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Hypoosmotic shock adaptation by prolactin involves upregulation of arginine vasotocin and osmotic stress transcription factor 1 mRNA in the cinnamon clownfish *Amphiprion melanopus*

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We cloned cDNA-encoding arginine vasotocin (AVT) from the brain of the cinnamon clownfish *Amphiprion melanopus*, and that was predicted to encode a protein of 153 amino acids. We examined changes in the expression of AVT mRNA in the brain and arginine vasotocin receptor (AVTR) mRNA and osmotic stress transcription factor 1 (OSTF1) mRNA in the gills of the cinnamon clownfish using quantitative real-time PCR in an osmotically changing environment (seawater (35 psu) → brackish water (BW, 17.5 psu) and BW with prolactin [PRL]). The expression of AVT, AVTR, and OSTF1 mRNA in the brain and gills increased after transfer to BW, and the expression was repressed by PRL treatment. AVT-immunoreactive cells were almost consistently observed in the telencephalon. The plasma Na^+ and Cl^- levels decreased in BW, but the level of this parameter increased in BW with PRL treatment during salinity change. These results suggest that AVT, AVTR, and OSTF1 play important roles in hormonal regulation in osmoregulation organs, and that PRL improves the hyperosmoregulatory ability of cinnamon clownfish in BW environment.

Keywords: arginine vasotocin; arginine vasotocin receptor; cinnamon clownfish; osmotic stress transcription factor 1; prolactin

1. Introduction

In teleost fish, osmoregulation during changes in salinity is associated with the movement of ions, such as Na^+ and Cl^-, and water molecules within gills, kidneys, and intestines (Evans 1993). In seawater (SW) fish, the external osmotic pressure is higher than the internal pressure and fish take in a large quantity of SW, absorb water through the intestines to replace water loss caused by osmotic stress, and discharge Na^+ and Cl^- ions through the gills (Moyle and Cech 2000). SW fish also absorb Na^+ and Cl^- ions through the kidneys and discharge them to the outside environment. Hormones and proteins, such as cortisol, prolactin (PRL), growth hormone (GH), Na^+/K^+-ATPase (NKA), arginine vasotocin (AVT), and aquaporins (AQPs), are involved in osmoregulation (Geering 1990; Warne and Balment 1995).

AVT is a nonapeptide hormone released by the neurohypophysis of teleost fish and other nonmammals (Acher and Chauvet 1995). AVT was also shown in the preoptic area (POA) of brain, and POA was shown in the pituitary, telencephalon, optic tectum, and cerebellum (Hur et al. 2011). It is involved in various physiological functions, such as maintaining blood pressure (Warne and Balment 1995), antidiuretic functions (Henderson and Wales 1974; Amer and Brown 1995), and osmoregulation (Warne and Balment 1995). This hormone is similar in function to arginine vasopressin (AVP), which was mammalian homolog of AVT and controlled the water retention (Acher 1996). Changes in the osmotic pressure in the body of a fish lead to changes in the concentration of AVT in the plasma (Kulczykowska 2001; Warne et al. 2005) and pituitary gland (Harding et al. 1997), suggesting that AVT serves an osmoregulatory function. In addition, although mammals have three types of AVP receptors (V_1, V_{1b}, and V_2), teleost fish have only one (V_1). This receptor has been cloned in white suckers *Catostomus commersonii* (Mahlmann et al. 1994), flounder *Platichthys flesus* (Warne 2001), and black porgy (Choi and An 2008).

Osmotic stress transcription factor 1 (OSTF1) is initially expressed under osmotic stress and has been reported in Mozambique tilapia (Fiol and Kültz 2005), Japanese eel (Tse et al. 2006), and black porgy (Choi and An 2008). OSTF1 was classified as an 'early hyperosmotically upregulated protein' (Fiol et al. 2006). The expression of OSTF1 mRNA in the gills of black porgy was found to be activated only under hypoosmotic stress (Choi and An 2008). Also, Choi and An (2008) reported that OSTF1 was a transcription factor specific to osmolality and is expressed only

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during osmotic stress, regardless of oxidative or water-temperature stress.

Recently, studies have examined the relation of OSTF1 and the ions regulated by salinity levels to water transporter hormone such as cortisol (McGuire et al. 2010), NKA (Choi and An 2008), and GH (Breves et al. 2010). However, with regard to AVT studies on teleost fish, little research has focused on the changes in gene expression between AVT and hypoosmotic acclimation hormones. Also, previous studies show the expression of arginine vasotocin receptor (AVTR) mRNA in the gills of teleosts during salinity changes (Heierhorst et al. 1989; An et al. 2008).

The production of clownfish in Korea is conducted completely in tank cultures. However, rainfall in Korea is highly concentrated during the rainy season in the summer, causing the salinity of the water to change. Recently, with the rapid growth of the aquaculture of ornamental fish such as the cinnamon clownfish *Amphiprion melanopus*, production has become an important component of aquaculture in Korea. Therefore, we observed the expression of AVT, AVTR, and OSTF1 in cinnamon clownfish as a SW ornamental fish and investigated the immunohistochemistry (IHC) of the brain after transferring the fish to brackish water (BW, 17.5 psu). Also, we investigated the expression of this gene after PRL (a freshwater [FW] acclimation hormone) injections to study the role of PRL when fish are transferred to a BW environment.

2. Materials and methods

2.1. Experimental fish

The immature cinnamon clownfish ($n = 80$, 5.6 ± 1.2 g) were purchased from the Center for Ornamental Reefs & Aquaria (CCORA, Jeju, Korea) and maintained in four 40-l circulating filter tanks prior to experiments.

Transfer of cinnamon clownfish from SW (35 psu) to BW was performed as follows: briefly, artesian water was poured into a square 40-l filter tank, the water was adjusted at BW, and then the fish were exposed for 48 h. The temperature was maintained at 28 ± 0.5°C, and the photoperiod was a 12:12 h light/dark cycle. No food was supplied during the experimental periods.

2.2. PRL treatment

PRL was dissolved in saline, and each fish was given an intraperitoneal injection of PRL (5 μg/g body mass [BM]); the sham group of fish was injected with a dissolved equal volume of saline (10 μl/g BM). After intraperitoneal injection, the fish were transferred from SW to BW.

2.3. Sampling of brains and gills

The brains and gills were selected from five fish in each group (SW, BW, BW + saline, and BW + PRL) for the following time periods: 0, 12, 24, and 48 h. The tissues were frozen immediately after collection in liquid nitrogen and stored at −80°C until total RNA extraction was performed. Blood was also taken from the caudal vasculature using a 1-ml heparinized syringe. After centrifugation ($10{,}000 \times g$, 4°C, 5 min), the plasma was stored at −80°C before analysis.

2.4. Phylogenetic analysis of AVT

Phylogenetic analysis was conducted using known teleost AVT amino acid sequences aligned using BioEdit software. The phylogenetic tree was constructed using the neighbor-joining method with the Mega 3.1 software package (CEFG, Tempe, AZ, USA).

2.5. Immunohistochemistry

Brain cells were detected immunohistochemically according to the methods described in Iwata et al. (2010) with modifications. Four l-μm thick rehydrated tissue sections were incubated overnight at 4°C with the rabbit anti-AVP (dilution 1:500, Bachem) and 30 min at 37°C with the secondary antibody (HRP-conjugated a-rabbit immunoglobulin, 1:100 dilution). The antibodies were diluted in 2% bovine serum albumin in TBS pH 7.6. EnVision (K4001; Dako, Glostrup, Denmark) and 3,3'-diaminobenzidine (DAB^+) (K3468; Dako) were used as the detection system. Slides were counterstained with Meyer'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).

2.6. Plasma parameter analysis

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

2.7. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, 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 means ± SD.

3. Results

3.1. Identification of AVT

Cinnamon clownfish AVT cDNA includes an open reading frame that was predicted to encode a protein of 153 amino acids (HQ441172) (supplementary data, Table 1).[1]

Using the blast algorithm (Blastp) of the National Center for Biotechnology Information, we found that AVT amino acids display high identity with those of other species. The amino acid sequences of AVT were compared with those deduced from the cDNA of other teleost species (Figure 1). The amino acid similarities were as follows: 90% with rock hind *Epinephelus adscensionis*, 89% with orange-spotted grouper *Epinephelus coioides*, 86% with Nile tilapia *Oreochromis niloticus*, and 89% with *Haplochromis burtoni*.

The AVT cDNAs found in cinnamon clownfish all consisted of the characteristic signal peptide (residues 1–19), specific AVT amino acids (residues 20–28), enzymatic processing site [Gly-Lys-Arg (GKR), residues 29–31], and neurophysin (residues 29–123). The neurophysins of AVT included a leucine-rich core segment (residues 139–143) that resembled the copeptin (residues 124–153) of teleost AVT (Figure 1).

3.2. Phylogenetic analysis

The phylogenetic tree obtained by Clustal analysis of the sequences described below is shown in Figure 2. AVT of the cinnamon clownfish was most similar to that of other fish species AVT (85–90%), and was shown in homolog about 48–50% with mammal AVP as the specific AVT and AVP amino acids.

3.3. Expression levels of AVT and AVTR mRNA

The expression levels of both AVT mRNA in the brain and AVTR mRNA in the gills were highest 24 h after transfer to BW (AVT and AVTR; approximately 65.2- and 3.3-fold compared with SW, respectively), and expression levels of AVT and AVTR in BW with PRL treatment were significantly lower than the untreated control group (AVT and AVTR; approximately 3.4- and 1.6-fold compared with BW, respectively; Figure 3). In the Western blot analysis, protein was detected in a size range corresponding to the predicted size for cinnamon clownfish AVT (approximately 38 kDa), and the expression pattern of protein resembled the pattern of the AVT mRNA expression in cinnamon clownfish brains (Figure 3; supplementary data, Table 1).

3.4. IHC of AVT

A significant increase in the number and area of AVT-immunoreactive (AVT-IR) neurons, as well as in the intensity of immunostaining, was seen in the telencephalon of BW clownfish compared with SW fish (Figure 4). The PRL-treated clownfish brain appeared to have less AVT-IR neurons compared with the BW telencephalon.

3.5. Expression of OSTF1 mRNA

The expression of OSTF1 mRNA in the gills is shown in Figure 5. In particular, OSTF1 mRNA was higher than the untreated control level at 12, 24, and 48 h after transfer to BW (approximately 46.2-fold compared with SW). In addition, OSTF1 mRNA in BW with

Table 1. Primers used for PCR amplification.

PCR	Genes	DNA sequences
RT-PCR	AVT-F	5'-GAT ACT GGG ATC AGA CAG TG-3'
	AVT-R	5'-GAT CTG TGT TCT CTG TCT CC-3'
	AVTR-F	5'-GCA TCC ACC TAT ATG ATG GTG-3'
	AVTR-R	5' -GTT CGA GCA GCT GTT GAG ACT-3'
	OSTF1-F	5'-TGC TAG CGT TGT GGC C-3'
	OSTF1-R	5'-ACT GGA ACT TCT CCA G-3'
RACE-PCR	AVT 5 RACE	5'-CTG TCC TCT CGT GGC CAC ATG CAG CAG-3'
	AVT 3 RACE	5'-TCC TTG ATC CCC ATG TGC GTC CTG GGA-3'
QPCR	AVT-F	5'-CTT CTT GCC CTT TCC TCT G-3'
	AVT-R	5'-TGT CTG ATC CCA GTA TCC G-3'
	AVTR-F	5'-TGT GCT ACG GGT TCA TCT-3'
	AVTR-R	5'-GTC CTC AGT TTG GCT CTT G-3'
	OSTF1-F	5'-AGA TCC TCA AAG AGC AGA TC-3'
	OSTF1-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'

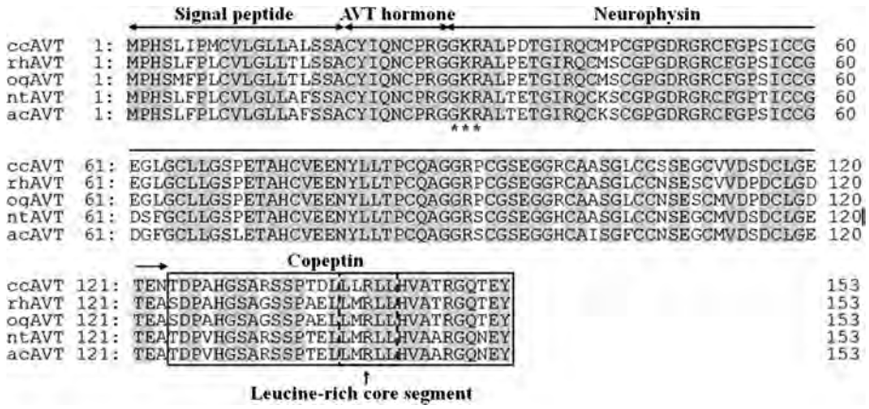

Figure 1. Comparison of the amino acid sequence of AVT. The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The amino acid sequences of cinnamon clownfish AVT (ccAVT, HQ441172), rock hind AVT (rhAVT, HQ141397), orange-spotted grouper AVT (ogAVT, GU831571), Nile tilapia AVT (ntAVT, XM3446089), and African cichlid fish AVT (acAVT, AF517935) optimally aligned to match identical residues, indicated by the shaded box. Signal peptide, AVT hormone, and neurophysin are indicated by arrowed solid lines, while copeptin is indicated by a box. The open column indicates the leucine-rich core segment.

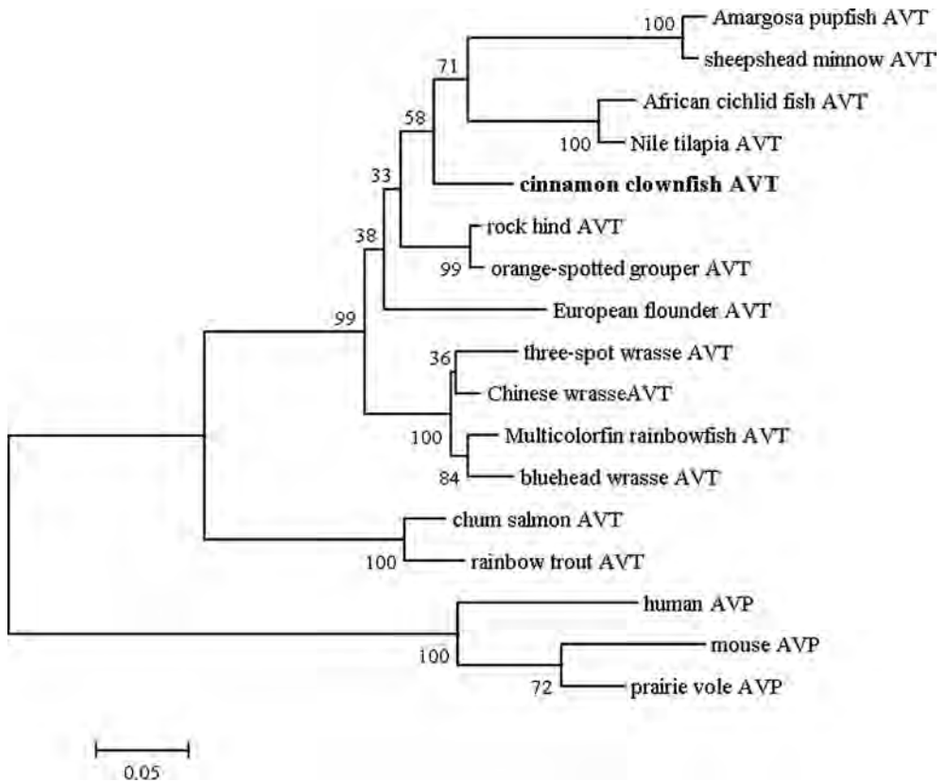

Figure 2. Phylogenetic tree based on an amino acid alignment for AVT in teleost fish. Bootstrap values (%) are indicated for 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences: cinnamon clownfish AVT (HQ441172), rock hind AVT (HQ141397), orange-spotted grouper AVT (GU831571), Nile tilapia AVT (XM3446089), African cichlid fish AVT (AF517935), Amargosa pupfish AVT (GU138978), sheepshead minnow AVT (JF431047), three-spot wrasse AVT (GU212657), Chinese wrasse AVT (DQ073098), chum salmon AVT (X17328), European flounder AVT (AB036517), multicolorfin rainbowfish AVT (DQ073094), Atlantic salmon AVT (BT049519), bluehead wrasse AVT (AY167033), human AVP (M25647), mouse AVP (AL731707), and prairie vole AVP (DP001209).

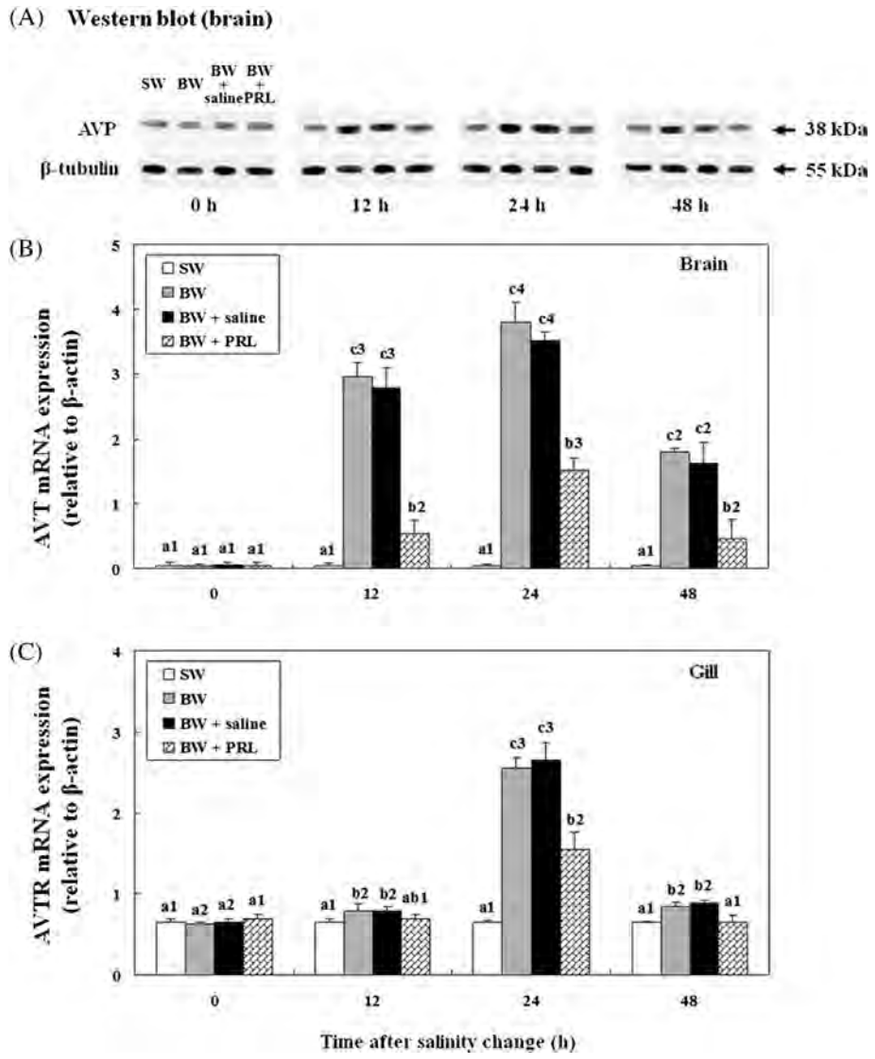

Figure 3. AVT and AVTR mRNA expression levels in the brain and gills of cinnamon clownfish during salinity change. (A) Western blot using AVP (polyclonal rabbit anti-[Arg^8]-vasopressin IgG, 38 kDa) to examine AVT expression in the brain of cinnamon clownfish during salinity change. The 55 kDa β-tubulin was used as the internal control. Expression of AVT mRNA levels in the brain (B) and AVTR mRNA levels in the gills (C) of cinnamon clownfish during salinity change using quantitative real-time PCR. The fish were placed in seawater (SW, 35 psu), brackish water (BW, 17.5 psu), and BW with prolactin (BW + PRL) for 0–48 h. Lower-case letters indicate significant differences among SW, BW, and BW + PRL within the same time after salinity change. The numbers indicate a significant difference from the control within the same salinity concentration and PRL treatment group ($P < 0.05$). Values are means + SD ($n = 5$).

PRL treatment was significantly lower than that in the untreated control group (supplementary data, Table 1).

3.6. Plasma Na^+ and Cl^- concentration

Plasma Na^+ and Cl^- were 162 ± 4.0 and 175 ± 4.2 mEq/l, respectively, at the start of the experiment, and they reached their lowest levels of 132 ± 5.3 mEq/l at 24 h and 164 ± 2.6 at 12 h after transfer to BW, respectively. However, plasma Na^+ in BW with PRL treatment was higher than that of BW, and the levels were similar with SW, but Cl^- was slightly increased in 48 h (Figure 6).

4. Discussion

We compared the expression changes in AVT, AVTR, and OSTF1 mRNA in a time course after the SW cinnamon clownfish were transferred to BW and also

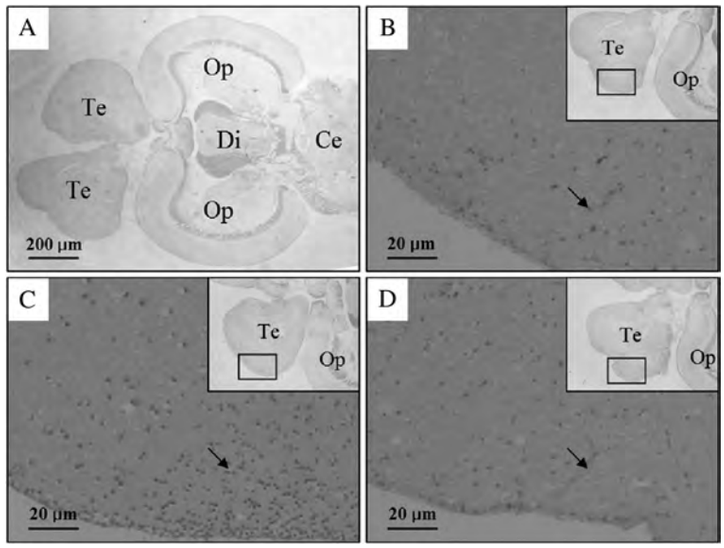

Figure 4. Immunohistochemical localization of brain AVT-immunoreactive (AVT-IR) cells in cross sections of cinnamon clownfish brains adapted to different salinities. (A) Whole brain, (B) seawater (SW, 35 psu), (C) 24 h after transfer to brackish water (BW, 17.5 psu), and (D) BW treated with prolactin (PRL) and 24 h after transfer to BW. Arrows indicate AVT-IR cells in the telencephalon. Te, telencephalon; Op, optic tectum; Ce, cerebellum; Di, diencephalons. Bar = 200 μm (A), 20 μm (B–D).

examined the activity and distribution of AVT-IR cells in the brain by IHC.

The deduced amino acid sequences of the cinnamon clownfish AVT included signal peptides, specific AVT hormone amino acids (^{20}CYIQNCPRG^{28}), an enzymatic processing site [Gly-Lys-Arg (^{29}GKR^{31})], and a leucine-rich core protein called neurophysin. The neurophysin acts as carrier protein in the transport of the hormone from the hypothalamus, the copeptin, and is thought to function as a PRL -releasing factor (Nagy et al. 1988) (Figure 1). The structure of the cinnamon clownfish AVT is similar to that of the white sucker (Heierhorst et al. 1989), European flounder (Warne et al. 2000), and three-spot wrasse *Halichoeres trimachlatus* (Hur et al. 2011).

Expression of AVT mRNA in the brain and AVTR mRNA in the gills was increased by salinity change (Figure 3), and considering that AVT is involved in

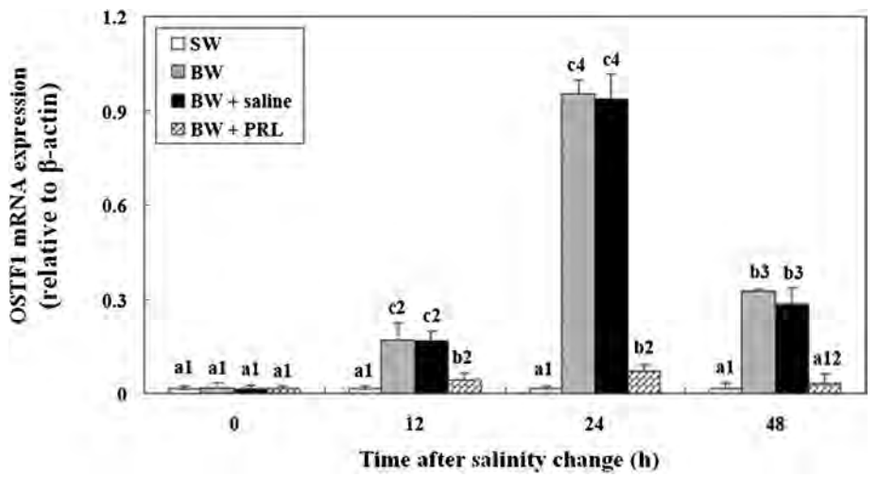

Figure 5. Expression of OSTF1 mRNA levels during salinity change in the gills of cinnamon clownfish using quantitative real-time PCR. The fish were placed in seawater (SW), brackish water (BW), BW with prolactin (BW + PRL) for 0–48 h. Lower-case letters indicate significant differences among SW, BW, and BW + PRL within the same time after salinity change. The numbers indicate a significant difference from the control within the same salinity concentration and PRL treatment group ($P < 0.05$). Values are means ± SD ($n = 5$).

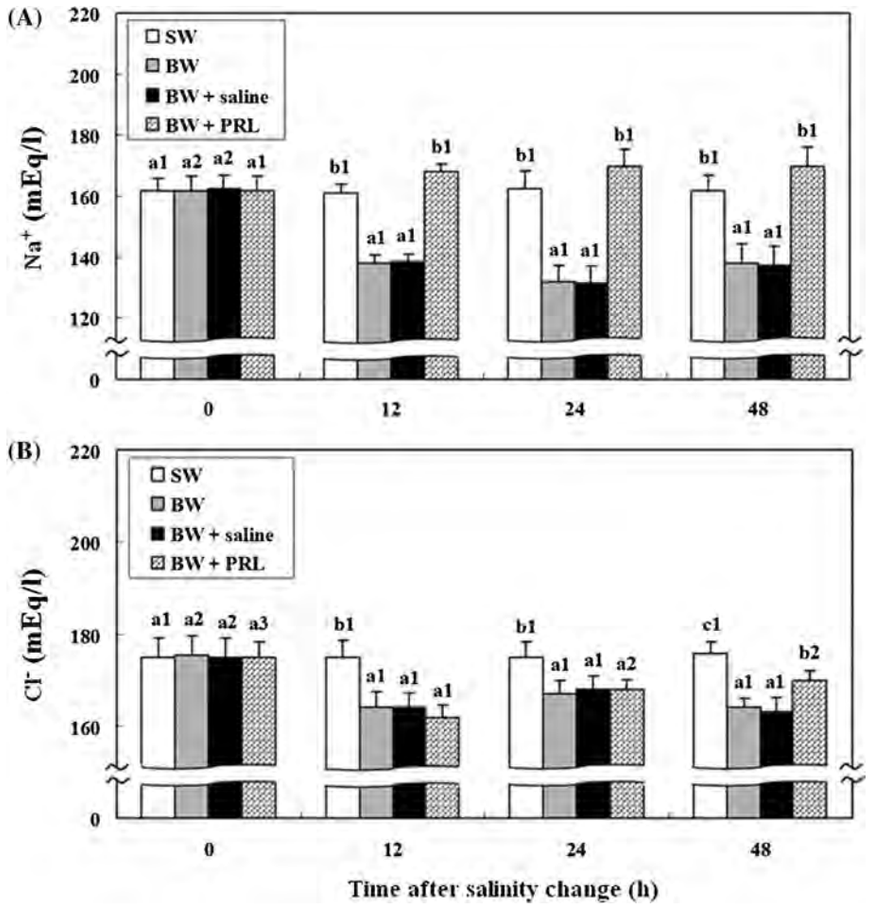

Figure 6. Effect of prolactin treatment on plasma Na^+ and Cl^- levels during salinity change in cinnamon clownfish. (A) Plasma Na^+ levels in seawater (SW), brackish water (BW), and BW with prolactin (BW + PRL) at different incubation times. (B) Plasma Cl^- levels in seawater (SW), brackish water (BW), and BW with prolactin (BW + PRL) at different incubation times. Lower-case letters indicate significant differences among SW, BW, and BW + PRL within the same time period after salinity change. The numbers indicate a significant difference from the control within the same salinity concentration and PRL treatment group ($P < 0.05$). Values are means $\pm$ SD ($n = 5$).

both the diuretic and antidiuretic actions of the kidneys (Amer and Brown 1995), the conclusion was made that osmotic pressure was regulated, and that AVTR was activated in the gills and kidneys to promote water excretion. This led to a decrease in the plasma osmolality and an imbalance in osmotic pressure. This result seems to be due to the increased expression of AVT mRNA to absorb the lost ions, such as Na^+ and Cl^-, into the body as the ions were discharged through the gills by osmolality in BW. This is in accordance with previous studies showing that the mRNA expression and activity of NKA were higher in BW than in SW in teleosts such as the sea bream (Marshall and Bryson 1998), black porgy (Choi and An 2008), sea bass (Giffard-Mena et al. 2008), and cinnamon clownfish (Park, Shin, Kil, et al. 2011). Also, in the Western blot analysis, the expression of the AVT protein was similar to that of AVT mRNA in the brain (Figure 3A).

The level of ions such as Na^+ and Cl^- decreased when the cinnamon clownfish were transferred to BW (Figure 6), in agreement with the results for various fish exposed to hypoosmotic conditions (Chang et al. 2007; An et al. 2008), and the decrease in osmolality may have been due to water flow into and the ion flow out of the body caused by osmolality, and a reduction of the gill permeability to these electrolytes. In teleost fish, the expression of these genes in FW is thought to decrease because the fish had adapted to some degree to the hypoosmotic environment. On the basis of this result, cinnamon clownfish can hyperosmoregulate through activation of these genes to adapt to BW.

In addition, OSTF1 mRNA expression was approximately 46.2 times higher in the BW than in SW

(Figure 5). Unlike OSTF1 expression in tilapia under hyperosmotic stress (Fiol and Kültz 2005), the expression of OSTF1 mRNA in black porgy (Choi and An 2008) occurred under hypoosmotic stress, suggesting fish subjected to osmotic stress under hyperosmolality and hypoosmolality, respectively, and that OSTF1 is activated not only in response to hyperosmolality, but also to hypoosmotic stress.

We investigated the expression of AVT, AVTR, and OSTF1 mRNA in BW after injection of PRL (5 μg/g BW) to understand the role of PRL as a FW-adapting hormone. In the case of PRL injection, the expression of mRNA was lower than in the control group (SW), and we found that expression may be repressed by PRL. Previous studies reported an AVT action via regulation of PRL secretion, and that PRL causes water excretion (Clarke and Bern 1980; Kleszczyńska et al. 2012). Therefore, AVT plays the same function with PRL, expression levels were decreased by injecting much of PRL and then PRL acted as feedback to maintain the homeostasis during being transferred to BW. This result agreed with previous studies (Kelly et al. 1999; Mancera et al. 2002), perhaps because PRL acts to repress NKA activity in gills to repress ion excretion in cinnamon clownfish exposed to BW, decreasing the expression of AVT and AVTR mRNA. Moreover, PRL was suggested to act on the kidneys to increase Na^+ reabsorption, increasing the plasma level of Na^+ (Clarke and Bern 1980). PRL is suggested to act through the hyperosmoregulatory ability of cinnamon clownfish in adapting to BW.

The expression of OSTF1 mRNA was reduced in cinnamon clownfish by PRL, suppressing water absorption when the fish were adapted in FW. This is because OSTF1 is activated not only in response to hyperosmolality, but also to hypoosmotic stress. Also, this result agreed with previous study (Park, Shin, Choi, et al. 2011), PRL is involved in FW adaptation, appeared to help fish adjust to hypoosmotic environments and reduce hypoosmotic stress.

We examined the expression and distribution of AVT-IR cells related with AVT expression in the brain during the BW environment and PRL treatment by IHC. The results showed that the distribution of AVT-IR cells and AVT mRNA and protein expression according to salinity changes were displayed in similar pattern.

In this study, expression of brain AVT-IR neurons significantly increased in number and area as well as in the intensity of immunostaining seen in the telencephalon when compared with SW (Figure 4). The PRL-treated cinnamon clownfish brain appeared to have less AVT-IR neurons in the telencephalon compared with the non-PRL-treated brain in BW. We found a distinct difference in the distribution of brain AVT-IR cells associated with salinity, and PRL controlled the expression of AVT in the brain of cinnamon clownfish.

In summary, cinnamon clownfish can hyperosmoregulate through activation of AVT and AVTR to adapt to BW, and OSTF1 was expressed in reaction to hypoosmotic stress. PRL regulates the hyperosmoregulatory ability and controls AVT, AVTR, and OSTF1 in cinnamon clownfish in BW. The distribution and expression of AVT proteins in the brain was examined by IHC and western blotting after exposure to BW. We suggest that cinnamon clownfish can hyperosmoregulate through the activation of these genes to adapt to BW.

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Note

1. Supplementary material can be found by clicking on the Supplementary Content tab at http://dx.doi.org/10.1080/19768354.2012.719547

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