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# Expression of aquaporin-3 and −8 mRNAs in the parr and smolt stages of sockeye salmon, *Oncorhynchus nerka*: Effects of cortisol treatment and seawater acclimation

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ABSTRACT

This study aimed to examine the role of 2 aquaporin (AQP) isoforms (AQP3, and −8) in sockeye salmon (*Oncorhynchus nerka*) in response to a hyperosmotic challenge from freshwater to seawater (SW) during the parr and smoltification (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 freshwater (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 from their food and via the gills. However, in seawater (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 aquaporins (AQPs), cortisol, prolactin, growth hormone, $Na^+/K^+$-ATPase (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 from a 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

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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 $Na^+/N^+$-ATPase 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 (*Oncorhynchus 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 smoltification (smolt) stages. Additionally, we investigated the presence of these AQPs in the gill, by immunohistochemistry (IHC), after transferring the fish to SW. Furthermore, we investigated the effect of injection of the fish with the SW-adapting hormone, cortisol, and then examined how AQPs interacted with cortisol during acclimation of the salmon to SW.

## 2. Materials and methods

### 2.1. Experimental fish

Sockeye salmon (*O. nerka*; parr, $n = 150$, 8.4 ± 1.0 g; smolt, $n = 150$, 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, we 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 °C, 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 [BW]); the sham group was injected with an equal volume of 0.9% physiological saline (10 μL/g BW). 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 phosphate buffered saline (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 (L-l5) 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 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). 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; 40 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. Quantitative PCR (QPCR)

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 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 [$\Delta\Delta Ct = 2^{\wedge}\text{-}(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$].

### 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 phenylmethylsulfonyl fluoride [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, we 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 immunocytochemically according to the methods described in Uchida et al. (2000), with modifications. For identification of branchial AQP3-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.

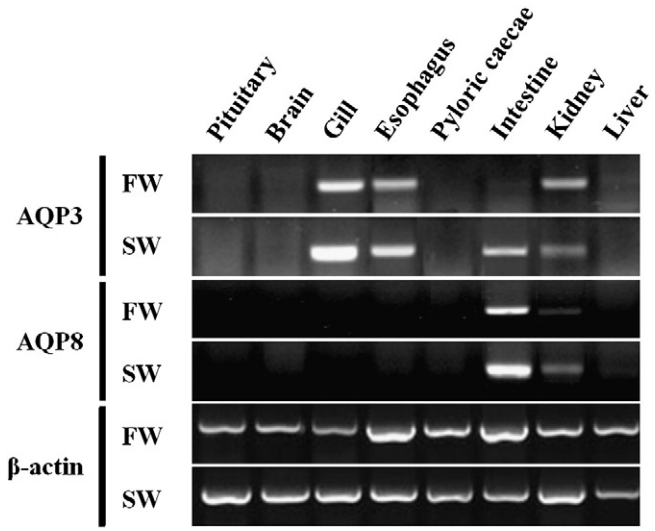


**Fig. 1.** Tissue distribution of parr sockeye salmon AQPs (pituitary, brain, gill, esophagus, pyloric caecae, intestine, kidney, and liver). RT-PCR analysis of AQP3 and AQP8 transcripts in different tissues collected from FW and SW fish, as shown in a 1.0% agarose electrophoresis gel with ethidium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

*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; $P < 0.05$).

### 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 ($P < 0.05$).

### 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

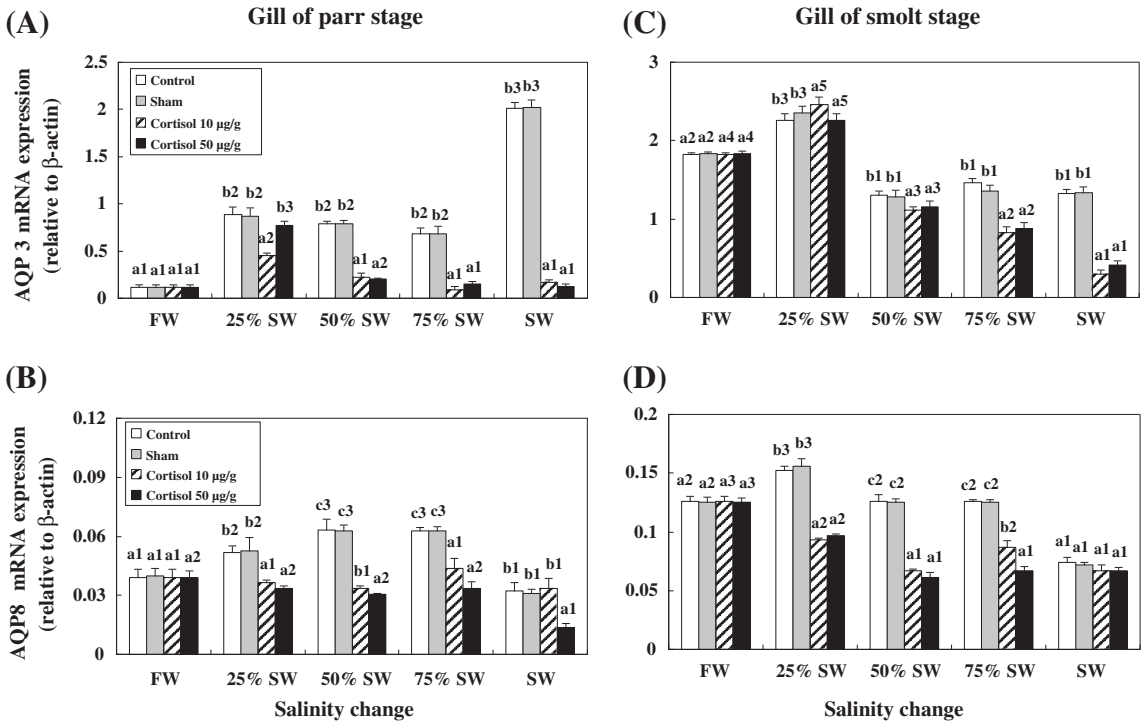


**Fig. 2.** Expression levels of AQP3 (A, C) and AQP8 (B, D) mRNAs in the gill of parr (A, B)/smolt (C, D) sockeye salmon after salinity transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu), and after cortisol injection, using quantitative real-time PCR. We reverse transcribed 3 μg of total RNA prepared from gill tissue 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 ($P < 0.05$).

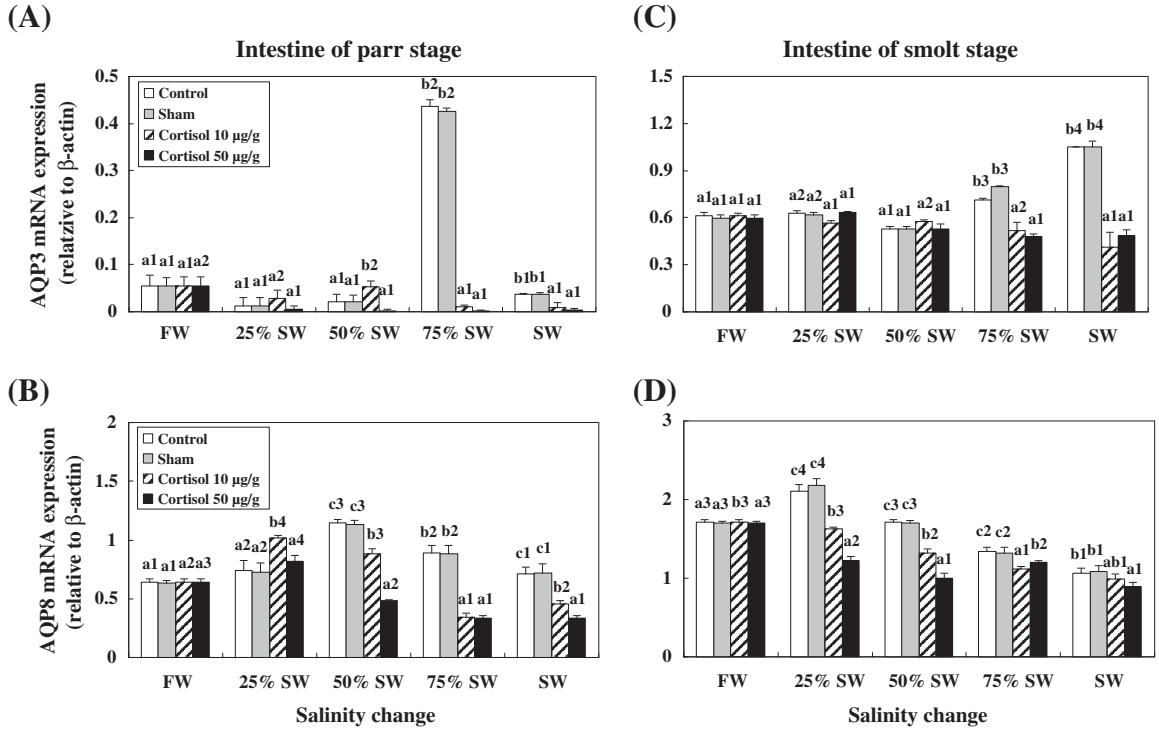


**Fig. 3.** Expression levels of AQP3 (A, C) and AQP8 (B, D) mRNA levels in the intestine of parr (A, B)/smolt (C, D) sockeye salmon after salinity transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu), and after cortisol injection, using quantitative real-time PCR. We reverse transcribed 3 μg of total RNA prepared from intestine specimens 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 ($P < 0.05$).

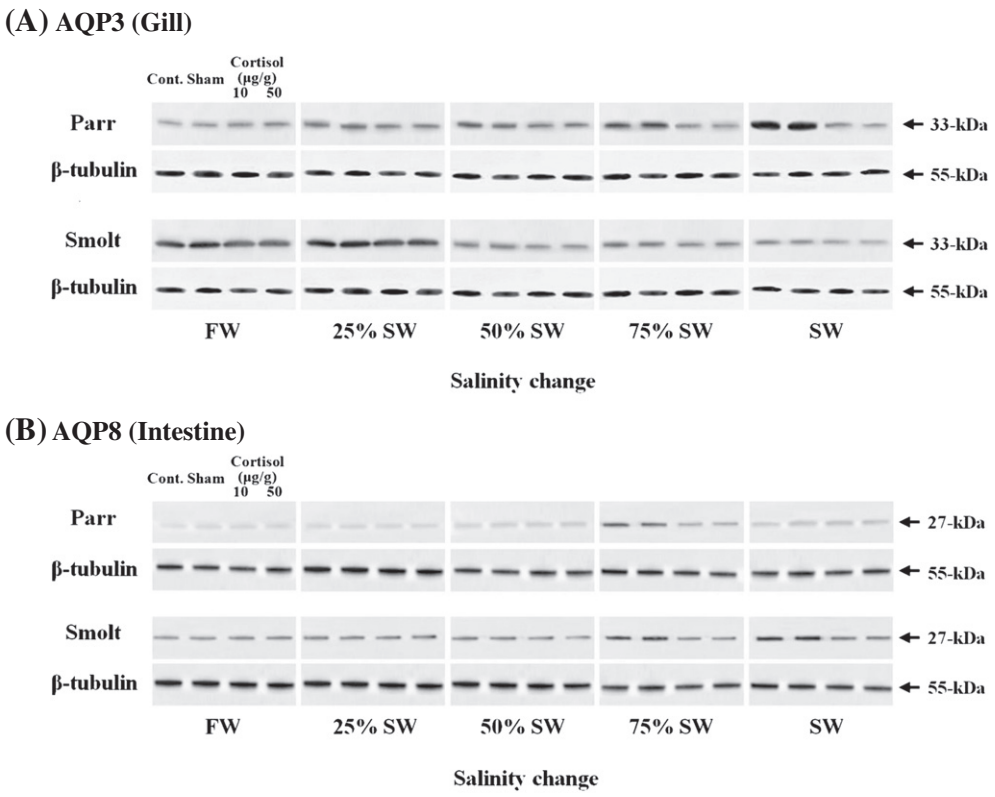


**Fig. 4.** Western blot of AQP3 (A) and AQP8 (B) protein expression in gills and intestines of parr/smolt sockeye salmon after salinity transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu), and after cortisol injection; β-tubulin (55-kDa) was used as the internal control.

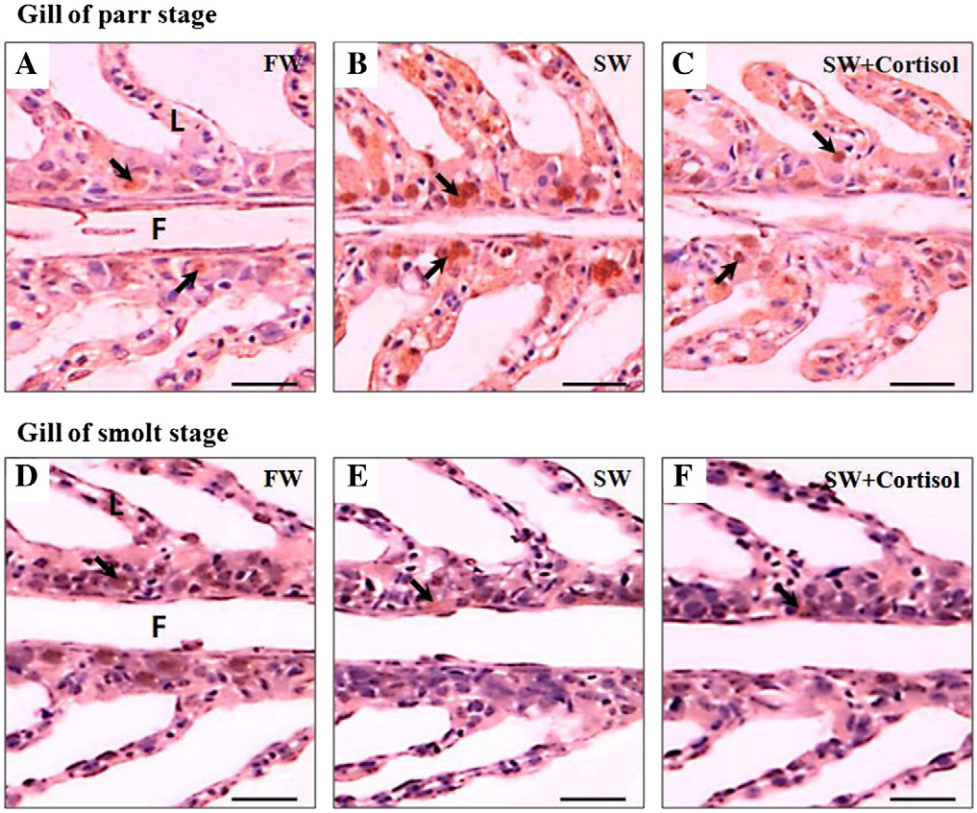


**Fig. 5.** Immunohistochemical localization of gill AQP3-immunoreactivity (AQP3-IR) in cross sections of parr/smolt sockeye salmon gill adapted to different salinities (FW and SW). (A and D): Freshwater (FW); (B and E): seawater (SW); (C and F): SW treated with cortisol (50 μg/g); (A, B, and C) indicate 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.

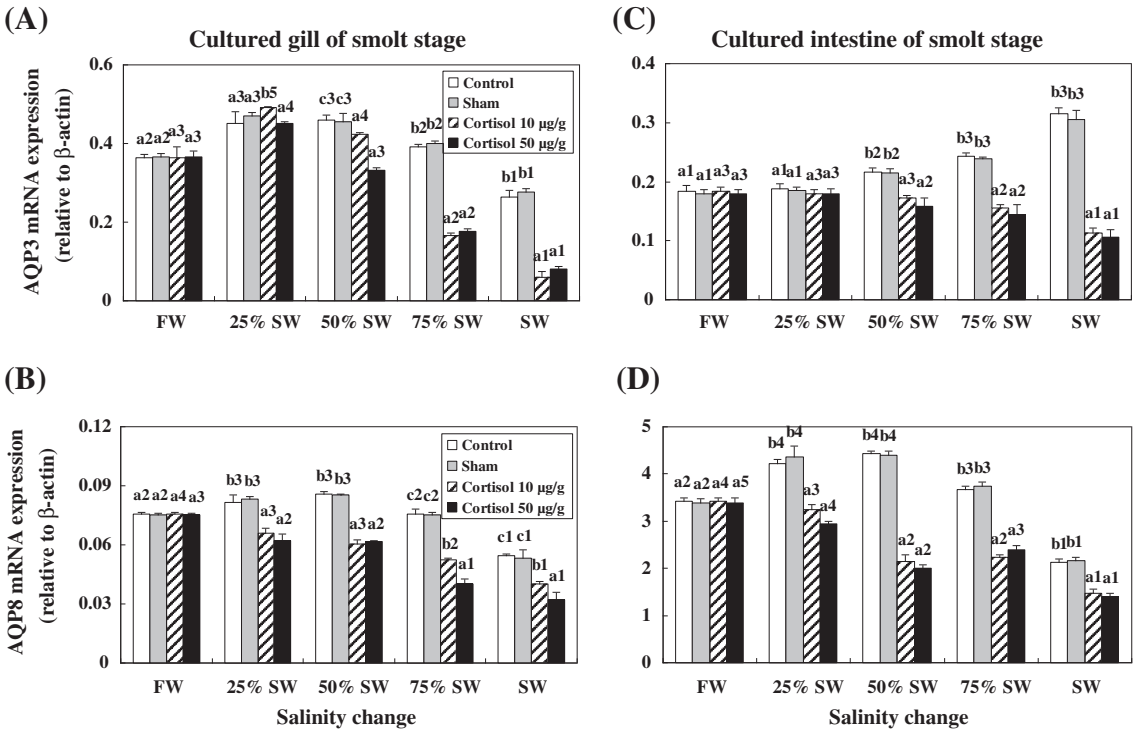


**Fig. 6.** Expression levels of AQP3 (A, C) and AQP8 (B, D) mRNAs from cultured gill (A, B) and intestine (C, D) samples of smolt sockeye salmon after salinity transfer (from freshwater [FW, 0 psu] to seawater [SW, 35 psu]), and after cortisol injection, using quantitative real-time PCR. We reverse transcribed 3 μg of total RNA prepared from cultured gill and intestine samples, 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 ($P < 0.05$).

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; $P < 0.05$).

## 4. Discussion

We examined the adaptation of sockeye salmon to various saline conditions with respect to their osmoregulatory capacity and the associated expression of AQPs. First, we 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). We 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 (*Anguilla 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, we 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. We

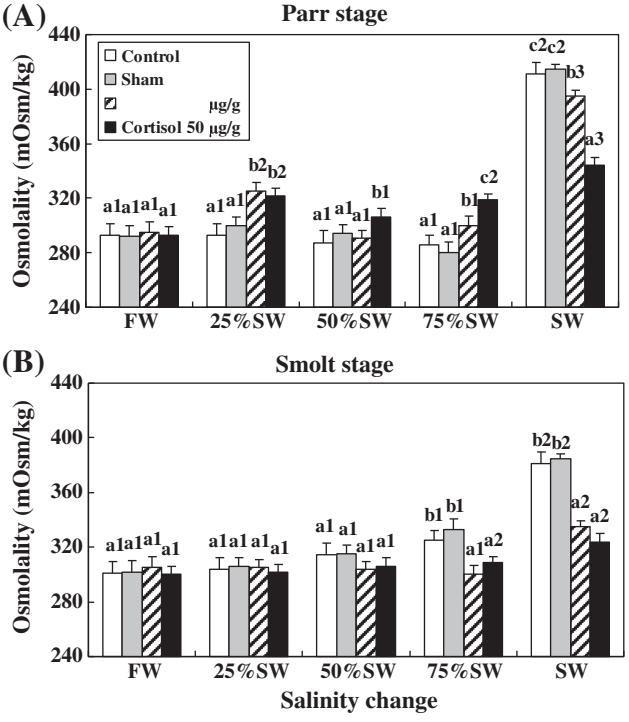


**Fig. 7.** Effect of cortisol treatment on plasma osmolality levels after salinity transfer (from freshwater [FW, 0 psu] to seawater [SW, 35 psu]), and after cortisol injection in parr (A)/smolt (B) sockeye salmon, and values are means ± SE ($n = 5$). Values with letters indicate significant differences 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 ($P < 0.05$).

therefore propose 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,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 our 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. We 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 our 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). We suggest that increased cortisol levels improve the osmotic-regulatory capacity from hypo-osmoregulation to hyper-osmoregulation in a SW environment.

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

We further investigated the expression of AQP mRNAs in SW-acclimated fish after injection of cortisol (10 or 50 μg/g BW) 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); we therefore 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). We propose that cortisol, the SW-acclimation hormone, induced ion adaptation in a hyper-osmotic environment, hence decreasing plasma osmolality.

In summary, we 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.

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