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Profiles of hypothalamus–pituitary–interrenal axis gene expression in the parr and smolt stages of rainbow trout, *Oncorhynchus mykiss*: Effects of recombinant aquaporin 3 and seawater acclimation

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ABSTRACT

The objective of this investigation was to quantify how the hypothalamus–pituitary–interrenal (HPI) axis in the rainbow trout, *Oncorhynchus mykiss* (parr/smolt), responds to salinity changes during transfer from freshwater (FW) to seawater (SW) and recombinant aquaporin 3 (rAQP3) injection. mRNA expression levels of HPI axis genes [corticotropic-releasing hormone (CRH) and adrenocorticotropic hormone (ACTHα and ACTHβ)] significantly increased when the fish were transferred from FW to SW (parr: 16.4-, 13.2-, 21.4-, and 11.9-fold higher than FW; smolt: 2.3-, 2.7-, 13.6-, and 6.2-fold higher than FW, respectively). Furthermore, and the plasma ACTH, Na^+, Cl^-, and K^+ levels were the highest at 50% SW. Moreover, these parameters were significantly lower in the rAQP3-treated group than those in the control (parr: 2.0-, 2.4-, 2.1-, and 2.0-fold lower than SW; smolt: 4.2-, 1.9-, 2.4-, and 2.3-fold lower than SW, respectively). Hence, HPI axis genes may play a role in SW adaptation during migration from FW to SW environments. We showed that there was a negative correlation between rAQP3, HPI axis genes, and ion levels when the fish were transferred to SW, with levels being significantly lower in the rAQP3-injected group. Hence, cortisol appears to be a stress hormone and plasma Na^+ and Cl^- levels significantly increased when the fish were transferred to SW, with levels being significantly lower in the rAQP3-treated group. These results indicate that rAQP3 modulates the HPI axis and ion transportation in rainbow trout.

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; Bentley, 2002). 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 external environment (Evans, 1993; Bentley, 2002).

In teleosts, water and osmolality homeostasis is mainly regulated by the hypothalamus–pituitary–interrenal axis (HPI axis). Hormones and proteins, such as corticotropic-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), cortisol, aquaporins (AQPs), prolactin, growth hormone, Na^+/K^+-ATPase (NKA), and arginine vasotocin, are involved in osmoregulation (Geering, 1990; Madsen and Bern, 1992; Warne and Balment, 1995; Huising et al., 2004).

CRH is the key factor in the HPI axis and causes the release of ACTH, which acts on the adrenal cortex to release cortisol. CRH can also directly stimulate cortisol release from the adrenal gland. This axis is organized and regulated through a series of negative feedback loops. The HPI axis is regulated by cortisol produced in the adrenal cortex, which uses negative feedback to inhibit both the hypothalamus and the pituitary gland (Bernier et al., 1999, Doyon et al., 2006). In addition, the increase in plasma cortisol levels following the activation of the HPI axis is probably required for central nervous system activation and higher blood glucose concentrations (Bamberger et al., 1996).

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, and 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 to increased plasma glucose levels (McCormick and Saunders, 1987). Rainbow trout (*Oncorhynchus mykiss*) also have the stage of smoltification and report on osmoregulation, and parr (fry) transform into migratory smolts (juveniles) (McLeese et al., 1994).

Cortisol is often referred to as an SW-adapting hormone because it is strongly implicated in the ability of fish to maintain water and

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electrolyte balance when in SW environments (Mommsen et al., 1999). Veillette et al. (1995) reported that cortisol levels regulate water absorption in the intestine during parr–smolt transformation in the Atlantic salmon, *Salmo salar*.

AQPs are a group of membrane proteins that form water transfer channels and are important in maintaining the water balance in the osmoregulatory organs that control body fluid homeostasis (Borgnia et al., 1999; Matsuzaki et al., 2002). Many studies have been performed on euryhaline teleosts to show that the AQP is related to the movement of the water (Kim et al., 2010; Tipsmark et al., 2010; Choi et al., 2013).

Currently, 13 types of AQPs have been found in a variety of organisms, from bacteria to mammals, and have been divided into three groups (aquaporins, aquaglyceroporins, and superaquaporins) on the basis of their genomic structure and amino acid homology (King et al., 2000; Verkman, 2005; Ishibashi et al., 2009). Out of these, AQP3 is a member of the aquaglyceroporin group that is permeable to water, glycerol, urea, and ammonia/ammonium; it is expressed in the gills of teleosts such as European sea bass, *Dicentrarchus labrax*, and Atlantic salmon (Giffard-Mena et al., 2007; Tipsmark et al., 2010). Moreover, AQP3 mRNA expression in tilapia, *Oreochromis mossambicus*, and the Atlantic salmon is increased in the kidneys of SW-adapted fish compared to those in FW-adapted fish (Watanabe et al., 2005; Tipsmark et al., 2010).

Recently, seawater aquaculture of rainbow trout has become more prevalent. Previous studies reported that seawater farmed salmonids was enhanced growth and delayed sexual maturation rate (Oppedal et al., 1999; Türker and Yıldırım, 2011). Therefore, this study was designed to determine the physiological changes that occur in juvenile rainbow trout (parr/smolt) during seawater adaptation.

Here, we investigated the effect of recombinant AQP3 (rAQP3) on the HPI axis when rainbow trout (parr/smolt) acclimated to FW were transferred to SW by injection in rainbow trout to 0.1 and 1.0 μg/g (body mass) of rAQP3. Furthermore, we determined the physiological response of the fish to the injection of rAQP3 by measuring AQP3 mRNA and protein expressions as well as changes in HPI axis gene expression (mRNA), including CRH, ACTH, and levels of plasma ACTH, cortisol, and glucose.

2. Materials and methods

2.1. Experimental fish

Rainbow trout (*O. mykiss*; parr, 2.4 ± 1.0 g; smolt, 105.6 ± 5.2 g) were purchased from the Ewhajung Trout Aquarium (Gyeongsangbuk-do, Korea) and reared in eight 50-L circulation filter tanks prior to the start of the experiments.

The transfer of rainbow trout from FW (0 psu) to SW (35 psu) was performed 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 12:12-h light–dark cycle.

2.2. Recombinant AQP3 treatment

To investigate the role of AQP3 in salinity changes in rainbow trout, the fish were treated with rAQP3 (TP301856; OriGene Technologies, Inc., MD, USA), and the expression of AQPs mRNA was measured. The fish were allowed to adapt in 40-L tanks and were then anesthetized with 0.005% eugenol (4-ally-2-methoxyphenol) prior to injection. Each fish was given an intraperitoneal injection of rAQP3 dissolved in saline [0.1 and 1.0 μg/g body mass (BM)]; fish in the sham group were injected with an equal volume of saline (10 μL/g BM). After the intraperitoneal injection, fish were transferred from FW to 25% SW.

2.3. Sampling

Ten fish from each group (FW, 25% SW, 50% SW, 75% SW, SW, and rAQP3 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 by using a 1-mL heparinized syringe. After centrifugation (10,000 ×*g*, 4 °C, 5 min), the plasma was stored at −80 °C until further analysis.

2.4. Kidney culture

Kidney samples were cut into pieces of approximately 1 × 1.5 mm and carefully placed (in duplicate) in 24-well culture plates containing pre-incubation medium (MEM with Hanks' salts, 5 mg/mL 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 sulfates in sterile 24-well culture dishes. Although explants were occasionally found to adhere to the bottom of the 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.

Recombinant AQP3 dissolved in 0.9% physiological saline was added to the culture medium at a ratio of 1/1000 (v/v), and the specified concentrations of rAQP3 (0.1 and 1.0 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.

2.5. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of AQP3 mRNA by using total RNA extracted from the rainbow trout. Primers for QPCR analysis were designed with reference to the known sequences of rainbow trout (GenBank accession nos.: AQP3, **KJ737372**; CRH, **AJ566334**; ACTHα, **NM1124718**; ACTHβ, **NM1124719**) and are shown in Table 1. QPCR amplification was performed on a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following conditions: 0.5 μL of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, SYBR Green and Taq polymerase in buffer (10 mM Tris–HCl [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein), in a total volume of 25 μL. QPCR was carried out as follows: one cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, and annealing at 55 °C for 20 s. Samples from each experimental group were run in triplicate to ensure consistency. As an internal control, QPCR analysis for β-actin was performed on the same samples, and all data are expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) level. All analyses were based on the Ct values of the PCR products. The calibrated ΔCt

Table 1
Primers used for amplification of PCR.

Genes	Primer	DNA sequences
AQP3	Forward	5′-TGA TGC GTT GTG GGA CTA-3′
	Reverse	5′-GCT GCT GTG CCT ATA ATC TG-3′
CRH	Forward	5′-GCA AGG TAA AGT CGG TAA CA-3′
	Reverse	5′-CGT CAG GTC CAA TGA GAT C-3′
ACTHα	Forward	5′-CGA CTC TGA CTC TCC TCC-3′
	Reverse	5′-CAC ACT GTT CTG CTG CTC-3′
ACTHβ	Forward	5′-CAA GGC TCA GAC CAA GGT A-3′
	Reverse	5′-TGA CCC ATC CGA TAG GAC-3′
β-Actin	Forward	5′-ATC TGG CAT CAC ACC TTC TA-3′
	Reverse	5′-CTT CTC CCT GTT GGC TTT G-3′

value (ΔΔCt) for each sample and internal controls (β-actin) was calculated [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$].

2.6. Western blot analysis

Total protein isolated from the brain of rainbow trout during the 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). Samples were sonicated, and concentration was determined using the Bradford method (Bio-Rad). Equivalent amounts of total protein (30 μg) were loaded into each lane of a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. As a reference, a protein ladder (Fermentas, Glen Burnie, MD, USA) was used. Samples were electrophoresed at 80 V through the stacking gel and at 150 V through the resolving gel until the bromophenol blue dye front had run off the bottom of the gel. The gels were then immediately transferred to a 0.2-μm PVDF 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. The membranes were incubated with a polyclonal rabbit anti-AQP antibody (AQP3, 1:1,000 dilution; Choi et al., 2013), followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (dilution 1:4,000; Bio-Rad, Hercules, CA, USA) for 60 min. The internal control was β-tubulin (dilution 1:2,000; ab6046, Abcam, Cambridge, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:4,000; Bio-Rad, Hercules, CA, USA) for 60 min. Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) and exposed for 30 s with a Molecular Imager® ChemiDoc™ XRS^{+} Systems (Bio-Rad, Hercules, CA, USA). The images were scanned using a high-resolution scanner, and the band density was estimated using a computer program (Image Lab™ Software, version 3.0, Bio-Rad). The ratio of internal control (β-tubulin)/AQP3 for each concentration was calculated and plotted against the concentration of the internal control.

2.7. Plasma parameter analysis

Plasma ACTH and cortisol levels were analyzed by immunoassay technique using ACTH ELISA kit (E15926Fh; Cusabio Biotech, Hubei, China) and cortisol ELISA kit (E08487f; Cusabio Biotech) according to the manufacturer's instructions.

An anti-antibody that was specific to the antibody of the hormones (ACTH and cortisol) for fish was pre-coated onto a microplate, following which 50 μL of plasma, 50 μL HRP, and 50 μL of the antibody were added to each well. The microplate was incubated for 2 h at 37 °C. The wells were washed at three times using a wash buffer, and the remaining buffer after the last wash was aspirated or decanted off, and 50 μL each of the substrates included in the ELISA kit was added to each well. Then the microplate with the substrate solutions was incubated for 15 min at 37 °C in the dark, during which the solutions changed from colorless or light blue to darker shades of blue. Following incubation, 50 μL of stop solution was added to each well, resulting in the change of color from blue to yellow. The optical density of the solution in each well was then determined within 10 min by using a microplate reader set to 450 nm.

Plasma glucose, Na^{+}, Cl^{-}, and K^{+} were analyzed using the Biochemistry Autoanalyzer (FUJI DRI-CHEM 4000i; FujiFilm, Tokyo, Japan).

2.8. 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 was used to compare differences in the data ($P < 0.05$). Values are expressed as mean ± standard error (SE).

3. Results

3.1. Effect of changing salinity and rAQP3 on the expression of AQP3 mRNA (in vivo and in vitro)

The expression of AQP3 mRNA significantly increased in the kidney of parr/smolt rainbow trout from FW to SW conditions and AQP3 mRNA expression in the rAQP3-injected group was significantly high than that in the control group (SW).

In explaining this results, the quantity of AQP3 mRNA significantly increased after the transition from FW to SW [approximately 16.4-fold (parr; Fig. 1A) and 2.3-fold (smolt; Fig. 1B) higher, respectively] (Fig. 1). Moreover, AQP3 mRNA expression in the rAQP3-injected group was significantly higher than that in the SW group (approximately 2.0-fold (parr) and 4.2-fold (smolt) higher, respectively) ($P < 0.05$).

Western blot analysis with the anti-AQP3 antibody revealed a protein that had a mass corresponding to that predicted for rainbow trout AQP3 (33 kDa). The expression pattern of the protein resembled that of the AQP3 mRNA expressed in the rainbow trout kidney.

Additionally, the expression of AQP3 mRNA significantly increased in cultured kidney tissue from parr and smolt rainbow trout [approximately 10.1-fold (parr; Fig. 2A) and 1.3-fold (smolt; Fig. 2B) higher, respectively] during salinity change (FW to SW conditions)(Fig. 2).

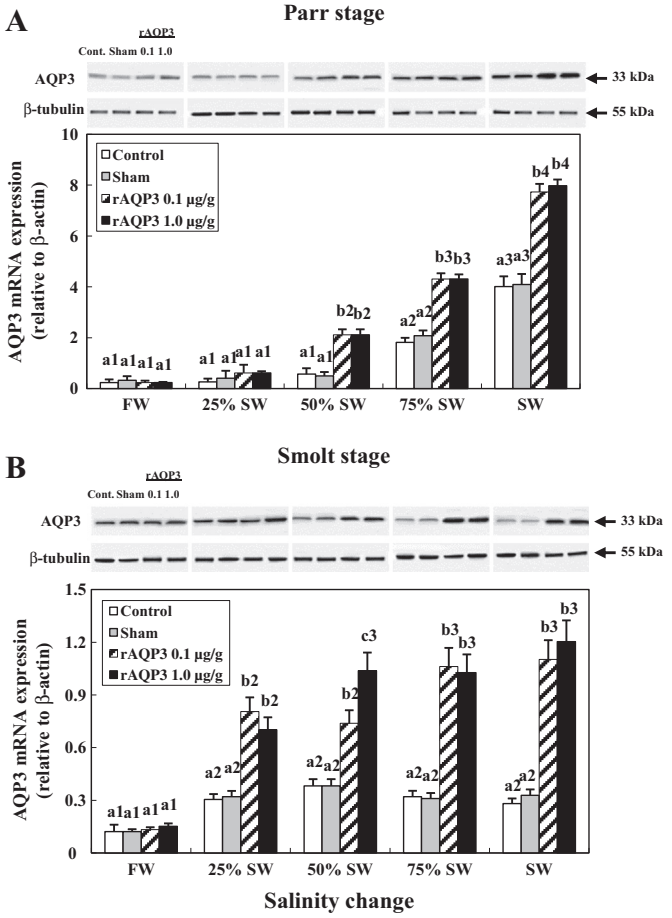

Fig. 1. AQP3 mRNA expression in the kidney of parr (A) and smolt (B) rainbow trout after salinity transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu) and rAQP3 injection, using quantitative real-time PCR. We reverse transcribed 3 μg of total RNA prepared from gill and amplified the sample using 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 means ± SE ($n = 10$). Values with the lowercase letters that differ indicate significant differences between rAQP3-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (control, sham, rAQP3 0.1, and rAQP3 1.0 μg/g) ($P < 0.05$).

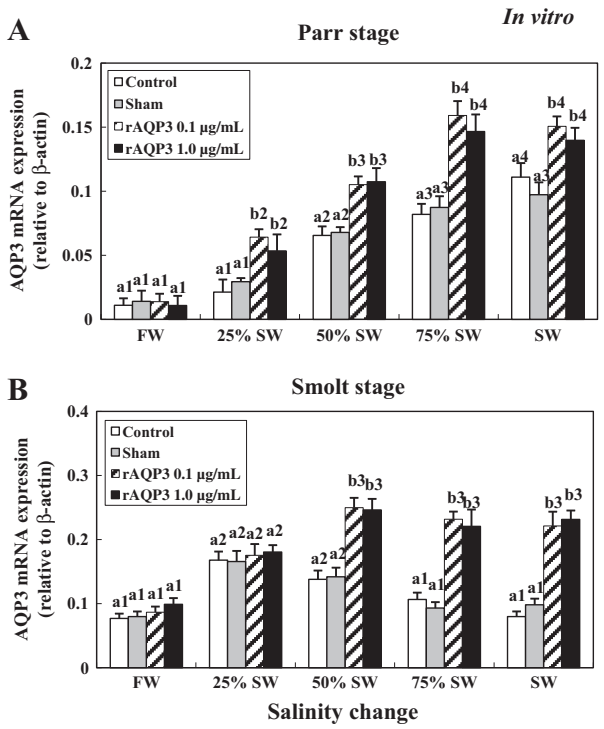

Fig. 2. AQP3 mRNA expression in cultured kidney cells (*in vitro*) from parr (A) and smolt (B) rainbow trout after salinity transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu) and rAQP3 treatment, using quantitative real-time PCR. Results are expressed as normalized fold expression (relative to control) with respect to β-actin levels for the same sample, and values are means ± SE ($n = 10$). Values with the lowercase letters that differ indicate significant differences between rAQP3-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (control, sham, rAQP3 0.1, and rAQP3 1.0 μg/mL) ($P < 0.05$).

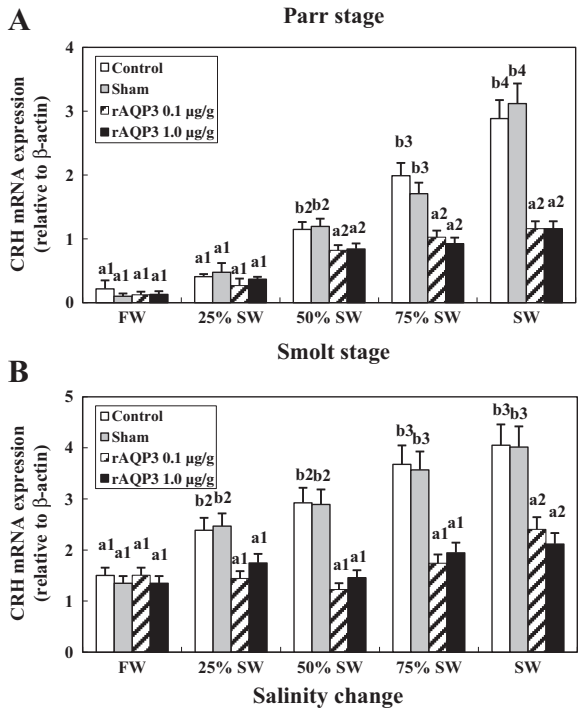

Fig. 3. CRH mRNA expression in the brain of parr (A) and smolt (B) rainbow trout after salinity transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu) and rAQP3 injection, using quantitative real-time PCR. Values with the lowercase letters that differ indicate significant differences between rAQP3-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (control, sham, rAQP3 0.1, and rAQP3 1.0 μg/g) ($P < 0.05$). All values are means ± SE ($n = 10$).

Moreover, AQP3 mRNA expression in the rAQP3 treatment group was significantly higher than that in the SW group (approximately 1.3-fold (parr) and 2.5-fold (smolt) higher, respectively) ($P < 0.05$).

3.2. Effect of changing salinity and rAQP3 injection on the expression of CRH mRNA

We observed the expression levels of CRH mRNA in the brain of parr/smolt rainbow trout during salinity change and rAQP3 injection (Fig. 3). The expression of CRH mRNA was higher in SW stages than that in FW and CRH mRNA expression in the rAQP3-injected group was significantly lower than that in the control group (SW). In the parr stage, CRH mRNA was significantly increased as the SW salinity levels increased (approximately 13.2-fold higher; Fig. 3A). In the smolt stage, CRH mRNA was significantly increased according to the increased salinity levels in SW (approximately 2.7-fold higher; Fig. 3B). Furthermore, the expression of CRH mRNA in the rAQP3-injected group was significantly decreased according to western blot analysis compared to the SW group [approximately 2.4-fold (parr) and 1.9-fold (smolt) lower, respectively] ($P < 0.05$).

3.3. Effect of salinity changes and rAQP3 injection on the level of ACTH mRNA and activity

The levels of ACTH mRNAs were determined in the pituitary, and plasma ACTH activity was assessed in the parr/smolt rainbow trout during salinity change and following rAQP3 injection (Figs. 4–6). Levels of ACTHs mRNA and activity were higher in SW stages than those in FW and was significantly lower in the rAQP3-injected group than those in the control group (SW). The expression of both ACTHα (Fig. 4) and

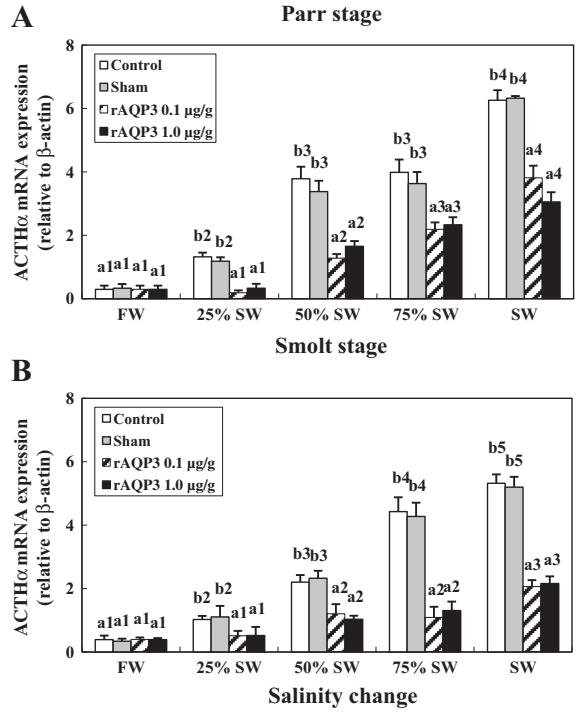

Fig. 4. ACTHα mRNA expression in the pituitary of parr (A) and smolt (B) rainbow trout after salinity transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu) and rAQP3 injection, using quantitative real-time PCR. Values with the lowercase letters that differ indicate significant differences between rAQP3-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (control, sham, rAQP3 0.1, and rAQP3 1.0 μg/g) ($P < 0.05$). All values are means ± SE ($n = 10$).

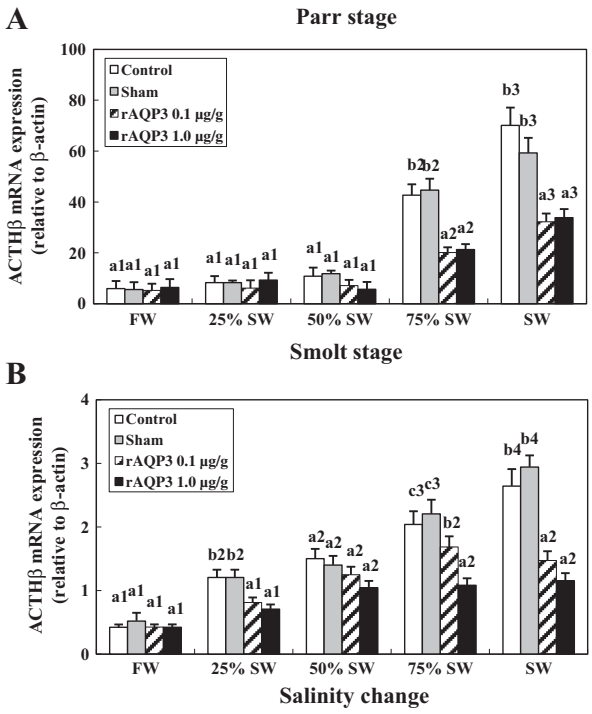

Fig. 5. ACTHβ mRNA expression in the pituitary of parr (A) and smolt (B) rainbow trout after salinity transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu) and rAQP3 injection, using quantitative real-time PCR. Values with the lowercase letters that differ indicate significant differences between rAQP3-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (control, sham, rAQP3 0.1, and rAQP3 1.0 μg/g) ($P < 0.05$). All values are means ± SE ($n = 10$).

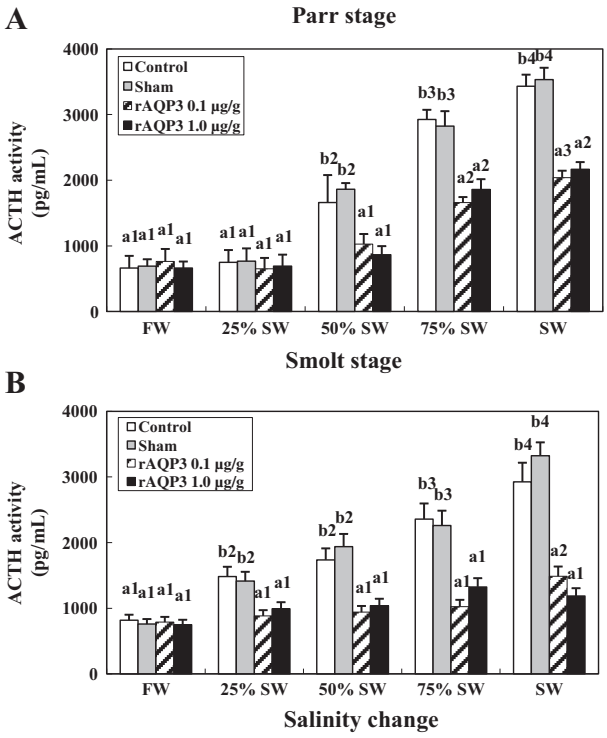

Fig. 6. Plasma ACTH activity following transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu), and following rAQP3 injection in parr (A) and smolt (B) rainbow trout. Values with the lowercase letters that differ indicate significant differences between rAQP3-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (control, sham, rAQP3 0.1, and rAQP3 1 .0 μg/g) ($P < 0.05$). All values are means ± SE ($n = 10$).

ACTHβ (Fig. 5) mRNAs was higher in SW than that in FW [approximately ACTHα: 21.4-fold (parr; Fig. 4A) and 13.6-fold (smolt; Fig. 4B); ACTHβ: 11.9-fold (parr; Fig. 5A) and 6.2-fold (smolt; Fig. 5B) fold higher, respectively]. Furthermore, the expression of ACTHα in the rAQP3-injected group was significantly decreased after the transfer compared to the SW group [approximately ACTHα: 2.1-fold (parr) and 2.4-fold (smolt); ACTHβ: 2.0-fold (parr) and 2.3-fold (smolt) lower, respectively] ($P < 0.05$).

Levels of plasma ACTH were 662.1 ± 40.8 (parr) and 819.1 ± 39.6 pg/mL (smolt) in fish at the start of the experiment and increased to 3433.4 ± 77.3 and 2938.2 ± 120.1 pg/mL for parr and smolt fish, respectively, after transfer to SW (Fig. 6). Furthermore, plasma ACTH levels in SW-acclimated parr and smolt rainbow trout administered rAQP3 treatment decreased to 2166.2 ± 2.0 (parr) and 1186.6 ± 1.9 pg/mL (smolt) after the SW transfer [approximately 1.58-fold (parr) and 2.5-fold (smolt) lower, respectively] ($P < 0.05$).

3.4. Effect of salinity changes and rAQP3 injection on the levels of plasma cortisol and glucose

We determined the plasma cortisol and glucose levels in fish during salinity changes by using the plate reader (Fig. 7). Plasma levels of cortisol and glucose significantly increased from FW to SW conditions, and plasma levels in the rAQP3-injected group were significantly decreased than control group (SW). The plasma cortisol (parr, 11.1 ± 1.1 and smolt, 24.9 ± 1.7 ng/mL) and glucose (parr, 36.5 ± 1.0 and smolt, 45.5 ± 1.2 mg/mL) levels were significantly increased from FW to SW (cortisol: parr, 86.3 ± 3.1 and smolt, 55.1 ± 2.5 ng/mL; glucose: parr, 117.3 ± 2.9 and smolt, 79.9 ± 2.7 mg/mL). Furthermore, plasma cortisol and glucose levels in the rAQP3-injected groups were significantly lower than those in the SW group [cortisol: approximately 1.9-fold (parr) and 1.3-fold (smolt) lower; glucose: approximately 2.0-fold (parr) and 1.4-fold (smolt) lower, respectively] ($P < 0.05$).

3.5. Effect of salinity changes and rAQP3 injection on the plasma levels of Na^+, Cl^-, and K^+ levels

We determined the plasma Na^+, Cl^-, and K^+ levels in fish during salinity change by using the plate reader (Fig. 8). Plasma levels of ion (Na^+, Cl^-, and K^+) significantly increased from FW to SW conditions and plasma levels in the rAQP3-injected group was significantly decreased than control group (SW). The plasma Na^+ (parr, 93.1 ± 8.1 and smolt, 123.8 ± 8.5 mEq/L), Cl^- (parr, 80.2 ± 7.9 and smolt, 110.5 ± 10.1 mEq/L), and K^+ (parr, 8.1 ± 0.9 and smolt, 3.9 ± 0.2 mEq/L) levels were significantly increased from FW to SW [Na^+: 203.2 ± 11.1 (parr) and 186.1 ± 10.6 (smolt) mEq/L; Cl^-: 200.5 ± 8.2 (parr) and 165.5 ± 5.9 (smolt) mEq/L; K^+: 18.9 ± 1.2 (parr) and 6.5 ± 0.5 (smolt) mEq/L, respectively]. Furthermore, plasma Na^+, Cl^-, and K^+ levels in the rAQP3-injected groups were significantly lower than those in the SW group [Na^+: approximately 1.5-fold (parr) and 1.3-fold (smolt) lower; Cl^-: approximately 1.8-fold (parr) and 1.3-fold (smolt) lower; K^+: approximately 1.4-fold (parr) and 1.2-fold (smolt) lower, respectively] ($P < 0.05$).

4. Discussion

Here, we examined the role of rAQP3 on the activity of the HPI axis by sequentially exposing rainbow trout in parr and smolt stages to SW at increasing levels of environmental salinity after injection of two concentrations of AQP3 (0.1 and 1.0 μg/g BM). First, to confirm that rAQP had physiological activity, we determined that AQP mRNA and protein expression levels were affected in the major osmoregulatory tissues, such as the kidney, following injection.

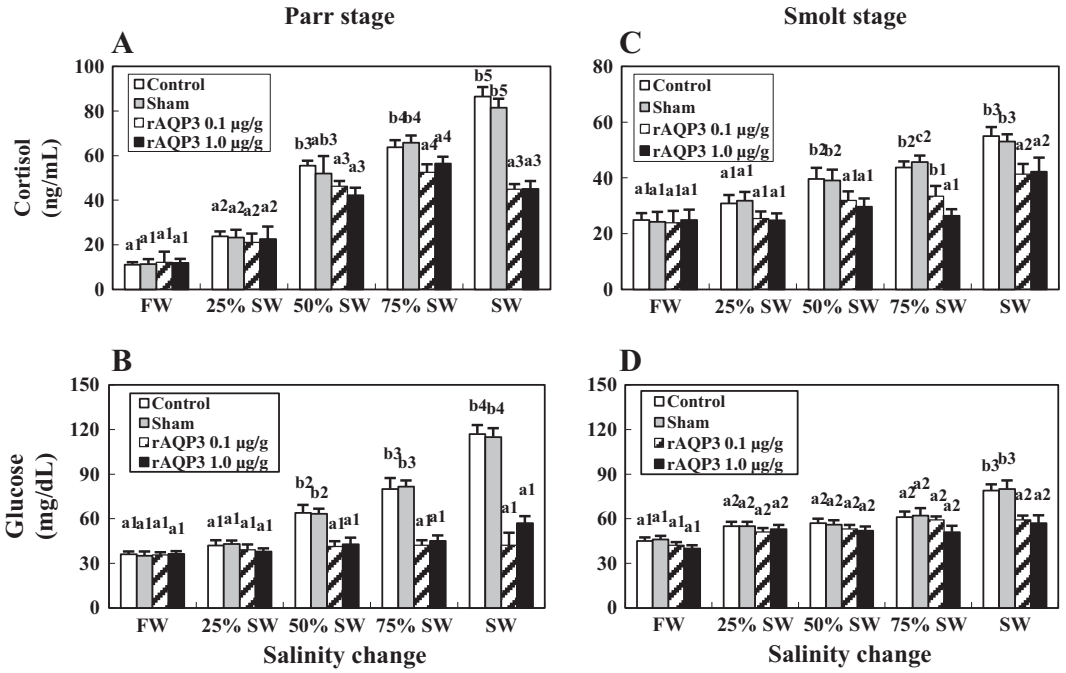

Fig. 7. Plasma cortisol (A, C) and glucose (B, D) levels following the transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu), and following rAQP3 injection in parr (A, B) and smolt (C, D) rainbow trout. Values with the lowercase letters that differ indicate significant differences between rAQP3-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (control, sham, rAQP3 0.1, and rAQP3 1.0 μg/g) ($P < 0.05$). All values are means ± SE ($n = 10$).

In the kidney, the expression of AQP3 mRNA and protein was significantly increased during acclimatization to an SW environment compared to an FW environment. Furthermore, AQP3 mRNA and protein expressions were significantly increased in the rAQP3-treated group than that in the untreated group. After rAQP3 (0.1 and 1.0 μg/mL) treatment, the results from *in vitro* cultured kidney cells revealed a similar pattern of expression to that observed *in vivo*. The results of this study are consistent with the findings from a previous study that showed that AQP3 mRNA expression was increased in the kidney of the Japanese eel, *Anguilla japonica* and Atlantic salmon (Kim et al., 2010; Tipsmark et al., 2010). Therefore, from the results of the present study, we inferred that AQP3 in the kidney plays an important role in the regulation of water homeostasis in conjunction with salinity changes in the environment. Furthermore, the expression of AQP3 mRNA increased more in the parr than that in the smolt stage of rainbow trout after rAQP3 treatment during adaptation from an FW to an SW environment. It is possible that at the parr stage, the rainbow trout are not yet able to adapt to SW; therefore, AQP3 mRNA expression will increase rapidly depending on exposure to changes in environmental salinity.

In this study, we investigated the effects of rAQP3 treatment on the HPI axis following changes in salinity and observed changes in the mRNA expression of HPI axis hormones, such as CRH and ACTH, in the pituitary gland and hypothalamus and plasma levels of ACTH, which increased during adaptation to an SW environment. However, changes in CRH and ACTH mRNA expressions, and activity of plasma ACTH, decreased following injection with rAQP3 compared with the untreated controls. In a similar study, Shin et al. (2014) reported that levels of cortisol and thyroid hormone (TH) were increased after transfer from an FW environment to an SW environment, although thyroid hormone levels were significantly decreased after cortisol treatment in the sockeye salmon, *Oncorhynchus nerka*. A previous study suggested that TH secreted after activation of the HPI axis are involved in the adaptation to the change in salinity and the steroid hormone cortisol has feedback actions on TH, which enhances the SW hypo-osmoregulatory capacity of fish. Similarly, the results of the present study show that levels of CRH and ACTH secreted from the HPI axis were reduced by the influence of rAQP3. Therefore, we considered that AQP3 is able to regulate the salinity adaptation through interaction with CRH and ACTH during the adaptation process to environmental salinity change.

Cortisol is a major corticosteroid hormone that affects the regulation of ion and water absorption in the gills and intestine and improves osmoregulation after a change in salinity, which requires an increase in AQP expression (Bamberger et al., 1996; McCormick, 2001; Martinez et al., 2005). In this study, we analyzed the levels of cortisol and glucose during transfer from the FW environment to the SW environment in the parr/smolt stages of rainbow trout. We found that levels of cortisol and glucose were increased in the parr/smolt stages during seawater adaptation. However, levels of cortisol and glucose were significantly lower in the treated rAQP3 group than that in the untreated group. In particular, lower concentrations of cortisol and glucose were observed in the parr stage than that in the smolt stage. In a similar study, Choi et al. (2013) reported that treatment of sockeye salmon to cortisol during the transfer from an FW environment to an SW environment led to decreased AQP mRNA and protein expression, especially in the parr stage. In this study, the levels of cortisol were different between the parr and smolt stages of rainbow trout. Thus, we determined that the smolt stage of rainbow trout acquired the ability to adapt to salinity changes. Furthermore, we suggest that rainbow trout in the smolt stage have enhanced hypo-osmoregulatory ability for SW environment adaptation through increased levels of cortisol.

In this study, plasma levels of Na^+, Cl^-, and K^+ ions were found to increase gradually during salinity changes, and that this response was prevented in the group rAQP3-treated group. Furthermore, we determined that AQP3 rapidly suppressed the influx of ions thorough interaction with cortisol and AQP3 following rAQP3 treatment.

In summary, we confirmed the following: (1) rAQP3 enabled the effective adaptation to seawater environments and altered the activity of the HPI axis in rainbow trout; and (2) AQPs, involved in water reabsorption and cortisol, and the SW-adapted hormone, act in a complementary manner and control osmoregulation by selective water permeation in rainbow trout when exposed to an SW environment adapted.

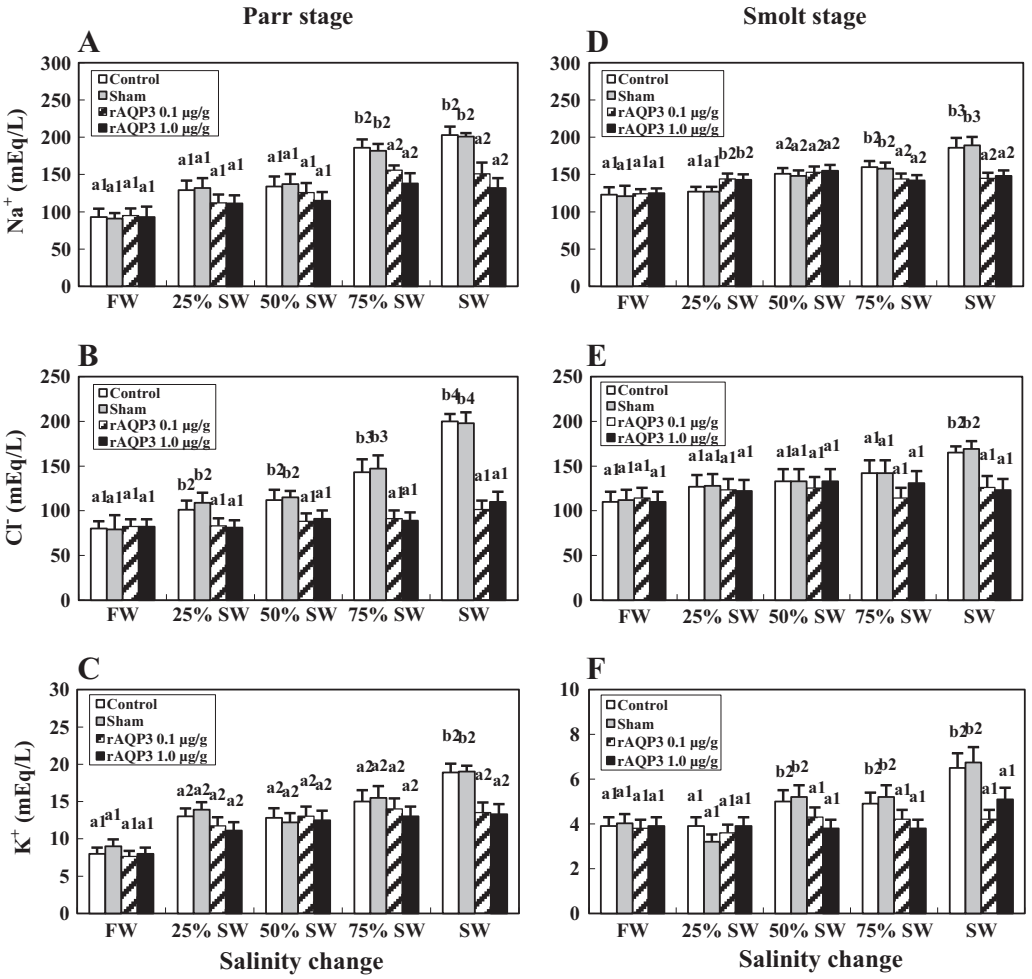

Fig. 8. Levels of plasma Na^+ (A, D), Cl^- (B, E), and K^+ (C, F) after transfer from freshwater (FW, 0 psu) to seawater (SW, 35 psu), and following rAQP3 injection in parr (A, B, C) and smolt (D, E, F) rainbow trout. Values with the lowercase letters that differ indicate significant differences between rAQP3-injected concentrations within the same salinity group. The numbers indicate significant differences between salinities within the same treatment group (control, sham, rAQP3 0.1, and rAQP3 1.0 μg/g) ($P < 0.05$). All values are means ± SE ($n = 10$).

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