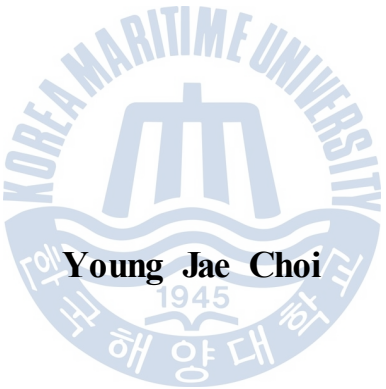
Thesis for the Degree of Master of Science

Molecular and Endocrinological Regulation on Seawater Acclimation in the Parr and Smolt Stages of Sockeye Salmon *Oncorhynchus nerka*



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by

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Molecular and Endocrinological Regulation on Seawater Acclimation in the Parr and Smolt Stages of Sockeye Salmon *Oncorhynchus nerka*

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CONTENTS

List of Table

List of Figures

List of Abbreviations

AQPs	aquaporins
AQP3-IR	aquaporins 3-immunoreactive
BM	body mass
BSA	bovine serum albumin
Ct	threshold cycle
ELISA	enzyme-linked immunosorbent assay
FW	freshwater
HPT	hypothalamic-pituitary-thyroid
HPI	hypothalamo-pituitary-interrenal
IHC	immunohistochemistry
MEM	minimum essential medium
MRCs	mitochondria-rich cells
NKA	Na^+/K^+-ATPase
PCR	polymerase chain reaction
QPCR	quantitative real-time polymerase chain reaction
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SW	seawater
smolt	smoltification
SE	standard error
TRs	thyroid hormone receptor
THs	thyroid hormones
TSH	thyroid stimulating hormone
TRH	thyrotropin-releasing hormone
T_4	thyroxine
T_3	triiodothyronine
TBS	tris-buffered saline

홍연어 *Oncorhynchus nerka* 치어의 해수적응을 위한 분자내분비학적 조절

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요 약

본 연구는 홍연어 *Oncorhynchus nerka* 치어가 해수 환경에 적응하는 동안 aquaporins (AQPs) 및 갑상선호르몬 수용체 유전자의 발현과 외인성 코티졸의 내분비학적 조절 메커니즘을 밝히고자 수행되었다.

1. 해수적응에 따른 AQPs 유전자의 발현과 외인성 코티졸의 상호작용

홍연어 치어의 삼투압 조절 메커니즘을 확인하기 위하여, 담수 환경에 서식하고 있는 parr 단계와 smoltification (smolt) 단계의 홍연어를 각각 염분 단계별 (0, 25, 50, 75, 100% 해수) 해수 환경에 노출시킨 후, 생리학적 반응을 조사하였다. 염분 변화에 따른 수분 이동에 중점을 둔 연구로, 수분 이동 통로의 구성에 관여하는 막 단백질인 AQP3와 8 mRNA 발현 및 각각의 단백질 발현량의 변화를 관찰하였으며, 대표적인 삼투압 조절 기관인 아가미에서 AQP3의 분포 및 발현량의 변화를 면역조직화학법으로 관찰하였다. 또한, 해수적응 호르몬인 코티졸을 처리하여 코티졸에 의한 AQPs (AQP3와 AQP8) mRNA 발현량의 변화를 비교하였다. 실험 결과, parr 단계의 홍연어 아가미와 장에서는 담수에서 해수로의 염분 변화에 따라 AQP3와 AQP8 mRNA 및 단백질 발현량 모두 유의적으로 증가하는 경향이 관찰되었다. 반면, smolt 단계에서는 AQP3와 AQP8 모두

25% 해수까지는 유의적으로 증가하였다가 감소하는 경향이 관찰되었으며, 담수 환경과 비교한 결과 오히려 해수 환경에서 유의적으로 낮은 발현량이 관찰되었다. 또한, 혈장 삼투질 농도는 parr 단계(411 ± 5.3 mOsm/kg) 보다 smolt 단계(381 ± 2.6 mOsm/kg)에서 상대적으로 높은 수준의 혈장 삼투질 농도를 유지하고 있었다. 코티졸을 복강 주사한 경우, parr와 smolt 단계 모두에서 AQP mRNA 및 단백질 발현량이 감소했으며, 혈장 삼투질 농도 또한 344 ± 4.0 mOsm/kg (parr)과 324 ± 4.2 mOsm/kg (smolt)으로 각각 감소하였다. 따라서 본 연구에서는 해수 환경에 노출하였을 때, 수분 재흡수에 관여하는 유전자인 AQP에 의해 해수 환경에서 선택적인 수분 투과를 통하여 어체내의 삼투압을 조절하고 있었으며, smolt 시기를 거치면서 고염분 환경에 적응할 수 있는 삼투능력이 향상/획득되었음을 확인할 수 있었다. 또한, 코티졸은 홍연어가 parr에서 smolt 단계로 성장해 가는 동안 수분의 이동을 촉진시키고, 고염분 환경 적응 시에 수분의 흡수를 증가시켜 AQP 유전자의 작용을 조절하고 있음을 확인할 수 있었다.

2. 해수적응에 따른 갑상선호르몬 수용체 유전자의 발현과 외인성 코티졸의 상호작용

담수 환경에 서식하고 있는 smolt 단계의 홍연어를 인위적으로 염분 단계별 (0, 25, 25, 50, 75, 100% 해수) 해수 환경에 노출시키면서 갑상선호르몬 수용체 mRNA 및 단백질 발현량의 변화를 관찰하였다. 또한, 혈장에서 triiodothyronine (T_3), thyroxine (T_4) 및 Na^+, Cl^-, K^+ 이온 농도의 변화를 확인하였다. 더욱이, 해수 적응 호르몬인 코티졸의 영향을 알아보기 위하여 코티졸을 처리하여 홍연어가 해수에 적응 시 갑상선호르몬 수용체와 코티졸과의 상관관계를 밝혀내고자 하였다. 연구 결과, 단계별 염분 변화 환경에 노출됨에 따라 갑상선호르몬 수용체 mRNA 및 단백질 발현 모두 유의적으로 증가하는 경향이 관찰되었으며, 혈장 내 T_3, T_4 농도 또한 증가하였다. 반면, 코티졸을 복강 주사한 실험구에서는 대조구에 비하여 갑상선호르몬 수용체 mRNA 및 단백질의 발현량 뿐만 아니라 혈장 내 T_3 및 T_4 농도 또한 감소하였다. 염분 농도가 증가함에 따라 Na^+, Cl^-와 K^+ 이온은 증가하는 경향을 보였으나, 코티졸을 처리한 실험구에서는 대조구보

다도 낮은 Na^+, Cl^-와 K^+ 이온 농도 값을 보였다. 본 연구 결과, 홍연어는 치어기 때 담수에 머물다가 성장을 위해 바다로 이동하면서 갑상선호르몬 및 갑상선호르몬 수용체가 분비되어 염분 변화 환경에 적응해 가면서 성장을 촉진시키는 것으로 사료된다. 또한, 동시에 코티졸이 세포막의 이온 수송 조절에 영향을 끼쳐, 체내 삼투압 조절 및 갑상선호르몬과의 피드백 작용을 수행하면서 해수 적응 능력을 증가시키는 것으로 판단된다.

Chapter 1.

General 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).

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). As an example, parr stage salmon discharge water from the intestine, but during the smoltification (smolt) period, these fish gain the seawater (SW) adaptation of absorbing water and ions (McCormick and Saunders, 1987). Increased salinity tolerance during smoltification is due at least in part to increased Na^+/K^+-ATPase (NKA) activity in the gill epithelium, as well as due to increased plasma glucose levels (McCormick and Saunders, 1987).

Aquaporins (AQPs) are a group of membrane proteins that form water transfer channels, which play an important role in maintaining the water balance in the osmoregulatory organs that control body fluid homeostasis (Borgnia et al., 1999; Matsuzaki et al., 2002). The role of AQPs in water movement has been studied in euryhaline teleosts that are adapted to freshwater (FW) and SW (Borgnia et al., 1999; Lignot et al., 2002; Aoki et al., 2003; Martinez et al., 2005). These three subunits are the AQPs group, which selectively moves water (AQPs 0, 1, 2, 4, 5, 6, and 8), the aquaglyceroporins group, which regulate movement of water, glycerol, and urea (AQPs 3, 7, 9, and 10), and the superaquaporins group, which displays low amino acid homologies and have indistinct characteristics (AQP 11, 12) (Verkman, 2005; Ishibashi et al., 2009).

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 commonly known as the SW adaptation hormone. However, in species such as salmon, whose life cycles include both SW and FW phases, cortisol is involved in FW adaptation via interaction with prolactin in conjunction with ionic regulation in FW as well as SW (Laurent and Perry, 1990; Zhou et al., 2004).

Therefore, parr and/or smolt sockeye salmon *Oncorhynchus nerka* were exposed to osmotic environmental changes for studying: I investigated the expression patterns of AQPs and TRs in sockeye salmon in response to a hyperosmotic challenge from FW to SW during the parr and smolt stages. Also examined the response of plasma osmolality; Na^+, Cl^-, and K^+ ions; T_3 and T_4; and cortisol levels to salinity changes. Furthermore, injected the SW acclimation hormone, cortisol, into fish to investigate its effect and then examined how AQPs and TRs interact with cortisol when sockeye salmon acclimate to SW.

Chapter 2.

Effects of exogenous cortisol on seawater acclimation in relation to expression of AQP mRNAs in the parr and smolt stages of sockeye salmon

Abstract

This study aimed to examine the role of 2 AQP isoforms (AQP3, and -8) in sockeye salmon *Oncorhynchus nerka* in response to a hyperosmotic challenge from FW to SW during the parr and smolt stages. AQP3 mRNA was primarily detected in the osmoregulatory organs, such as gills, while AQP8 mRNA was primarily found in the intestine. These results suggested that AQP isoforms play a role in osmoregulation in specific osmoregulatory organs. Similarly, AQP3 mRNA expression in the gills [mean values: 1.06 ± 0.05 (parr) and 1.29 ± 0.07 (smolt)] was significantly higher than AQP8 mRNA levels (parr: 0.04 ± 0.003; smolt: 0.14 ± 0.004), and in the intestine, AQP8 mRNA expression (parr: 0.89 ± 0.007; smolt: 1.91 ± 0.03) was significantly higher than AQP3 mRNA levels (parr: 0.24 ± 0.006; smolt: 0.83 ± 0.005); these expression patterns were similar *in vivo* and *in vitro*. Additionally, AQP mRNA levels were lower in cortisol treated than in control groups. Therefore, these results suggest that AQPs play important roles in the water absorption mechanisms associated with multiple AQP isoforms, and that cortisol enhances the hypo-osmoregulatory capacity of fish in SW, and also controls the expression of AQPs in a hyperosmotic environment.

1. Introduction

In teleost fish, osmoregulation during salinity changes is associated with the movement of ions, such as Na^+ and Cl^-, and water molecules within the gills, kidneys, and intestines (Evans, 1993; Veillette et al., 2005; Hwang and Lee, 2007; Evans, 2008). In FW fish, the internal osmotic pressure is higher than the external pressure, and these fish are challenged by the continuous osmotic gain of water and loss of salt to the dilute surroundings. They counteract this by producing large volumes of dilute urine, retaining ions in the kidney, along with compensatory uptake of ions fromtheir food and via the gills. However, in SW fish, the external osmotic pressure is higher than the internal pressure, and fish take in a large quantity of SW, absorbing water through the intestines to replace water loss caused by osmotic stress, and then discharge ions through the gills. SW fish also absorb Na^+ and Cl^- ions through the kidneys and discharge these to the outside environment (Evans, 1993; Bentley, 2002).

Hormones and proteins, such as AQPs, cortisol, prolactin, growth hormone, NKA, and arginine vasotocin, are involved in osmoregulation (Geering, 1990; Madsen and Bern, 1992; Warne and Balment, 1995).

AQPs are a group of membrane proteins that form water transfer channels, which play an important role in maintaining the water balance in the osmoregulatory organs that control body fluid homeostasis (Borgnia et al., 1999; Matsuzaki et al., 2002). The role of AQPs in water movement has been studied in euryhaline teleosts that are adapted to FW and SW (Borgnia et al., 1999; Lignot et al., 2002; Aoki et al., 2003; Martinez et al., 2005). So far, 13 types of AQPs have been cloned froma variety of organisms, from bacteria to mammals (King et al., 2000). AQPs are divided into three subunits on the basis of their genomic structure and amino acid homology.

These three subunits are the AQPs group, which selectively moves water (AQPs 0, 1, 2, 4, 5, 6, and 8), the aquaglyceroporins group, which regulate movement of

water, glycerol, and urea (AQPs 3, 7, 9, and 10), and the superaquaporins group, which displays low amino acid homologies and have indistinct characteristics (AQP 11, 12) (Verkman, 2005; Ishibashi et al., 2009).

AQP3, an aquaglyceroporin, is a channel that is permeable to water, glycerol, urea, and ammonia/ammonium; it is expressed in gills and kidney of teleosts, such as European sea bass *Dicentrarchus labrax* (Giffard-Mena et al., 2007), and Atlantic salmon *Salmo salar* (Tipsmark et al., 2010). Moreover, AQP3 mRNA expression is increased in the kidneys of SW fish compared to those of FW fish [e.g. tilapia *Oreochromis mossambicus* (Watanabe et al., 2005) and Atlantic salmon (Tipsmark et al., 2010)].

AQP8 is an AQP that is almost exclusively expressed in the mucosal layer of the intestines, emphasizing the particular significance of this pore in water uptake in Atlantic salmon that are SW-adapted (Tipsmark et al., 2010). Furthermore, Tipsmark et al. (2010) have reported that AQP8 mRNA expression peaks during smoltification in Atlantic salmon.

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). As an example, parr stage salmon discharge water from the intestine, but during the smoltification period, these fish gain the SW adaptation of absorbing water and ions (McCormick and Saunders, 1987). Increased salinity tolerance during smoltification is due at least in part to increased NKA activity in the gill epithelium, as well as due to increased plasma glucose levels (McCormick and Saunders, 1987).

Cortisol is often referred to as a SW-adapting hormone, because it is strongly implicated in the ability of fish to maintain water and electrolyte balance when in SW environments (Mommsen et al., 1999), and because increased cortisol levels contribute to the regulation of smoltification (Prunet et al., 1989; Nagae 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.

Here, experimental sockeye salmon *O. nerka*, which had been acclimated to FW, were transferred to SW to examine changes in AQP3 and -8, at mRNA and protein levels, in response to a hyperosmotic challenge by transference from FW to SW during the parr and smolt stages. Additionally, I investigated the presence of these AQPs in the gill, by immunohistochemistry (IHC), after transferring the fish to SW. Furthermore, the effect of injection of the fish with the SW-adapting hormone, cortisol were investigated. Then examined how AQPs interacted with cortisol during acclimation of the salmon to SW.

2. Materials and methods

2.1. Experimental fish

Sockeye salmon (parr, 8.4 ± 1.0 g; smolt, 14.6 ± 1.2 g) were reared at Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University, Japan, in outdoor tanks supplied with a continuous flow of spring water at an ambient temperature and under natural photoperiod conditions (Japan). Fish, both baseline and experimental specimens, were collected in February 20, 2012 (parr) and April 12, 2012 (smolt), respectively. As baseline specimens, I used the first 150 sockeye salmon that were landed each day; these were immediately killed for physiological biopsy, which involved measuring all variables, plus an extensive sequence of additional sampling. Experimental fish were maintained in 4 square 40-L tanks.

Sockeye salmon were transferred from FW (0 psu) to SW (35 psu) by following a specific protocol. Briefly, at first, the tanks contained ground water; to this, SW was added in stages to gradually convert all the water in the tanks into SW. In this process, the fish were sequentially maintained at 25% SW, 50% SW, and 75% SW for 24-h periods. The temperature was maintained at 12 ± 0.5℃, and the photoperiod was maintained at a 12 h:12 h light－dark cycle.

2.2. Cortisol treatment

To investigate the role of cortisol in the response to salinity changes in sockeye salmon, fish were treated with hydrocortisone-21-hemisuccinate (cortisol; Sigma, St Louis, MO, USA), and the expression of AQP mRNAs was measured. Fish were allowed to adapt in 40-L tanks contained ground water and were then anesthetized with 0.005% eugenol (4-allyl-2-methoxyphenol) prior to injection.

Cortisol was dissolved in 0.9% physiological saline, and each fish was given an intraperitoneal injection of cortisol [10 and 50 μg/g body mass (BM)]; the sham group was injected with an equal volume of 0.9% physiological saline (10 μL/g BM). Afterwards, fish were transferred from FW to 25% SW.

2.3. Tissue culture

2.3.1. Gill tissue culture

Preparation of the gill filaments for organ culture followed similar methods as described in McCormick and Bern (1989). The second gill arch was excised from the gill basket and washed in Dulbecco's modified Ca^{2+}/Mg^{2+}-free PBS (mM) (2.6 KCl, 1.5 KH_2PO_4, 137 NaCl, 8 Na_2HPO_4). Gill filaments were separated from the gill arch immediately above the septum, and were gently dispersed by passage through a pipette. Gill filaments were washed three times in PBS containing 200 IU/mL penicillin, 200 pg/mL streptomycin, and 5 mg/mL amphotericin B. Then, two filaments were placed in 1 mL of sterile Leibovitz-15 media supplemented with L-glutamine (Gibco Ltd., NY, USA), containing 100 IU penicillin, 100 mg streptomycin, and 2.5 pg amphotericin B, in sterile 24-well culture dishes and the dishes were incubated at 20 ± 1°C. The cultured gills were sampled at 24-h intervals as the water was changed from 25% SW to 100% SW; each sample was centrifuged (20°C, × 10,000 g, 15 s), and the supernatant was removed and stored at −80°C until RNA extraction.

Hydrocortisone-21-hemisuccinate (corticosterone; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.9% physiology saline was added to the culture medium in a 1/1000 (v/v) ratio, and the specified concentrations of cortisol (10 and 50 μg/mL) were added. Each sample was centrifuged (20°C, 10,000 × g, 15 s), and then the supernatant was removed and stored at −80°C until RNA extraction.

2.3.2. Intestine tissue culture

Each intestinal region was cut into pieces of approximately 1×1.5 mm and carefully placed (in duplicate) in 24-well culture plates containing pre-incubation medium [minimum essential medium (MEM) with Hanks' salts, 5 mg/mL bovine serum albumin (BSA), 250 U/mL penicillin G, and 250 μg/mL streptomycin sulfate, adjusted to pH 7.8]. After 1-h, the medium was replaced with MEM containing Earle's salts (pH 7.8), 4 mg/mL BSA, 292 μg/mL L-glutamine, 50 U/mL penicillin

G, and 50 μg/mL streptomycin sulfate in sterile 24-well culture dishes. Although explants were occasionally found to adhere to the bottom of the plate wells, they typically remained unattached during culture. The cultured intestine was sampled at 24-h intervals during the transition of fish from FW to 100% SW; each sample was centrifuged (20°C, 10,000 × g, 15 s), and the supernatant was removed and stored at −80°C until required for RNA extraction.

Corticosterone dissolved in 0.9% physiology saline was added to the culture medium in a 1/1000 (v/v) ratio, and the specified concentrations of cortisol (10 and 50 μg/mL) were added. Each sample was centrifuged (20°C, 10,000 × g, 15 s), and then the supernatant was removed and stored at −80°C until RNA extraction.

2.4. Sampling

Five fish from each group (FW, 25% SW, 50% SW, 75% SW, SW, and cortisol injection group) were randomly selected for blood and tissue sampling. Immediately after collection of the tissue specimens, samples were frozen in liquid nitrogen and stored at −80°C until total RNA extraction was performed. Additionally, blood was taken from the caudal vein using a 1-mL heparinized syringe. After centrifugation (4°C, 10,000 × g, 5 min), the plasma was stored at −80°C.

2.5. Identification of AQPs

The primers used for reverse transcription polymerase chain reaction (RT-PCR) amplification of AQP mRNAs were designed from highly conserved regions of other teleost fish (Table 1). Total RNA was extracted from the brain and gills using TriReagent (MRC, Cincinnati, OH, USA). Polymerase chain reaction (PCR) amplification was performed using Takara Ex Taq (Takara, Tokyo, Japan) according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 98°C for 2 min; 30 cycles consisting of denaturation at 98°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 60 s; followed by 7

min at 72°C for the final extension. Amplified PCR products were assessed by electrophoresis on 1% agarose gel containing ethidium bromide (Biosesang, Sungnam, Korea). The PCR products were purified and then cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). Vectors were propagated in DH5α (RBC Life Sciences, Seoul, Korea) and plasmid DNA extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and subjected to nucleotide sequencing using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.6. Tissue distribution of AQP mRNAs

To examine tissue distribution of the mRNA of the 2 AQP investigated in this study, total RNA were extracted from the pituitary, brain, gill, esophagus, pyloric caecae, intestine, kidney, and liver, as described above. Total RNA was extracted from the tissues using Tri-Reagent (MRC, Cincinnati, OH, USA). Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Bioneer, Seoul, Korea) according to the manufacturer's instructions. PCR amplification was performed with specific primer sets (Table 1) with a 2 × Taq Premix I (Solgent, Seoul, Korea). PCR was carried out as follows: initial denaturation at 95°C for 2 min; then, 40 cycles each consisting of denaturation at 95°C for 20 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min; followed by 7 min at 72°C for the final extension. Amplification of β-actin mRNA was used to verify the quality of the RT products, using a primer set specific for sockeye salmon β-actin cDNA (Table 1). The amplified PCR products were electrophoresed on 1% agarose gels, detected by staining with ethidium bromide, and visualized by illumination with UV light.

2.7. QPCR

Quantitative real-time polymerase chain reaction (QPCR) was performed to determine the relative expression of AQP mRNAs using total RNA extracted from

sockeye salmon. Primers for QPCR were designed with reference to the known sequences of sockeye salmon (GenBank accession nos.: AQP3, **JX183096**; AQP8, **JX183098**), and are shown in Table 1. PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was carried out as follows: 1 cycle of denaturation at 95°C for 5 min, then, 35 cycles each consisting of denaturation at 95°C for 20 s, and annealing and extension at 55°C for 20 s. For each experimental group, triplicate PCR runs were performed to ensure consistency. As an internal control, β-actin was also amplified for each sample, and all data were expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products. In two QPCR assays, the intra- and interassay coefficients of variation for AQP3 and AQP8 mRNAs were less than 8%.

Table 1. Primers of AQPs used for amplification of PCR

PCR	Genes	DNA sequences
RT-PCR	AQP3-F	5′-CTC ATC CTT GTG ATG TTT GG-3′
	AQP3-R	5′-TGC CAA TCA CCT GGT CAA AG-3′
	AQP8-F	5′-GGC TGT ACT GTC ATC ATC AA-3′
	AQP8-R	5′-CTT CAT GAG AAT ACG TGT C-3′
	β-actin-F	5′-TCG AGC ACG GTA TTG TGA CC-3′
	β-actin-R	5′-CGG AAC CTC TCA TTG CCG AT-3′
QPCR	AQP3-F	5′-TGT GCT ACG GGT TCA TCT-3′
	AQP3-R	5′-GTC CTC AGT TTG GCT CTT G-3′
	AQP8-F	5′-AGA TCC TCA AAG AGC AGA TC-3′
	AQP8-R	5′-GTT CTT CAG CAG GTA GTT CTC-3′
	β-actin-F	5′-GGA CCT GTA TGC CAA CAC TG-3′
	β-actin-R	5′-TGA TCT CCT TCT GCA TCC TG-3′

2.8. Production of AQPs polyclonal antibody

To obtain the antigen of the sockeye salmon AQP3 and AQP8 antibody, a synthetic peptide was synthesized at Cosmo Genetech (Seoul, Korea) and was coupled to BSA. AQP3 protein was attached to the N-terminal of the maltose binding protein, and the purified proteins were analyzed by 10−15% SDS-PAGE alongside a protein marker (Fermentas, ON, Canada). Similarly, the AQP8 peptide chain was designed against highly conserved regions of AQP8 amino acid sequences from sockeye salmon and other teleosts (residues 65 − 85; N-ter-RLQPALVHGLAVAVMVACMAE-C-ter). A rabbit was injected with 100 μg of BSA-conjugated synthetic peptide in Freund's complete adjuvant, and was boosted at 2-week intervals by subcutaneous injection of 200 μg of BSA-conjugated synthetic peptide in Freund's incomplete adjuvant. The rabbit was bled 1 week after the fifth injection. Antiserum was purified with an affinity peptide column coupling, using the same peptide as described above (Lu et al., 2000). This antibody recognized full-length sockeye salmon AQPs, obtaining bands at 33 kDa (AQP3) and 27 kDa (AQP8). The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$].

2.9. Western blot analysis

Total protein isolated from the brain of sockeye salmon during salinity change was extracted using protein extraction buffer [5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL PMSF, and 0.15 mg/mL leupeptin], sonicated, and quantified using the Bradford method (Bio-Rad). Total protein (30 μg) was loaded into each lane of a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. As a reference, I used a protein ladder (Fermentas, Glen Burnie, MD, USA). 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 moved off the gel. The gels were then immediately transferred to a 0.2 μm polyvinylidene diflouride membrane (Bio-Rad, Hercules, CA, USA) 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, followed by washing in TBS. Membranes were incubated with polyclonal rabbit anti-AQPs (AQP3, 1:1000 dilution, and AQP8, 1:4000 dilution) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (dilution 1:4000; Bio-Rad) for 60 min. The internal control was β-tubulin (dilution 1:2000; ab6046, Abcam, Cambridge, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:4000; Bio-Rad) for 60 min. Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) with a 30 s exposure, using a Molecular Imager® ChemiDoc™ XRS+ Systems (Bio-Rad). The membrane images were scanned by a high resolution scanner and the band density estimated using a computer program (Image Lab™ Software, version 3.0, Bio-Rad). The ratio of internal control (β-tubulin)/AQP3 or AQP8 for each concentration was calculated and plotted against the concentration of the internal control.

2.10. IHC

Gills were detected immunohistochemically according to the methods described in Uchida et al. (2000), with modifications. For identification of branchial aquaporins 3-immunoreactive (AQP3-IR) cells, the first gill arch was removed, fixed in 4% paraformaldehyde for 24 h at 4°C, dehydrated in ethanol, and then embedded in paraffin. Four 1-μm-thick rehydrated tissue sections were incubated overnight at 4°C with primary rabbit anti-AQP3 antibody (1/500 dilution), and then with the secondary antibody (HRP-conjugated anti-rabbit immunoglobulin, 1/100 dilution) for 30 min at 37°C. The antibodies were diluted in 2% BSA in TBS (pH 7.6). EnVision+ (K4001; Dako, Glostrup, Denmark), and finally, antibody binding was visualized by applying 3,3´-diaminobenzidine (DAB+, K3468; Dako) (brown color) were used as detection system. Slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted with Canada balsam for observation under a light microscope (DM 100; Leica, Wetzlar, Germany); images were captured with a digital camera (DFC 290; Leica).

2.11. Plasma parameter analysis

Plasma osmolality was examined using a Vapor Pressure Osmometer (Vapro 5600, Wescor Co., Logan, UT, USA).

2.12. Statistical analysis

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

3. Results

3.1. Tissue distribution of AQP mRNAs

Fig. 1 shows the tissue-specific expression patterns of sockeye salmon AQP3 and AQP8 mRNAs in fish acclimated to FW and SW. AQP3 mRNA was primarily detected in the gills of sockeye salmons that had been acclimated to either FW or SW. AQP8 mRNA was primarily detected in the intestines of SW-acclimated sockeye salmon.

3.2. Expression of AQP mRNAs and cortisol injection of parr/smolt sockeye salmon

QPCR analyses were used to show the relative expression levels of AQP3 and AQP8 mRNAs in the tissue (gill and intestine) of parr/smolt sockeye salmon during salinity change. Expression of both AQP mRNAs in all tissues was higher in all stages of SW- than in FW-acclimated salmon (Figs. 2 and 3). In the gills of parr stage salmon, AQP3 mRNA levels were significantly increased according to the increase in salinity levels; however, AQP8 mRNA levels peaked at 75% SW, and then decreased in 100% SW (Fig. 2A and B). In the gills of smolt stage salmon, AQP mRNAs peaked at 25% SW, and then decreased as salinity increased to 100% SW (Fig. 2C and D). In the intestine of parr stage salmon, AQP mRNAs were significantly increased at 75% SW (AQP3) or 50% SW (AQP8) and then decreased as salinity increased to 100% SW (Fig. 3A and B). In the intestine of smolt stage salmon, AQP3 mRNA was significantly increased as salinity increased to 100% SW; however, AQP8 mRNA peaked at 25% SW, and then decreased as salinity increased to 100% SW (Fig. 3C and D). Furthermore, the expression of AQPs in the cortisol-injected group was significantly decreased after the transfer to SW compared to the control group (Figs. 2 and 3; $P < 0.05$).

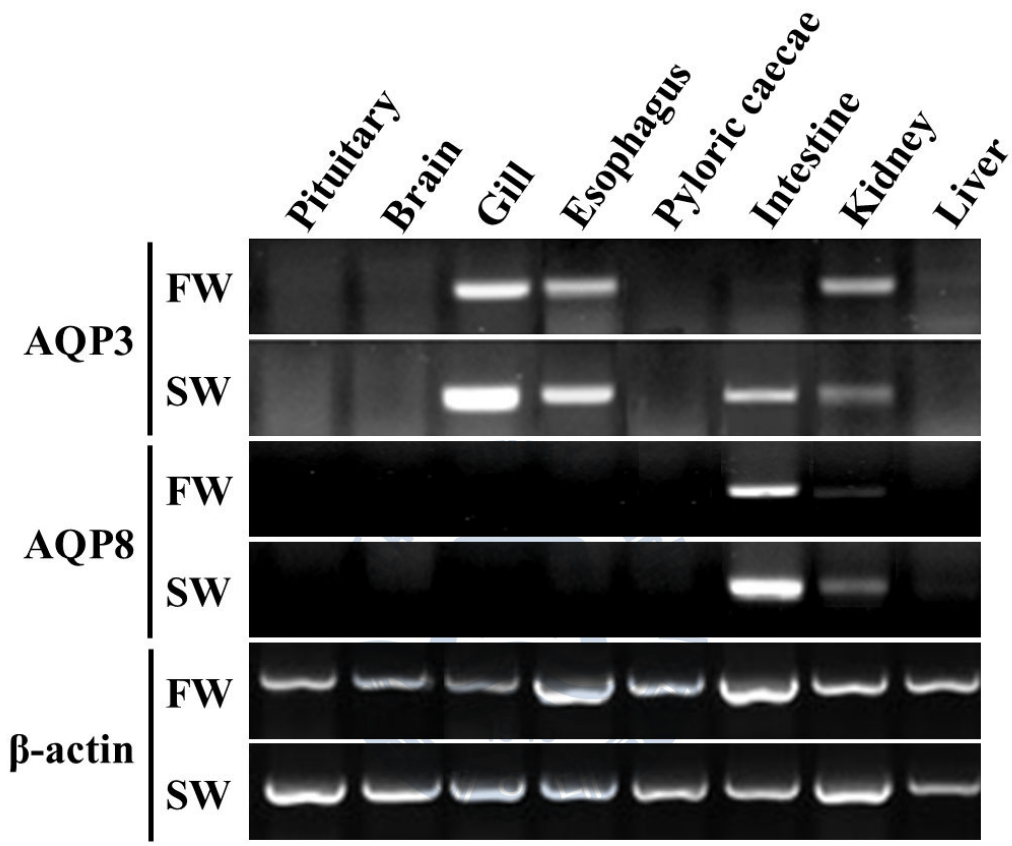

Fig. 1. Tissue distribution of parr sockeye salmon AQPs (pituitary, brain, gill, esophagus, pyloric caecae, intestine, kidney, and liver). RT-PCR analysis of AQP3 and AQP8 transcripts in different tissues collected from FW and SW fish, as shown in a 1.0% agarose electrophoresis gel with ethidium bromide.

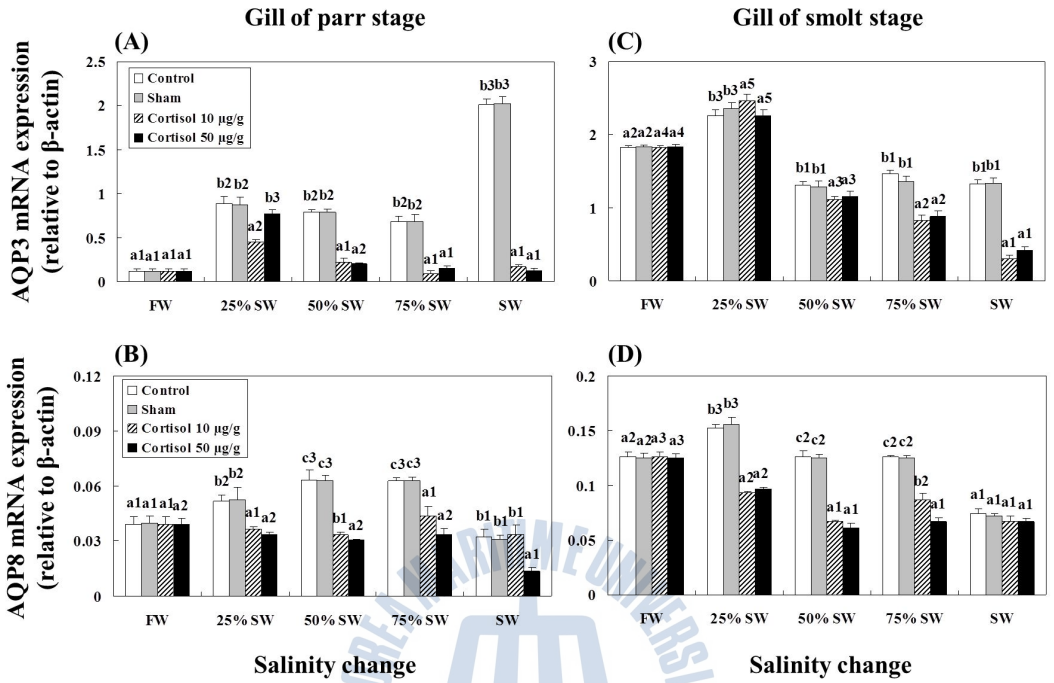

Fig. 2. Expression levels of AQP3 (A, C) and AQP8 (B, D) mRNAs in the gill of parr (A, B)/smolt (C, D) sockeye salmon after salinity transfer from FW (0 psu) to SW (35 psu), and after cortisol injection, using quantitative real-time PCR. Three micrograms of total RNA prepared from gill tissue was reverse-transcribed and amplified the samples using gene-specific primers. Results are expressed as normalized fold-expression (relative to control) with respect to β-actin levels for the same sample, and values are given as means ± SE (n = 5). Values with letters indicate significant differences for the FW→SW transition, within the same time after salinity change. The numbers indicate a significant difference from the control within the same salinity and the cortisol treatment group ($P < 0.05$).

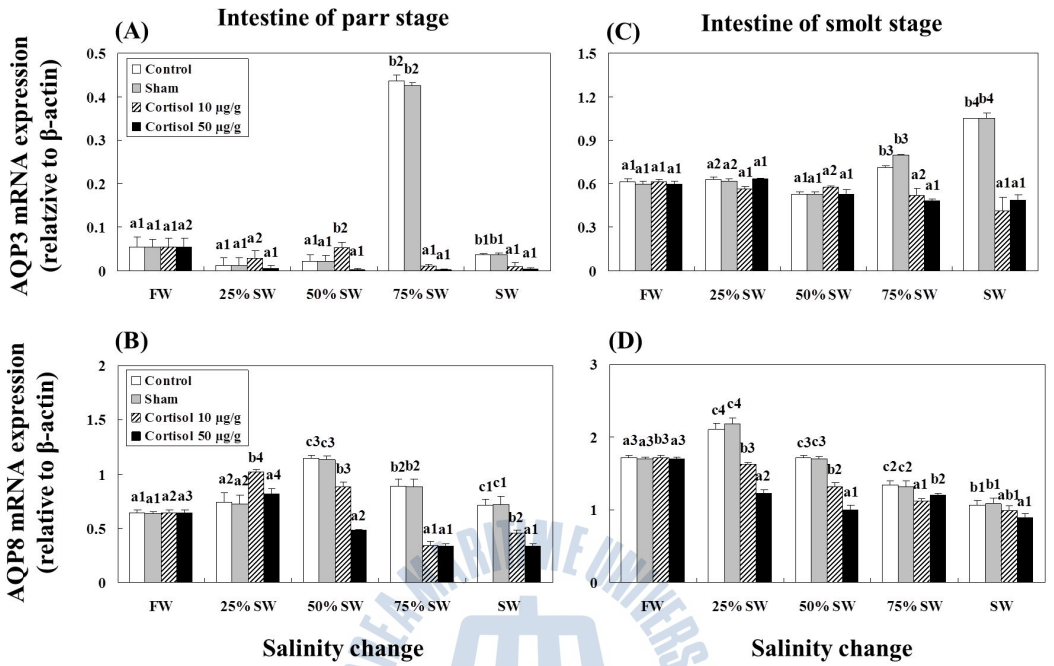

Fig. 3. Expression levels of AQP3 (A, C) and AQP8 (B, D) mRNA levels in the intestine of parr (A, B)/smolt (C, D) sockeye salmon after salinity transfer from FW (0 psu) to SW (35 psu), and after cortisol injection, using quantitative real-time PCR. Three micrograms of total RNA prepared from intestine was reverse-transcribed and amplified the samples using gene-specific primers. Results are expressed as normalized fold-expression (relative to control) with respect to β -actin levels for the same sample, and values are presented as means ± SE (n = 5). Values with letters indicate significant differences at the FW→SW transition, within the same time after salinity change. The numbers indicate a significant difference from the control within the same salinity and the cortisol treatment group ($P < 0.05$).

3.3. Expression of AQP protein levels in SW- and FW-acclimated fish, and after cortisol injection of parr/smolt sockeye salmon (*in vivo*)

In western blot analysis, AQP proteins were detected at a size corresponding to the predicted size for sockeye salmon AQPs (AQP3: approximately 33 kDa, and AQP8: approximately 27 kDa), and the expression of these respective proteins correlated with the AQP3 and AQP8 mRNA levels in parr/smolt sockeye salmon gill and intestines (Fig. 4). More particularly, AQP3 (in the gills) and AQP8 protein levels (in the intestines) were higher in SW- than in FW acclimated fish. Furthermore, the expression of AQPs in the cortisol-injected group decreased after the transfer to SW, as compared to the control group.

3.4. IHC of AQP3 in the gill

An increase in the intensity and area of AQP3-IR staining in IHC was observed in the mitochondria-rich cells (MRCs) of SW-acclimated sockeye salmon gills, compared to that in FW-acclimated fish (Fig. 5). The gills of the cortisol-treated sockeye salmon appeared to have less AQP3-IR compared to that of SW-acclimated control fish.

3.5. Expression of AQP mRNAs and cortisol injection of smolt sockeye salmon in cultured gill and intestine (*in vitro*)

QPCR analyses showed the relative expression levels of AQP3 and AQP8 mRNAs in the cultured tissue (gill, intestine) of smolt sockeye salmon during salinity change (Fig. 6). The AQP3 and AQP8 mRNA expression was significantly higher in the cultured gill and intestine tissue derived from SW than from FW fish. Moreover, the expression of AQP mRNAs in the cortisol-injected group was significantly decreased after the transfer to SW, compared to the control group ($P < 0.05$).

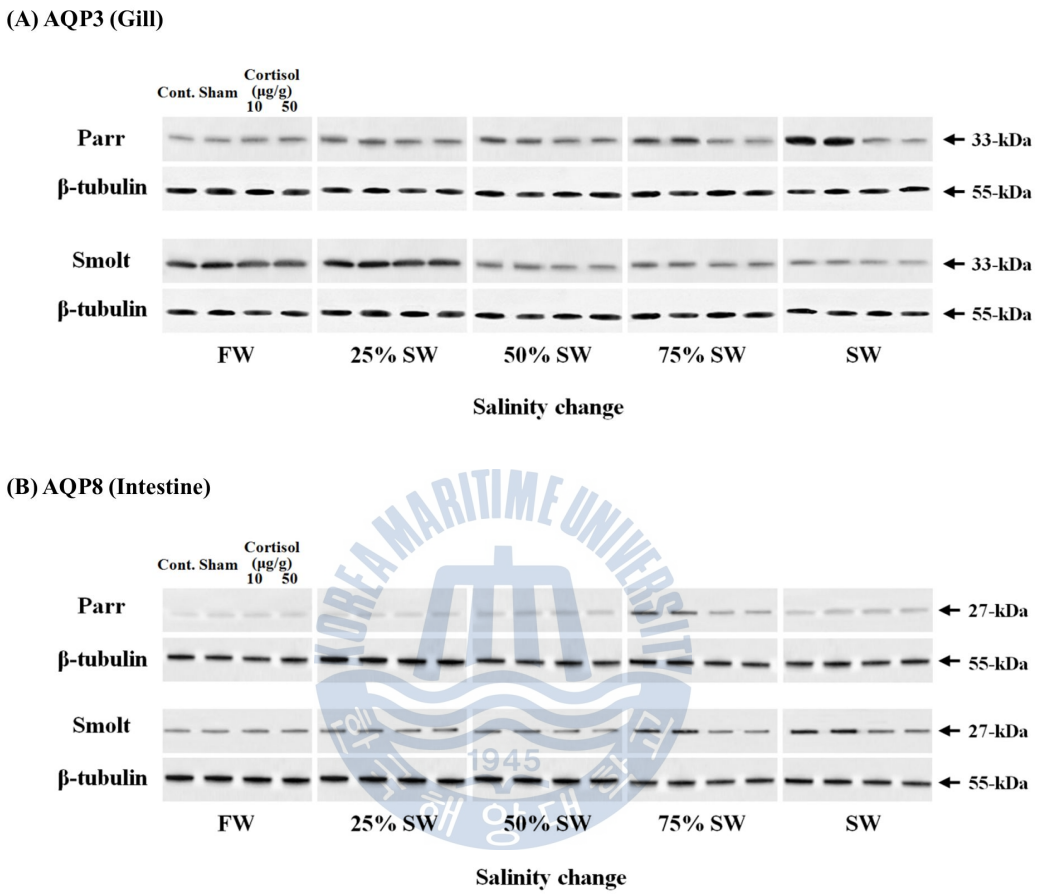

Fig. 4. Western blot of AQP3 (A) and AQP8 (B) protein expression in gills and intestines of parr/smolt sockeye salmon after salinity transfer from FW (0 psu) to SW (35 psu), and after cortisol injection; β-tubulin (55 kDa) was used as the internal control.

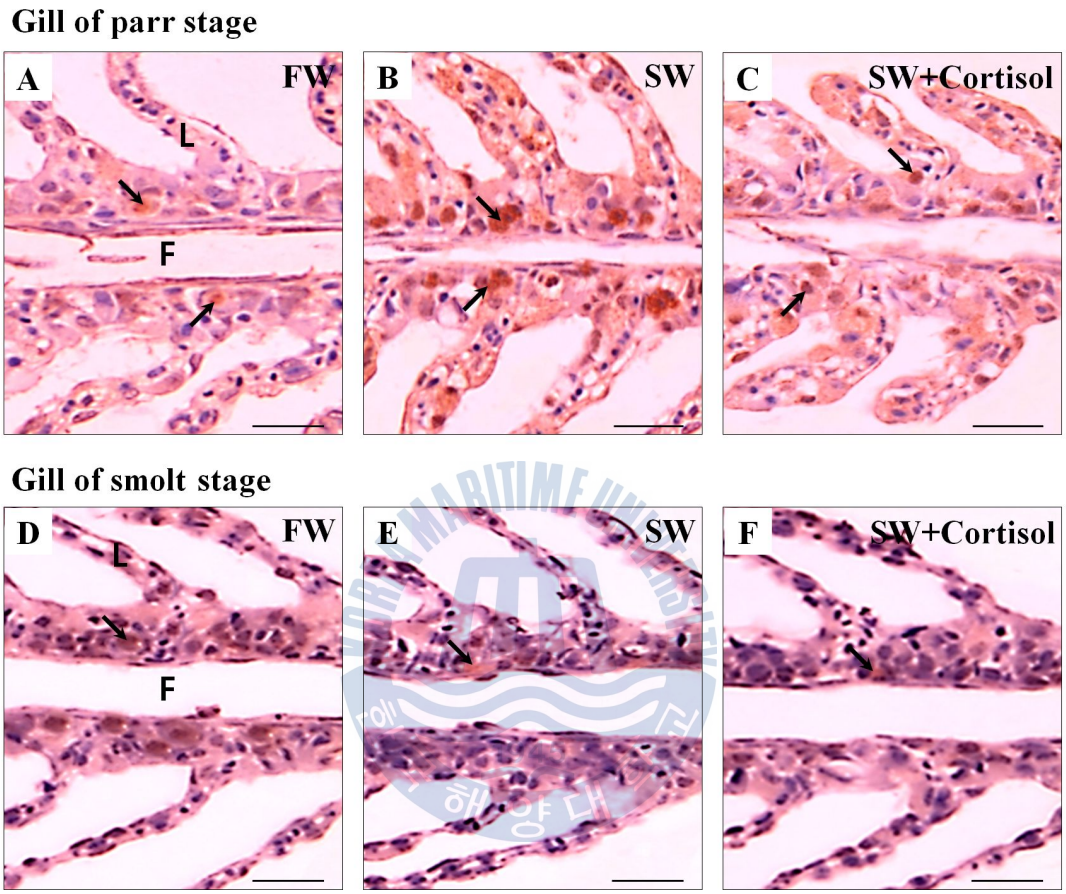

Fig. 5. Immunohistochemical localization of gill AQP3-IR in cross sections of parr/smolt sockeye salmon gill adapted to different salinities (FW and SW). (A and D): FW; (B and E): SW; (C and F): SW treated with cortisol (50 μg/g); (A, B, and C) indicate parr, and (D, E, and F) indicate smolt sockeye salmon. Arrows indicate AQP3-IR cells, which are stained brown. F = filament; L = lamellae. Bar = 10 μm.

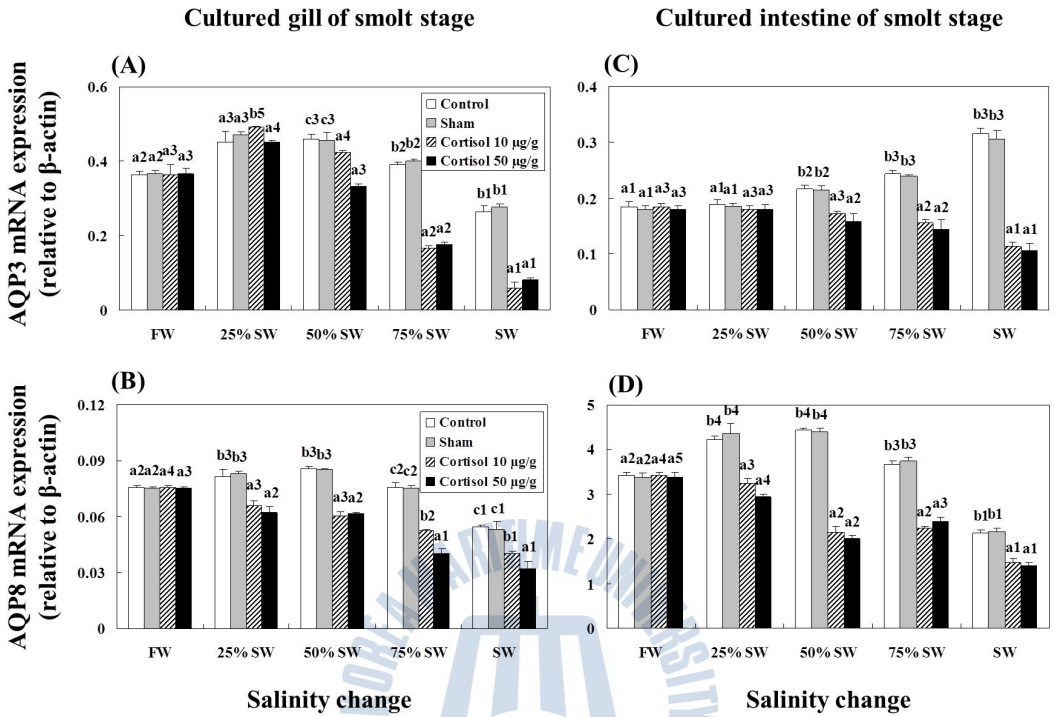

Fig. 6. Expression levels of AQP3 (A, C) and AQP8 (B, D) mRNAs from cultured gill (A, B) and intestine (C, D) samples of smolt sockeye salmon after salinity transfer from FW (0 psu) to SW (35 psu), and after cortisol injection, using quantitative real-time PCR. Three micrograms of total RNA prepared from gill and intestine samples were reverse-transcribed and amplified the samples using gene-specific primers. Results are expressed as normalized fold-expression (relative to control) with respect to β-actin levels for the same sample, and values are presented as means ± SE (n = 5). Values with letters indicate significant differences at FW→SW transition within the same time after salinity change. The numbers indicate a significant difference from the control within the same salinity and cortisol treatment group ($P < 0.05$).

3.6. Plasma osmolality

Plasma osmolality of sockeye salmon were 293 ± 7.0 mOsm/kg for parr, and 301 ± 7.2 mOsm/kg for smolt fish at the start of the experiment. Osmolality reached its highest level of 411 ± 5.3 mOsm/kg and 381 ± 2.6 mOsm/kg for parr and smolt fish, respectively, after transfer to SW. However, plasma osmolality in SW-acclimated parr and smolt sockeye salmon given cortisol treatment decreased to 344 ± 4.0 mOsm/kg (parr) and 324 ± 4.2 mOsm/kg (smolt) after the transfer (Fig. 7; $P < 0.05$).

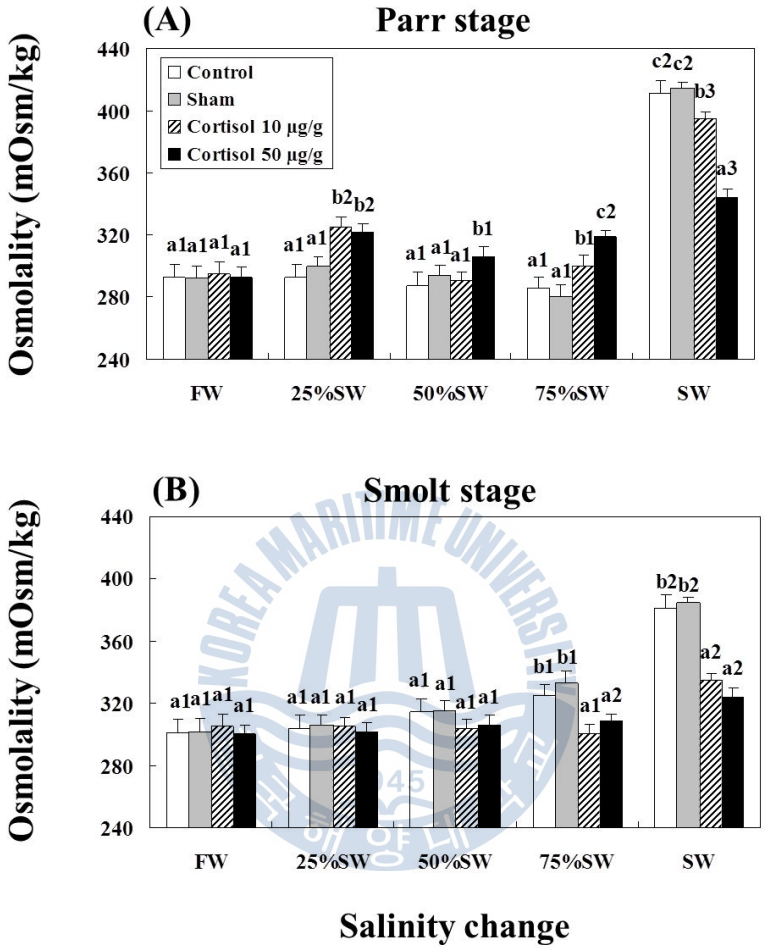

Fig. 7. Effect of cortisol treatment on plasma osmolality levels after salinity transfer from FW (0 psu) to SW (35 psu), and after cortisol injection in parr (A)/smolt (B) sockeye salmon, and values are means ± SE (n = 5). Values with letters indicate significant differences between FW to SW and cortisol injection within the same time-range after salinity change. The numbers indicate a significant difference from the control within the same salinity and cortisol treatment group ($P < 0.05$).

4. Discussion

I examined the adaptation of sockeye salmon to various saline conditions with respect to their osmoregulatory capacity and the associated expression of AQPs. First, I compared the expression and activity of AQP3 and AQP8 over time after migrating sockeye salmon were transferred from FW to an artificially hyper-osmotic environment (FW→SW). I observed that AQP3 mRNA (in the gills) and AQP8 mRNA (in the intestines) were expressed at significantly higher levels in the SW environment than in the FW environment (Fig. 1).

These results indicate that AQP3 mRNA levels increase during the salinity change from FW to SW, similar to a previous study in European eel *Anguilla anguilla* (Martinez et al., 2005) and gilthead sea bream *Sparus aurata* (Raldúa et al., 2008). Moreover, previous studies have shown that AQP8 mRNA levels increased in the intestines of Atlantic salmon (Tipsmark et al., 2010) and Japanese eel *A. japonica* (Kim et al., 2010) upon transfer to SW. The increased expression of AQP3 and AQP8 mRNAs during SW-acclimation suggested that AQPs are involved in the hyper-osmoregulatory ability of sockeye salmon during adaptation to SW.

Similarly, I observed that the expression of AQP3 and AQP8 mRNAs and protein levels in the gills of parr stage sockeye salmon increased in SW after the salinity of the environment was artificially changed (Figs. 2 and 4). Tipsmark et al. (2010) reported that chloride cells change the structure for water flow and increase the flux of water molecules in order to decrease the osmotic difference between the internal and external environment during salinity changes. Therefore, I proposed that water flux increases in the cells of the gills due to increased AQPs mRNA expression in a SW environment.

Furthermore, AQP3-IR staining significantly increased in the MRCs of the gills of parr stage sockeye salmon during SW acclimation when compared with those of FW-acclimated fish (Fig. 5). Teleost gills have MRCs, which control the absorption

of NaCl and calcium in the FW-environment, and secrete Cl^- in the SW environment (Evans, 1993; Marshall, 2003; Marshall and Grosell, 2006), MRCs also play an important role in maintaining acid–base balance, excretion of ammonium and ammonia, and excretion of urea to maintain urea homeostasis (Marshall and Grosell, 2006). This result agreed with those of a previous study (Jung et al., 2012), which showed that AQP3 levels are increased in the MRCs of killifish *Fundulus heteroclitus* gills during transfer from a FW to a SW environment.

In contrast, expression of AQP3 and AQP8 mRNA levels were significantly increased in the gills of smolt stage salmon that had been acclimated to 25% SW, and then decreased with salinity increases towards 100% SW (Figs. 2C, D, and 4A). AQP3 and AQP8 mRNA expression levels increased in order to maintain homeostasis during SW-acclimation; then, expression of AQPs decreased in parr stage salmon that had been acclimated to 50% SW as sockeye salmon had gained significant osmoregulatory ability. Furthermore, Sundell et al. (2003) have reported that cortisol levels increased significantly at the time of smoltification in Atlantic parr stage salmon; hence, these results suggested that teleost fish gain the ability for osmoregulation during the smolt stage.

The expression of AQP3 mRNA increased in the intestine of parr and smolt stage sockeye salmon during SW adaptation (Fig. 3A and C). Previous studies also indicate that AQP mRNAs are significantly increased in the intestine of SW- compared to FW-acclimated euryhaline fish [European eel (Martinez et al., 2005), European sea bass (Giffard-Mena et al., 2007), and gilthead sea bream (Raldúa et al., 2008)]; thus, AQPs appear to be engaged in the absorption of water through the intestine. Moreover, AQP3 is present in the epithelial cells of the intestine, and control the inflow of salt via water absorption (Aoki et al., 2003; Martinez et al., 2005; Cutler et al., 2007; Raldúa et al., 2008). Similarly, results of these study suggest that AQP3 mRNA expression is increased in order to facilitate absorption of water from the surface of the intestine.

In contrast, expression of AQP3 mRNA increased rapidly in parr and smolt stage sockeye salmon acclimated to 75% SW; overall expression levels were

significantly higher in the smolt than in the parr stage salmon. This indicates that the intestines of parr stage sockeye salmon have the ability to discharge the excess water absorbed from FW, but during the smoltification period, the intestines gain the ability to adapt to SW, such as absorption of water and ions, due to increased expression of AQP3. I propose that at the parr stage, sockeye salmon are not yet equipped with the ability to adapt to SW conditions; hence, AQP3 mRNA expression is increased rapidly, depending on exposure to environmental salinity changes.

The expression of AQP8 mRNA increases in the intestine of both parr and smolt stage sockeye salmon acclimated to SW, when compared to FW-acclimated fish (Figs. 3 and 4). AQP8 reconstituted into lipid bilayers facilitates neutral ammonia transport, with a two-fold preference over water molecules (Saparov et al., 2007). In Atlantic salmon, AQP8 peaked during smoltification (Tipsmark et al., 2010). The results of these study suggest that AQP8 mRNA is highly expressed in the intestine during SW-acclimation. AQP8 enhances the hypo-osmoregulatory ability required for adaptation to a SW environment. On the other hand, expression of AQP8 mRNA decreases in the gills during the smolt stage (Fig. 2), which suggests that sockeye salmon has then obtained the osmoregulatory ability during transition from parr to smolt stage.

Moreover, plasma osmolality was maintained at higher levels in the parr stage than in the smolt stage upon exposure to a SW environment (Fig. 7). Parr stage fish are considered to be more able to adapt to external salinity changes than smolt stage fish. In a previous study, it was reported that cortisol levels increase during smoltification (Sundell et al., 2003). I suggest that increased cortisol levels improve the osmotic-regulatory capacity from hypo-osmoregulation to hyper- osmoregulation in a SW environment.

Also investigated changes in AQP mRNAs expression in cultured osmoregulatory tissue, such as gills and intestine (Fig. 6); the observed patterns were similar to those found *in vivo* experiments. Therefore, I suggest that AQPs act directly to regulate water inflow and osmotic pressure in the osmoregulatory organs of teleost fish during salinity change.

I further investigated the expression of AQP mRNAs in SW acclimated fish after injection of cortisol (10 or 50 μg/g BM) to elucidate the role of cortisol as a SW-adapting hormone. After cortisol injection, expression of AQP mRNAs was lower than in the control group (FW); thus, AQP expression may be repressed by cortisol. Cortisol, a major corticosteroid hormone, affects the regulation of ion and water absorption in the gills and intestine, and improves osmoregulatory capacity after a change in salinity, which requires an increase in AQP expression (McCormick, 2001; Martinez et al., 2005). In the present study, AQP mRNAs and protein expression levels are reduced in the parr and smolt stages after treatment with cortisol (Figs. 2－6); Therefore, I propose that cortisol inhibits the expression of AQPs (Cutler et al., 2007). This result agreed with those of a previous study (Martinez et al., 2005; Cutler et al., 2007), which indicated that the mRNA and protein expression levels of AQP1 and AQP3 were decreased in European eel during a salinity change from FW to SW after cortisol injection.

Furthermore, plasma osmolality levels in the cortisol injection group were significantly decreased during transfer from FW to SW in the parr/smolt stage of sockeye salmon (Fig. 7). I propose that cortisol, the SW-acclimation hormone, induced ion adaptation in a hyper-osmotic environment, hence decreasing plasma osmolality.

In summary, I hypothesize the following. (1) AQPs are involved in osmoregulation by selectively controlling water permeation in sockeye salmon in a SW environment. (2) Sockeye salmon gain the ability to adjust osmolality to saline environments during smoltification. (3) Cortisol increases water transfer during transition from the parr to the smolt stages, enhancing the SW hypo-osmoregulatory capacity of fish, and also controls expression of AQPs.

Chapter 3

Effects of exogenous cortisol on seawater acclimation in relation to expression of TR mRNAs in the parr and smolt stages of sockeye salmon

Abstract

The objective of this investigation was to quantify how TR of the sockeye salmon *O. nerka*, respond to salinity changes from FW to SW conditions. 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. These results 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.

1. 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 *O. nerka* juveniles stay in FW for 16－18 months to grow into smolts that have the ability to tolerate 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 hypo-osmoregulatory 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 (Hoar, 1988), 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 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 HPT axis. For instance, the hypothalamus stimulates the production and release of the TSH by the pituitary (MacKenzie et al., 2009). In turn, TSH stimulates the secretion of T_4 by the thyroid, which is subsequently converted to the more biologically active T_3 (MacKenzie et al., 2009). The biological actions of THs are mediated through the 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, I investigated the response of TR mRNA and protein expressions when experimental sockeye salmon acclimated to FW were transferred to SW. Also examined the response of plasma T_3 and T_4; Na^+, Cl^-, and K^+ ions; and cortisol levels to salinity changes. Furthermore, I 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.

2. Materials and methods

2.1. Experimental fish

One-year-old sockeye salmon (average length 9.5 ± 0.5 cm; mass 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. I 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.

2.2. 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 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.

2.3. 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.

2.4. QPCR

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 iQTM 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 b-actin, and all data were expressed relative to the corresponding b-actin calculated Ct levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls was calculated as ΔΔCt = 2^−(Δ $Ct_{sample} - \Delta Ct_{internal\ control}$).

2.5. 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 PMSF, 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 diflouride membrane (Bio-Rad) at 85 V for 1.5 h at 4°C. Thereafter, the membranes were blocked with 5% milk in 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 antirabbit IgG secondary antibodies (dilution, 1:2,000; Bio-Rad) for 60 min. The internal control was β -tubulin (dilution, 1:5,000; 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.

2.6. Plasma parameter analysis

Plasma T_3 and T_4 levels were analyzed using enzyme immunoassay with the T_3 and T_4 immunoassay 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; Fuji- Film, Tokyo, Japan).

2.7. 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.

3. Results

3.1. 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. 8A and 9A).

3.2. 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 TS and TS levels in cortisol-injected groups were significantly lower than in the controls (Fig. 10).

3.3. 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. 11).

3.4. 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. 12).

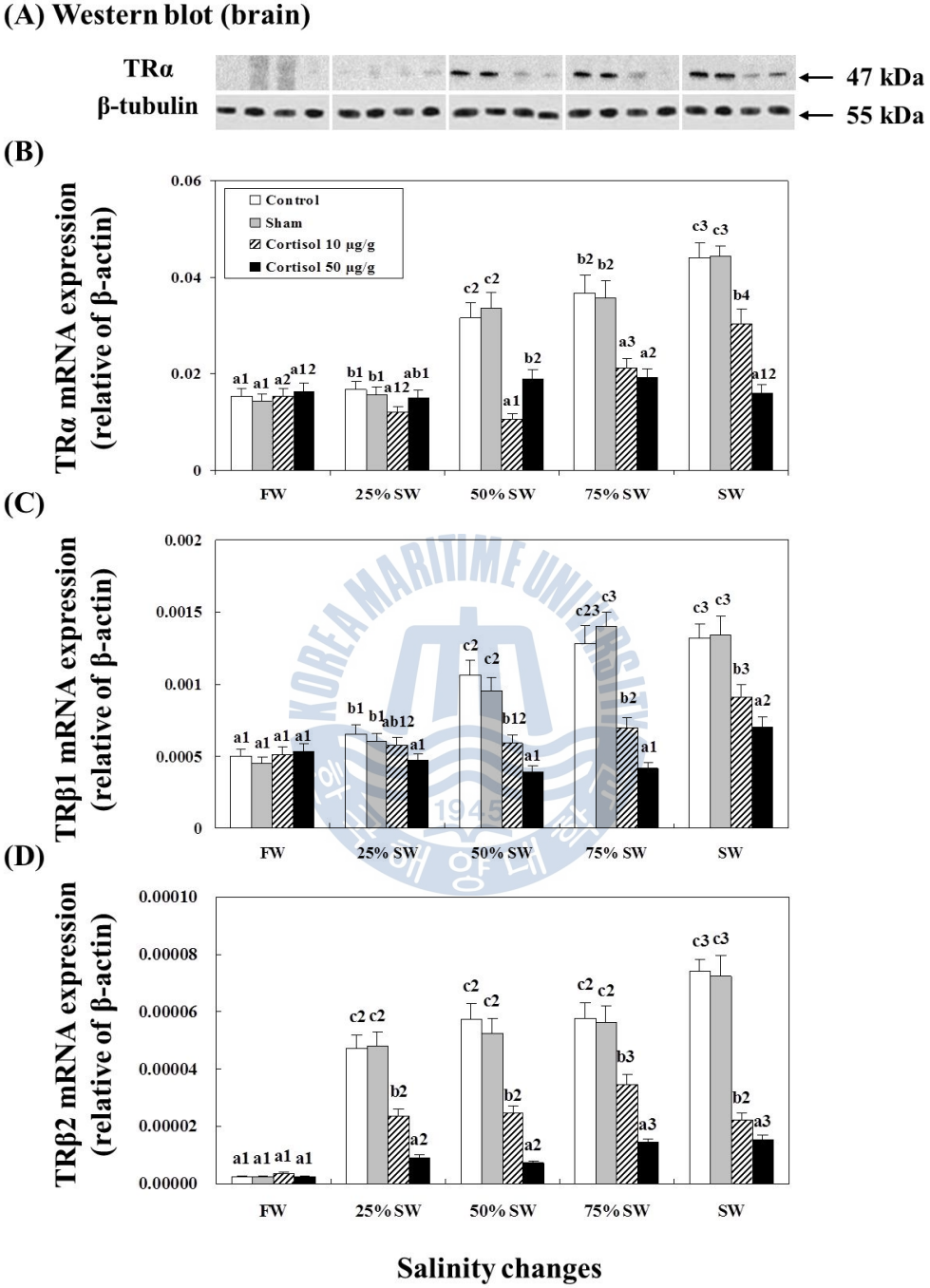

Fig. 8. TRα, TRβ1, and TRβ2 mRNA expression levels in the brain of 4 groups of sockeye salmon: without injection (control), injected with 10 and 50 μg/g cortisol, and injected with saline water (sham), which were transferred from FW to SW. (A) Western blot analysis using TRα (47 kDa) to examine protein expression in the brain of sockeye salmon; the internal control was 55 kDa β-tubulin. (B) TR α, (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 10 and cortisol 50 μg/g) ($P < 0.05$). All values are mean ± SE ($n = 5$).

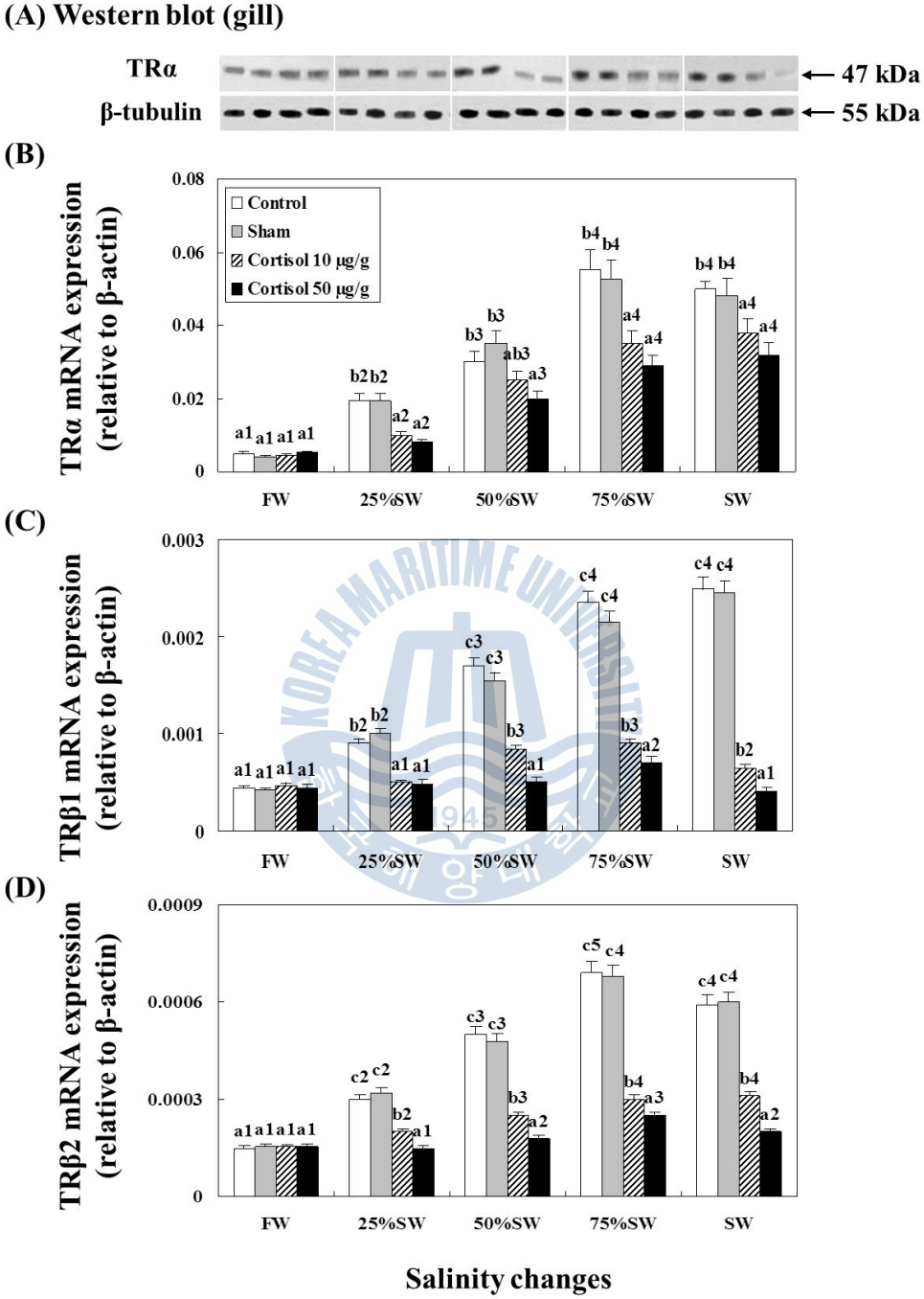

Fig. 9. (A) Western blot analysis using TRα (47 kDa) to examine protein expression in the gill of sockeye salmon; the internal control was 55 kDa β-tubulin. (B) TRα, (C) TRβ1, and (D) TRβ2 mRNA expression levels in the gill of 4 groups of sockeye salmon: without injection (control), injected with 10 and 50 μg/g cortisol, and injected with saline water (sham), which were transferred from FW to SW.

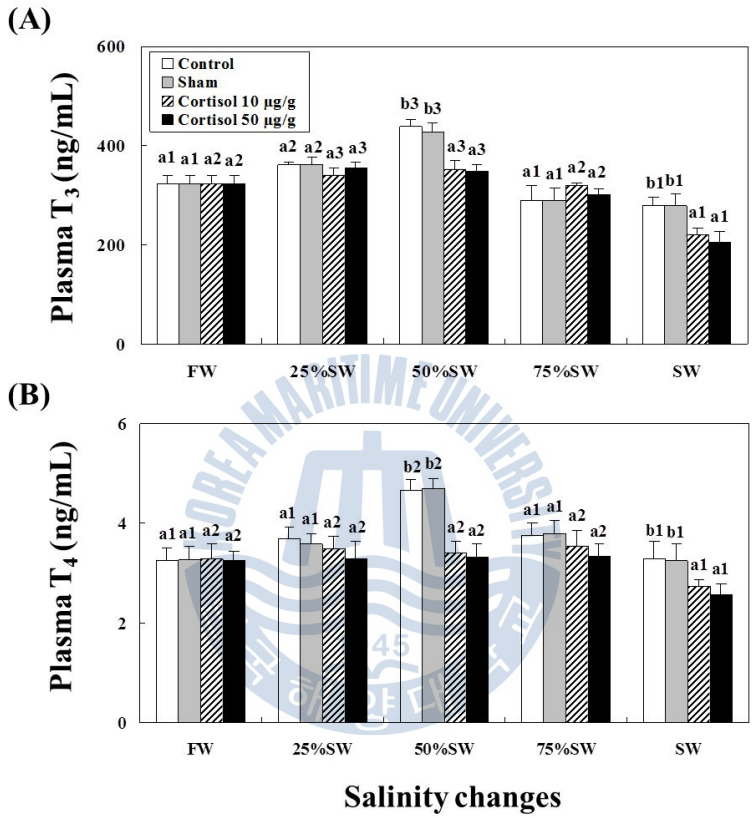

Fig. 10. Plasma T_3 and T_4 of 4 groups of sockeye salmon: without injection (control), injected with 10 and 50 μg/g cortisol, and injected with saline water (sham), which were transferred from FW to SW.

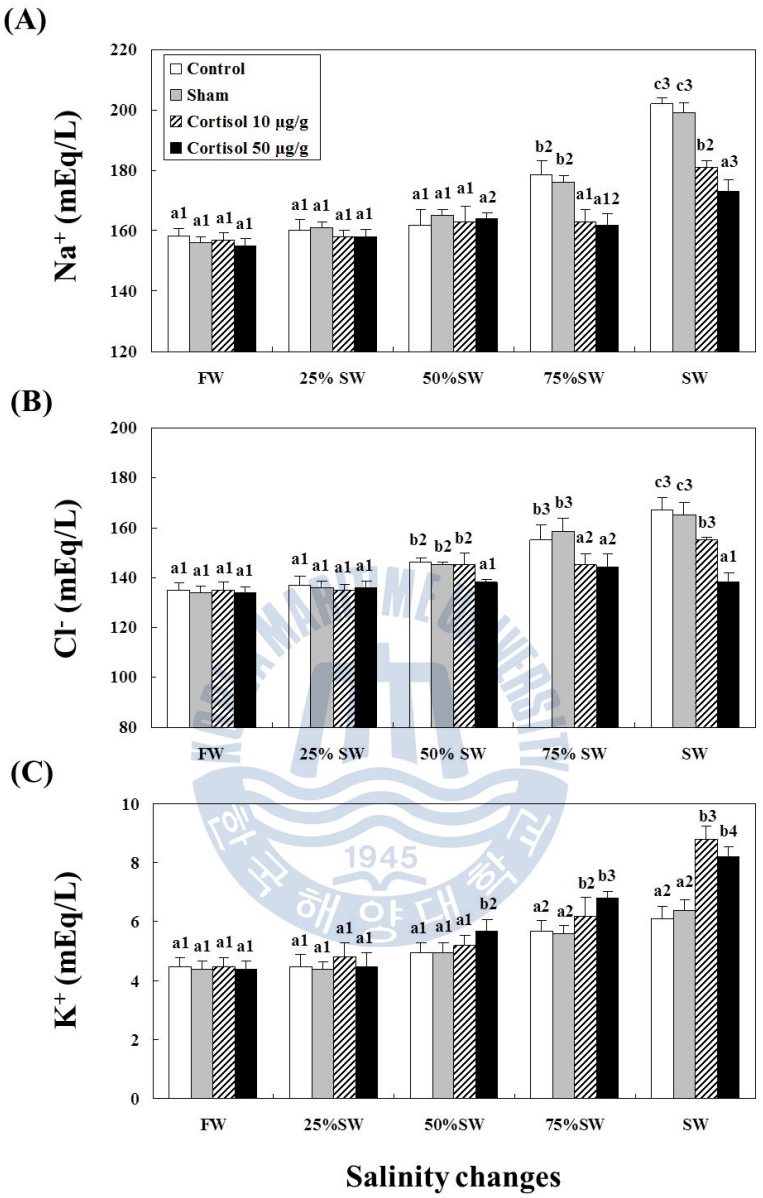

Fig. 11. Plasma Na^+, Cl^-, and K^+ in 4 groups of sockeye salmon: without injection (control), injected with 10 and 50 μg/g cortisol, and injected with saline water (sham), which were transferred from FW to SW.

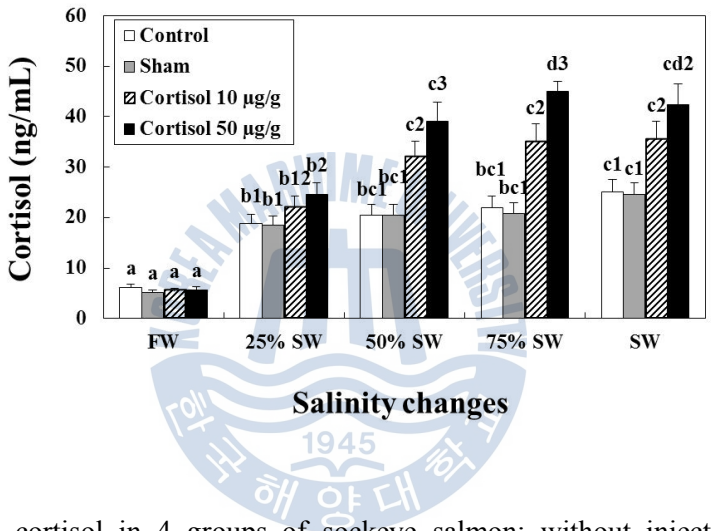

Fig. 12. Plasma cortisol in 4 groups of sockeye salmon: without injection (control), injected with 10 and 50 μg/g cortisol, and injected with saline water (sham), which were transferred from FW to SW.

4. Discussion

The present study, examines 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. 8 and 9). 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 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. This study shows 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 *O. 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 (Brent, 1996); therefore, it is important to promote growth as sockeye salmon acclimate to salinity during the downstream migration into SW.

In this study, observation was made about the 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 TRH genes (Langlois et al., 1997). This observation indicates that TRβ2 is pivotal to the negative regulation of TSH and TRH (Nakano et al., 2004); hence, hypothesized that TRβ2 expression levels are lower than those of other TR isoforms. Furthermore, I 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 *O. masou* and amago salmon *O. 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, results 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. 10). 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 SW 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, I 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 SW 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, results 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. 11). Furthermore, plasma cortisol levels in cortisol injection groups were significantly higher than those in the control groups (Fig. 12). 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, I 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, I hypothesized that injected cortisol acted as a stress hormone by increasing K^+ levels.

In summary, I 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.

Chapter 4.

General Discussion

The osmoregulatory capacity in sockeye salmon exposed to osmotic environmental change was investigated in this study.

I. I examined the adaptation of parr/smolt sockeye salmon to various saline conditions (FW→SW) with respect to their osmoregulatory capacity and the associated expression of AQPs. Firstly, I observed that the expression of AQP3 and AQP8 mRNAs and protein levels in parr stage sockeye salmon increased in SW after the salinity of the environment was artificially changed. The increased expression of AQP3 and AQP8 mRNAs during SW-acclimation suggested that AQPs are involved in the hyper-osmoregulatory ability of sockeye salmon during adaptation to SW. Furthermore, MRCs play an important role in maintaining acid–base balance, excretion of ammonium and ammonia, and excretion of urea to maintain urea homeostasis. AQP3-IR staining significantly increased in the MRCs of the gills of parr stage sockeye salmon during SW acclimation when compared with those of FW-acclimated fish. In contrast, expression of AQP3 mRNA increased rapidly in parr and smolt stage sockeye salmon acclimated to 75% SW; overall expression levels were significantly higher in the smolt than in the parr stage salmon, which suggests that sockeye salmon has then obtained the osmoregulatory ability during transition from parr to smolt stage. After cortisol injection, expression of AQP mRNAs and protein expression levels are reduced in the parr and smolt stages after treatment with cortisol. Cortisol increases water transfer during transition from the parr to the smolt stages, enhancing the SW hypo-osmoregulatory capacity of fish, and also controls expression of AQPs.

II. I examined how sockeye salmon smolt adapted to various saline conditions (FW →SW) 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. TH is known to be a hormone that is specifically related to osmoregulation and the acclimation of fish to FW and SW conditions. Salinity acclimation increases THs levels, as both T_3 and T_4 are known for osmotic and metabolic actions in fish. I 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). Additionally, I 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. Furthermore, plasma cortisol levels in cortisol injection groups were significantly higher than those in the control groups. This result shows the methodological validity of cortisol injection and that cortisol plays a role in osmoregulation by salinity changes.

So, I hypothesized that AQPs and TRs are involved in osmoregulation by selectively controlling water permeation in sockeye salmon in a SW environment. Furthermore, cortisol increases water transfer during transition from the parr to the smolt stages, enhancing the SW hypo-osmoregulatory capacity of fish, and also controls expression of AQPs and TH.

The factors outlined above can be used as molecular and physiological responses markers in osmoregulatory for anodromous salmonid exposed to osmotic environmental changes, and thus be used to set environmental standards for salmonids farming.

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그리고 대학원 과정 동안 전공분야의 학문적 지식을 알려주신 조성환 교수님과 박인석 교수님 그리고 논문 심사를 맡아주신 장영진 교수님께 다시 한번 감사의 말씀을 올립니다.

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마지막으로 언제나 바른길로 인도하시고, 용기를 주시고, 아낌없이 모든 것을 주시는 사랑하는 아버지, 어머니, 할머니께 감사의 말씀 올리며 항상 지금처럼만 건강하셔서 옆에서 저를 지켜봐 주셨으면 좋겠습니다.

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