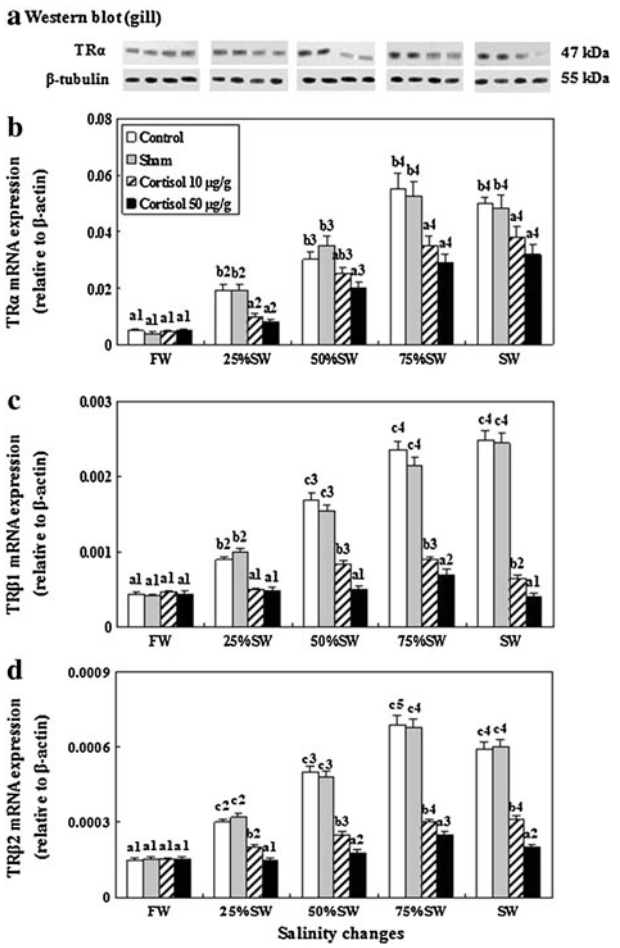

Fig. 2 **a** Western blot analysis using TRα (47 kDa) to examine protein expression in the gill of sockeye salmon; the internal control was 55 kDa β-tubulin. **b** TRα, **c** TRβ1, and **d** TRβ2 mRNA expression levels in the gill of 4 groups of sockeye salmon: without injection (*control*), injected with 10 and 50 μg/g cortisol, and injected with saline water (*sham*), which were transferred from freshwater (*FW*) to seawater (*SW*)

and T_3 levels in cortisol-injected groups were significantly lower than in the controls (Fig. 3).

Plasma Na^+, Cl^-, and K^+ levels. A significant 1.5-fold increase in Na^+, Cl^-, and K^+ levels was recorded from FW to SW. Furthermore, significantly lower plasma Na^+ and Cl^- levels were recorded in the cortisol-injected group than in the control group; however, significantly higher K^+ levels were recorded in the cortisol-injected group than in the control group (Fig. 4).

Plasma cortisol levels. Plasma cortisol levels were recorded from FW to SW, and the levels were significantly increased after changes in salinity. Furthermore, the levels in cortisol-injected groups were higher than in the control group (Fig. 5).

Discussion

In the present study, we examined how sockeye salmon smolt adapted to various saline conditions with respect to their osmoregulatory capacity and associated hormone mechanisms. The expression of TRs in mRNA and protein levels in the brain and gills were recorded with exposure to increasing levels of salinity (Figs. 1, 2). In mammals, it is generally believed that thyroid activity is regulated through the HPT axis. This "central control model" involves the hypothalamic secretion of thyrotropin-releasing hormone (TRH) that stimulates the release of TSH from the

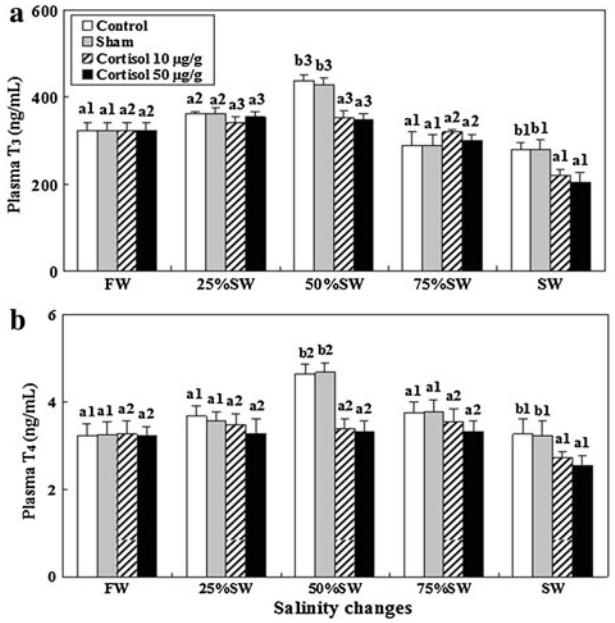

Fig. 3 Plasma T_3 and T_4 of 4 groups of sockeye salmon: without injection (*control*), injected with 10 and 50 μg/g cortisol, and injected with saline water (*sham*), which were transferred from freshwater (*FW*) to seawater (*SW*)

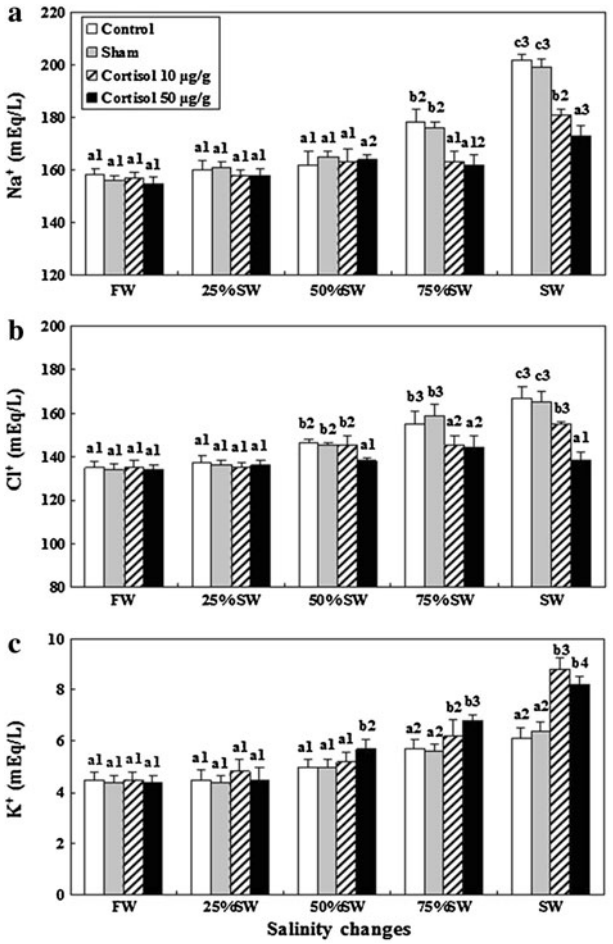

Fig. 4 Plasma Na^+, Cl^-, and K^+ in 4 groups of sockeye salmon: without injection (*control*), injected with 10 and 50 μg/g cortisol, and injected with saline water (*sham*), which were transferred from freshwater (*FW*) to seawater (*SW*)

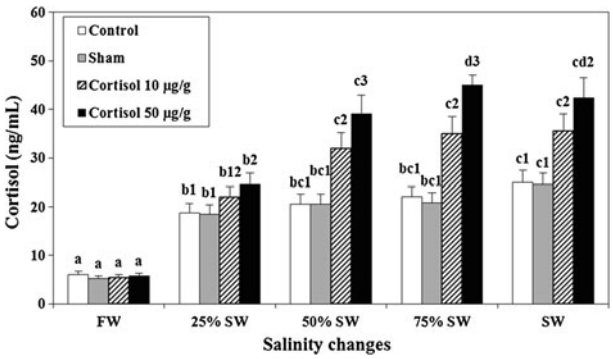

Fig. 5 Plasma cortisol in 4 groups of sockeye salmon: without injection (*control*), injected with 10 and 50 μg/g cortisol, and injected with saline water (*sham*), which were transferred from freshwater (*FW*) to seawater (*SW*)

pituitary. TSH, in turn, stimulates the secretion of T_4 which is subsequently converted to the more biologically active T_3. According to this model, thyroid status is determined by the quantity of free T_4, which is maintained by a balance between pituitary stimulation and negative feedback from the thyroid hormones acting on the hypothalamus and the pituitary gland (Eales and Brown 1993). It is of considerable importance to determine the reciprocal actions of this endocrine factor on smolting. We examined that the biological actions of THs were increased by combining with their receptor, TR for SW acclimation of juvenile sockeye salmon. TH is known to be a hormone that is specifically related to osmoregulation and the acclimation of fish to FW and SW conditions (Schreiber and Specker 2000). As well as the coho salmon, expression levels of the TRα and TRβ genes changed in the metamorphosing fish. Kawakami et al. (2003) have observed that both the TRα and TRβ genes were expressed at high levels during metamorpholic climax, but timing of peak and overall pattern were different. In addition, they have found that the TRβ2 gene is expressed specifically in the brain, and suggested that the TRβ2 plays an important role in the regulation of the HPT axis (Kawakami et al. 2003). For instance, Peter (2007) reported that TH significantly increases when tilapia, *Oreochromis mossambicus*, are exposed to various saline environments; several other studies have also reported the importance of TH in the SW acclimation of fish (Peter 2011). Salinity acclimation increases THs levels, as both T_3 and T_4 are known for osmotic and metabolic actions in fish (Leatherland 1994). Prolonged T_4 treatment has been shown to increase the number of chloride cells and gill NKA activity in Atlantic salmon, *Salmo salar*, smolts (Madsen and Korsgaard 1989). Peter et al. (2000) reported

that the physiological levels of T_4 and T_3 increase plasma Na^+ and Cl^- in tilapia, suggesting a role for THs in ion uptake in this fish. Furthermore, TR is involved in growth (Brent1996); therefore, it is important to promote growth as sockeye salmon acclimate to salinity during the downstream migration into SW.

In this study, we observed significantly lower expression levels of TRβ2 than of TRα and TRβ1. The levels of TRβ2 have been shown to express greater T_3-dependent repression than TRα and TRβ1 in the negative regulation of the thyrotropin-releasing hormone gene (Langlois et al. 1997). This observation indicates that TRβ2 is pivotal to the negative regulation of TSH and TRH (Nakano et al. 2004); hence, we hypothesized that TRβ2 expression levels are lower than those of other TR isoforms. Furthermore, we found that TR mRNA isoform and protein expression levels in the cortisol injection groups were significantly lower than those in the control (not injected). TH and cortisol have long been known as major hormones associated with smoltification, and their practical functions have been investigated on its progress (Ban 2005). For example, in masu salmon, *Oncorhynchus masou*, and amago salmon, *Oncorhynchus masourhodurus*, administration of TH induces morphological changes such as body silvering, fin margin blackening, or reduced condition factor (Soyano et al. 1988). Weisbart et al. (1987) reported a negative correlation between increased plasma cortisol and reduced T_4 levels in Brook trout, *Salvelius fontinalis*, which occurred after injection or following fish transfer from FW to 30 % SW. In this study, we recorded that the plasma T_3 and T_4 levels were significantly increased at 50 % SW and then decreased; however, the plasma levels of the cortisol injection group were significantly lower than that of the control (not injected) (Fig. 3). Corticosteroids decrease plasma concentrations of THs (Kühn et al. 1998).

Hence, cortisol and TH have synergistic effects on the development of SW tolerance in sockeye salmon. Because it is well known that cortisol, TH, and other hormones increase abruptly in accordance with smoltification, researchers and managers must consider the interrelationships among these hormones when performing endocrinological investigations concerned with the development of SW tolerance in anadromous fishes (Ban 2005). Also, there may be a negative correlation between cortisol and TH.

The smolt phase in salmonids requires structural and functional adaptations of the gills, which are accompanied by increased levels of THs, growth hormone, and cortisol (Young et al. 1989).

Cortisol is secreted from the hypothalamo-pituitary–interrenal (HPI) axis, and it has been shown to be involved in the regulation of acute stress response in teleost in addition to roles in osmoregulation (Huising et al. 2004). Also, cortisol plays a key role in energy substrate mobilization, including enhanced liver metabolic capacity, which is critical for coping with seawater adaptation (Vijayan et al. 1996). Therefore, when the plasma cortisol is elevated gradually, and then inhibited the cortisol release by HPI axis feed-back (Vijayan et al. 1996). According to these theories, we hypothesize that when the cortisol levels reach to some point, it is inhibited by the negative feedback of the HPI axis and then TRs are decreased in the brain and gill. Cortisol is related to various factors in osmoregulation functions; it means that injected cortisol is concerned with TRs and other factors related to osmoregulation, so TRs' roles relatively decrease during seawater acclimation.

In addition, sockeye salmon and Atlantic salmon usually smoltify during the spring (Shrimpton et al. 2000). If sockeye and Atlantic salmon smolts are prevented from entering SW, they will experience a loss of SW tolerance and will most likely smoltify the following spring (Shrimpton et al. 2000). Therefore, sockeye salmon used in this study were collected for the experiments from 20 February, at start of spring. The levels of TRs mRNA expression and activity were significantly increased by salinity changes in this period. These results suggest that salinity changes, as one of the factors to smoltification for seaward migration, upregulate the levels of TRs to acclimation in SW.

Additionally, we recorded a significant increase in Na^+, Cl^-, and K^+ when the fish were transferred to SW; however, Na^+ and Cl^- in the cortisol injection group were significantly lower than those in the control (Fig. 4). Furthermore, plasma cortisol levels in cortisol injection groups were significantly higher than those in the control groups (Fig. 5). This result shows the methodological validity of cortisol injection and that cortisol plays a role in osmoregulation by salinity changes. Cortisol is important in mobilizing energetic substrates, including enhanced liver metabolic capacity, which is critical for coping with SW adaptation (Vijayan et al. 1996; Mommsen et al. 1999). Therefore, we hypothesized that cortisol, which is an SW adaptation hormone, contributes to the inhibition of rapid ion inflow to maintain osmolality in smolt sockeye salmon. In contrast, the K^+ levels in the cortisol injection group were significantly higher than those in the control. K^+ is an ion that responds to stress (Fan et al. 2011), with its levels increasing as a stress response to salinity changes. Hence, we hypothesized that injected cortisol acted as a stress hormone by increasing K^+ levels.

In summary, we hypothesize the following: (1) TH is released and then TR increases when fish move from FW to SW conditions, which enhances acclimation to environmental salinity changes in sockeye salmon; and (2) the steroid hormone cortisol regulates ion transporters of osmoregulation and has feedback actions on TH, which enhances the SW hypo-osmoregulatory capacity of fish.

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