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FULL PAPER

Monitoring of Na^+/K^+-ATPase mRNA expression in the cinnamon clownfish, *Amphiprion melanopus*, exposed to an osmotic stress environment: profiles on the effects of exogenous hormone

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Abstract We examined changes in the expression of Na^+/K^+-ATPase mRNA in the gills of the cinnamon clownfish using quantitative real-time PCR in an osmotically changing environment [seawater (35 psu; practical salinity unit, 1 psu ≈ 1‰) → brackish water (17.5 psu) and brackish water with prolactin]. The expression of Na^+/K^+-ATPase mRNA in gills was increased after the transfer to brackish water, and the expression was repressed by prolactin treatment. Also, activities of gill Na^+/K^+-ATPase and plasma cortisol levels increased after the transfer to brackish water and were repressed in brackish water with prolactin treatment. Na^+/K^+-ATPase-immunoreactive cells were almost consistently observed in the gill filaments, but absent from the lamella epithelia. The plasma osmolality level decreased in brackish water, but the level of this parameter increased in brackish water with prolactin treatment during salinity change. These results suggest that the Na^+/K^+-ATPase gene plays an important role in osmoregulation in gills, and prolactin improves the hyperosmoregulatory ability of cinnamon clownfish in a brackish water (hypoosmotic) environment.

Keywords *Amphiprion melanopus* · Cortisol · Na^+/K^+-ATPase · Osmotic stress · Prolactin

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Introduction

For fish, salinity is an important environmental factor. Salinity changes cause a variety of physiological stresses and affect growth, reproduction, metabolism, osmoregulation and immunity (Ackerman et al. 2000). In salinity changes, the plasma osmolality is changed by the movement of water, and Na^+ and Cl^- ions in osmoregulatory organs such as the gills, kidney and intestine (Evans 1993), so fish try to maintain body homeostasis regarding the change of osmolality caused by salinity changes.

Although there are many hormones and proteins involved in maintaining homeostasis in the body, particularly involving the osmolality of these proteins and hormones, Na^+/K^+-ATPase (NKA) is one of the most important enzymes associated with ion regulation in gills of fish because it energizes the branchial excretion of Na^+ and Cl^- from marine teleosts (McCormick 1995; Marshall and Bryson 1998; Evans et al. 1999). This NKA is important not only to maintain homeostasis, but also to provide a driving force for many transport systems in a variety of osmoregulatory organs, including the gills of fish (McCormick 1995).

Immunocytochemical studies have reported that NKA occurs mainly in the epithelia of the kidney tubules (Ura et al. 1996) and in mitochondria-rich cells of the branchial epithelium in euryhaline teleosts (Wilson and Laurent 2002). Also, most studies show a similar positive correlation

between environmental salinity and gill NKA activity (Sakamoto et al. 2001), which forms the well-established 'diadromid paradigm' (Marshall and Bryson 1998; Lee et al. 2000). However, other studies have shown a negative correlation between NKA activity and external salinity in several euryhaline species of non-estuarine marine teleosts such as flounder, sea bream and mullet (Marshall and Bryson 1998), which does not support the paradigm. Therefore, it is well known that NKA is related to the movement of water and excretion of ions in the osmoregulation of fish.

The cinnamon clownfish is a popular seawater ornamental fish. Recently, with the rapid growth of the aquaculture of ornamental fish such as clownfish, production has become an important component of aquaculture in Korea. The production of clownfish in Korea is completely conducted in tank cultures. However, rainfall is highly concentrated in the rainy season of summer, causing the salinity of the water to change.

Therefore, we observed the expression of NKA, NKA activity and histological changes in cinnamon clownfish using the immunohistochemistry of the gills after the transfer of fish to brackish water (BW, 17.5 psu) environments. Also, we investigated the expression of this gene, osmolality and plasma cortisol levels after prolactin (PRL) injections to study the role of PRL when the fish were transferred to a BW environment.

Materials and methods

Fish and experimental conditions. Cinnamon clownfish ($n = 60$, 5.6 ± 1.2 g) were purchased from the Center for Ornamental Reefs and Aquariums (CCORA, Jeju, Korea) and maintained in two 40-l circulation filter tanks prior to experiments in the laboratory.

Transfer of cinnamon clownfish from seawater (SW, 35 psu) to brackish water (BW, 17.5 psu) was performed as follows: briefly, underground water was poured into square 40-l circulation filter tanks, and the water was adjusted to 17.5 psu; then the fish were exposed for 48 h. The temperature was maintained at 28 ± 0.5°C, and the photo period was a 12:12-h light-dark cycle. Fish were fed a commercial marine aquarium fish feed (Jeilfeed Co., Kyoungnam, Korea) twice a day.

Prolactin treatment. To investigate the roles of PRL in salinity changes in cinnamon clownfish, the fish were treated with PRL (Sigma, USA), and the expression of NKA mRNA was measured. The fish were allowed to adapt in 40-l circulation filter tanks in the laboratory and were then anesthetized with tricaine methane sulfonate (MS-222, Sigma, USA) prior to injection. 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 an intraperitoneal injection, the fish were transferred from SW to BW.

Sampling. The gills were selected from five fish in each group (SW, SW + saline, BW + saline, BW and BW + PRL) at the following time periods: 0, 12, 24 and 48 h. Immediately after gill collection, the gills were frozen 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 *g*, 4°C, 5 min), the plasma was stored at −80°C before analysis.

Quantitative real-time PCR. qPCR was conducted to determine the relative expression of NKA mRNA using the total RNA extracted from gills of cinnamon clownfish. Primers for qPCR were designed with reference to the known sequences of cinnamon clownfish as follows: GenBank accession nos. NKA: HM768896, β-actin: JF273495): NKA forward primer (5′-GCT CCG TCA ACG ATC TGG AAG AC-3′), NKA reverse primer (5′-AGG CTG TGT GGC ATG TGA ACT C-3′), β-actin forward primer (5′-GCG ACC TCA CAG ACT ACC TCA TG-3′) and β-actin reverse primer (5′-AAG TCC AGG CCA ACA TAG CAG AG-3′. PCR amplification was conducted using an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and the iQ^{TM} SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The qPCR condition was one cycle of denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data were normalized to the β-actin calculated threshold-cycle (Ct) level. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR, qPCR data from three replicate samples were analyzed with the software of the cycler system to estimate the transcript copy numbers for each sample.

Na^+/K^+-ATPase activity. NKA activity was measured according to the methods described by Uchida et al. (1996) with modifications. Gills (approximately 4–6 primary gill filaments from just above the septum) were collected from the anesthetized fish, immersed in 100 μl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80°C. The filaments were thawed, homogenized in SEI buffer containing 0.1% deoxycholic acid and centrifuged at 5,000 *g* for 30 s to remove insoluble materials. The supernatant was assayed for NKA activity and protein content. Homogenate samples (10 μl) were added to a 200-μl assay mixture with and without 0.5 mmol/l ouabain in 96-well microplates at 25°C and read at 340 nm for 10 min with intermittent mixing.

Protein content of the sample was determined by Protein Assay Standard II (Bio-Rad, USA). The NKA activity was calculated as the difference in ATP hydrolysis between the presence and absence of ouabain, and expressed as μmoles of ADP per mg protein per hour.

Western blot analysis. Total protein isolated from gills of cinnamon clownfish 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 per lane onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. For reference, a protein ladder (Fermentas) was used. Samples were electrophoresed at 80 V through the stacking gel and 150 V through the resolving gel until the bromophenol blue dye front had run off of the gel. The gels were then immediately transferred to a 0.2-μm polyvinylidene diflouride (PVDF) membrane (Bio-Rad) at 85 V for 1.5 h at 4°C. Thereafter, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min followed by washing in TBS. Membranes were incubated with monoclonal mouse antibody to α-subunits of chicken NKA (dilution 1:1,000, α5, Development Studies Hybridoma Bank, USA) followed by horseradish peroxidase conjugated anti-mouse IgG secondary antibody (dilution 1:2,000, Bio-Rad) for 60 min. Bands were detected using the standard ECL as well as the more sensitive ECL systems (ECL Advance; GE Life Sciences, Sweden) and exposure to autoradiography sensitive film for 2 min.

Immunohistochemistry. Gill chloride cells were detected immunocytochemically according to the methods described in Uchida et al. (2000) with modifications. For identification of branchial Na^+/K^+-ATPase-immunoreactive (NKIR) cells, the first gill arch was removed, fixed in 4% paraformaldehyde for 24 h at 4°C, dehydrated in ethanol and embedded in paraffin. Four-μm-thick rehydrated tissue sections were incubated overnight at 4°C with the primary monoclonal mouse antibody (1/500 dilution α5, USA) and 30 min at 37°C with the secondary antibody (HRP-conjugated α-mouse immunoglobulin, 1/100 dilution). The antibodies were diluted in 2% BSA in TBS pH 7.6. EnVision + (Dako, K4001, Glostrup, Denmark) and 3,3′-diaminobenzidine (DAB^+) (Dako, K3468) were used as the detection system. Slides were counterstained with Mayer's hematoxylin. Negative control experiments, in which PBS was used instead of the primary antibody, were conducted (data not shown) to confirm the above positive results.

Plasma parameter analysis. Plasma osmolality was examined using a Vapor Pressure Osmometer (Vapro 5520, Wescor Co., Logan, UT). Plasma cortisol was analyzed using a radioimmunoassay kit (Diagnostic Systems Laboratories, Atlanta, GA).

Statistical analysis. All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA followed by post hoc Duncan's multiple range test was used to compare the differences in the data ($P < 0.05$).

Results

qPCR for NKA mRNA expression. The expression of NKA mRNA in the gills was highest at 24 h after transfer to BW (approximately 46.2- and 37.3-fold compared to SW, respectively). Also, in Western blot analysis, NKA protein was detected in a size corresponding to the predicted size for cinnamon clownfish NKA (approximately 100 kDa), and this protein was similar to mRNA expression (Fig. 1).

Gill Na^+/K^+-ATPase activity. The activity of gill NKA was 14.1 ± 0.7 μmoles ADP/mg protein/h at the start of the experiment, and then it reached its highest level of 18.2 ± 1.3 μmoles ADP/mg protein/h at 24 h after transfer to BW. However, activity of NKA in BW with PRL treatment was lower than that in BW (Fig. 2).

Changes in the distribution of gill chloride cells. Gill NKIR cells of SW and BW with PRL treatment and BW-acclimated fish were distributed in filaments and the interlamellar region, yet none were found in lamellae (Fig. 3). We found the distinct difference in distribution of gill NKIR cells associated with salinity and PRL treatment was visualized using immunolocalization of the α-subunit on paraffin sections.

Plasma osmolality level. Plasma osmolality was 342.0 ± 5.5 mOsm/kg at the start of the experiment, and then it reached its lowest level of 308.8 ± 6.4 mOsm/kg at 24 h after transfer to BW. However, plasma osmolality in BW with PRL treatment was higher than that of BW at 24 and 48 h after the transfer (Fig. 4).

Plasma cortisol level. The plasma cotisol level was 5.5 ± 0.5 ng/ml at the start of the experiment, and then it reached its highest level of 18.1 ± 1.5 ng/ml at 24 h after the transfer to BW. However, the plasma cortisol level in BW with PRL treatment was significantly lower than that in BW (Fig. 5).

Discussion

We compared the expression changes of NKA mRNA as time passed after the SW cinnamon clownfish was transferred to BW, and also examined the activity and distribution of NKA in gills by immunohistochemistry.

There are many types of NKAs in euryhaline fish, especially a reciprocal shift in the expression of two isoforms of the gill NKA α-subunit when the surrounding

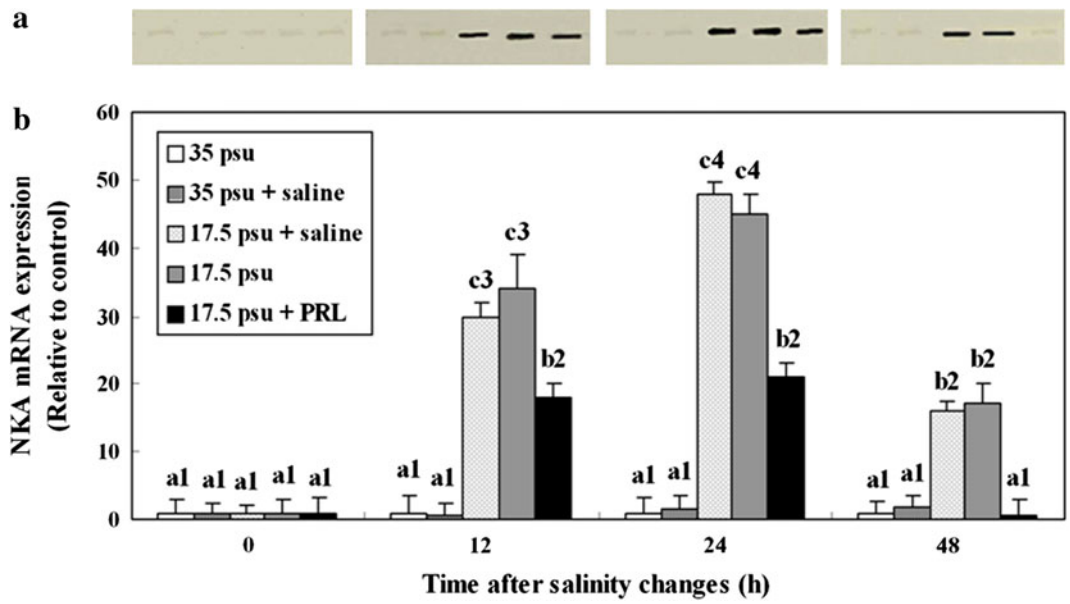

Fig. 1 Western blot of Na^+/K^+-ATPase (NKA) α protein expression after salinity transfer from seawater (SW, 35 psu) to brackish water (BW, 17.5 psu) and BW with prolactin (PRL) in cinnamon clownfish (**a**), and expression of NKA mRNA level in gill of cinnamon clownfish during salinity change using quantitative real-time PCR (**b**). We reverse transcribed 3 μg of total RNA prepared from gills 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. Values with *letters* indicate significant differences between 35, 17.5 psu and 17.5 psu + 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 ($n = 5$)

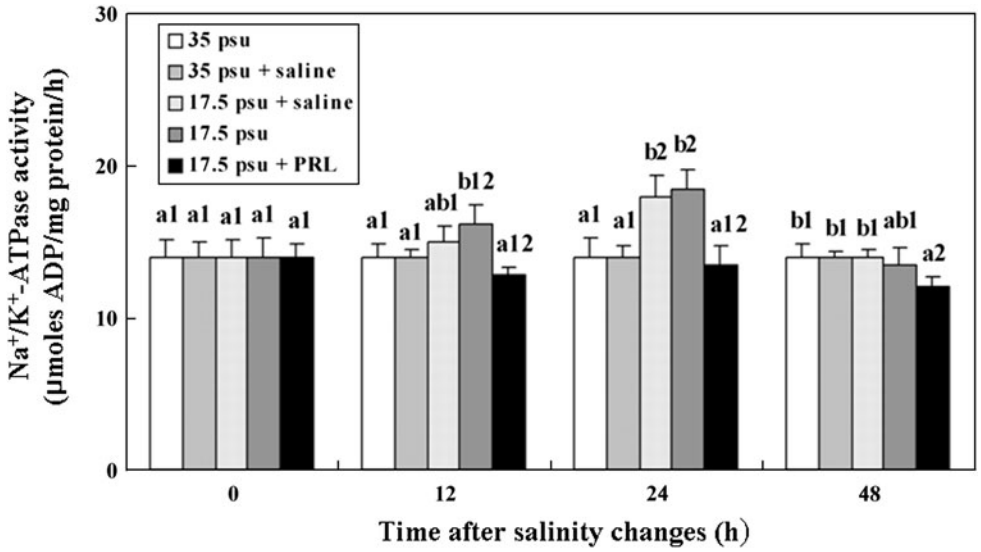

Fig. 2 Effect of prolactin treatment on gill Na^+/K^+-ATPase (NKA) activity after salinity transfer from seawater (SW, 35 psu) to brackish water (BW, 17.5 psu) and BW with prolactin (PRL) in cinnamon clownfish. Values with *letters* indicate significant differences between 35, 17.5 psu and 17.5 psu + 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 ($n = 5$)

salinity changes: α1a (freshwater isoform) and α1b (seawater isoform) (Richards et al. 2003; Bystriansky et al. 2006; Madsen et al. 2009). The α-subunit isoforms strongly influence the kinetic properties of NKA (Richards et al. 2003), and especially the NKA α1a subtype increased markedly after SW to BW transfer. So we observed the expression variation using NKA α1a subtype mRNA when the salinity changed.

The expression of NKA and activity of NKA in gills of cinnamon clownfish increased when the fish were

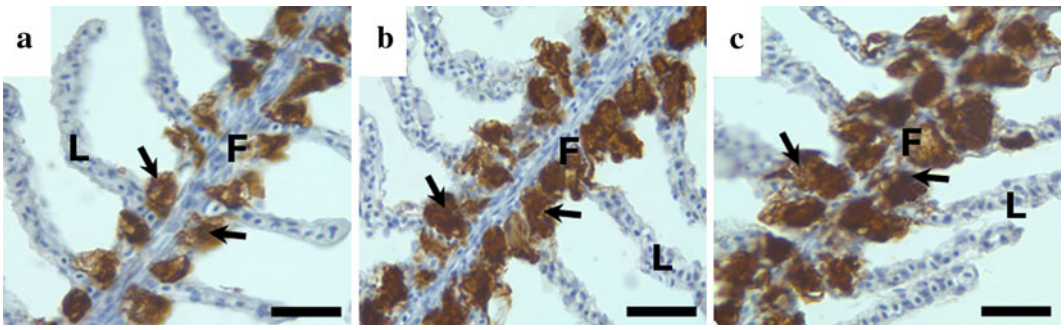

Fig. 3 Immunohistochemical localization of gill Na^+/K^+-ATPase immunoreactive (NKIR) cells recognized by α5 antibody in cross sections of cinnamon clownfish gill adapted to different salinity. **a** Seawater (SW, 35 psu), **b** 24 h transferred to brackish water (BW, 17.5 psu) treated with prolactin (PRL), **c** 24 h transferred to BW. *Arrows* indicate NKIR cells. *F* filament, *L* lamellae. *Bar* = 10 μm

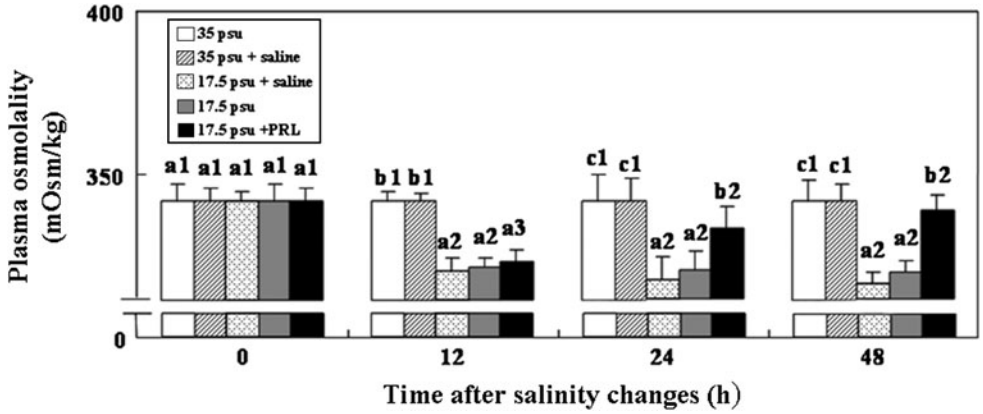

Fig. 4 Effect of prolactin treatment on plasma osmolality levels after salinity transfer from seawater (SW, 35 psu) to brackish water (BW, 17.5 psu) and BW with prolactin (PRL) in cinnamon clownfish. Values with *letters* indicate significant differences between 35, 17.5 psu and 17.5 psu + PRL within 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 ($n = 5$)

transferred to BW. This result seems to be because the expression of NKA and activity of NKA were increased to absorb the lost ions into the body because 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 some teleosts such as milkfish (Lin et al. 2003), black porgy (Choi and An 2008) and sea bass (Giffard-Mena et al. 2008), so these studies support our results. Also, in Western blot analysis, the expression of NKA protein was similar to the mRNA expression of NKA and activity of NKA.

Enzyme activity is known to be controlled by gene expression and the activation of enzymes (Chambers and Matrisian 1997). In general, NKA consists of an α-β protein complex and the ion pump in chloride cells; NKA is located on the cytoplasmic side of the extensive tubular system (Hootman and Philpott 1979). Associated with the ion pump is an increase in gill NKA activity (McCormick 1995) and an increase in NKA gene expression (Uchida et al. 1996). In this study, NKA mRNA expression increased significantly in hypoosmotic environments (Richards et al. 2003; Bystriansky et al. 2006; Madsen et al. 2009). Consistent with these observations, we obtained results showing that NKA activity, NKA protein and NKA mRNA levels in the gill increased significantly when cinnamon clownfish were transferred from SW to BW. However, there was a discrepancy in the degree of elevation between the mRNA and the activity and protein levels estimated by immunostaining of gill sections (Fig. 1 vs. Figs. 2, 3). Although the reason for this discrepancy is not clear, it may suggest a complex regulation of the NKA system as previously reported by Lin et al. (2003) and Wang et al. (2009).

In this study, expression of gill NKA is ascribed to NKIR cells distributed in the epithelia. NKIR cells in SW- and BW-acclimated cinnamon clownfish (Fig. 3), as well as in tilapia, occurred only in gill filaments (Uchida et al. 2000;

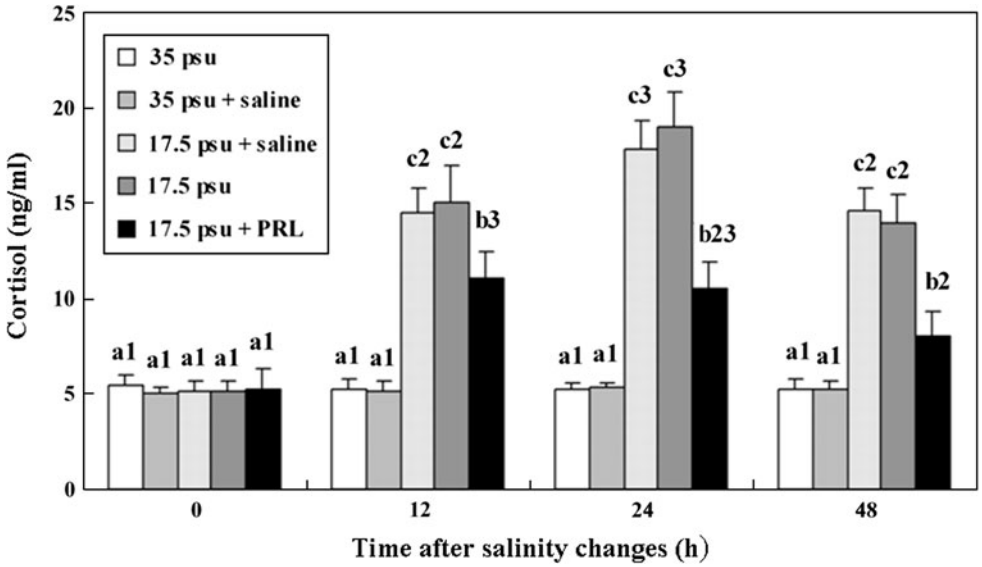

Fig. 5 Effect of prolactin treatment on plasma cortisol level in cinnamon clownfish after salinity transfer from seawater (SW, 35 psu) to brackish water (BW, 17.5 psu) and BW with prolactin (PRL). Values with *letters* indicate significant differences between 35, 17.5 psu and 17.5 psu + PRL within the 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 ($n = 5$)

Lee et al. 2003). NKIR cells are normally abundant in filament epithelia of both freshwater (FW) and SW teleosts (Wilson and Laurent 2002), and effective at secreting ions in hypertonic SW as well as taking up ions in hypotonic FW (Marshall 2002). Thus, utilizing the multi-ionoregulatory functions of existing NKIR cells in filaments may reflect the rapid physiological demands of the cinnamon clownfish, which resides in environments with changing salinities.

The level of osmolality decreased when the cinnamon clownfish were transferred to a BW environment, agreeing with the results for various fish exposed to BW (Lin et al. 2004; Chang et al. 2007; An et al. 2008), so the decrease in osmolality may be because of the water flow into and the ion flow out of the body caused by osmolality.

We investigated the expression of NKA mRNA and activity of NKA in BW after injection of PRL (5 μg/g BM) to understand the role of PRL as a FW-adapting hormone. In case of PRL injection, the expression of NKA and activity of NKA were lower than in the control group (SW), and we found that this may be repressed by PRL. This result agreed with the previous studies (Sakamoto et al. 1997; Mancera et al. 2002); this may be because PRL acts to repress NKA activity in gills to repress the ion excretion in cinnamon clownfish exposed to BW, so it seems to decrease the expression of NKA and activity of NKA. Moreover, PRL has been suggested to act on the kidney to increase Na^+ reabsorption and then increase the levels of Na^+ in plasma (Clarke and Bern 1980). Although the plasma osmolality levels decreased with PRL injections to levels higher than those in BW, we thought that this was because PRL suppressed the ion excretion. In addition, considering the recovery of the expression of NKA mRNA at 48 h after PRL injection, it is suggested that PRL acts through the hyperosmoregulatory ability of cinnamon clownfish in BW.

In this study, the plasma cortisol level of fish injected with PRL was significantly lower after injection with PRL than in the control fish. Chang et al. (2007) suggested that the transfer from SW to FW induced an increase in plasma cortisol that reached its peak within 24 h and then started to decrease, at which time PRL mRNA increased. This result suggests that exogenous PRL reduces stress responses during acclimation of marine teleosts to BW.

Moreover, we found an imbalance of osmolality and inflow of ions into the body through the increase of NKA in the gills of cinnamon clownfish exposed to BW. Also, in cinnamon clownfish exposed to BW we could determine the distribution of NKA in the gills using immunohistochemistry, and the immunostaining intensity of PRL treatment was weaker than in BW. Generally, enzyme activity is controlled by mRNA gene expression and activation of enzymes (Chambers and Matrisian 1997); based on this theory, NKA activity and expression by immunohistochemistry are significantly increased according to NKA mRNA expression, and we hypothesized that a biological effect of NKA occurred to acclimate cinnamon clownfish to the BW environment.

In addition, we found a decrease in plasma osmolality and ion levels. The hyperosmoregulatory ability of PRL suppresses ion extraction and water absorption in fish adapting to BW.

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References

Ackerman PA, Forsyth RB, Mazur CF, Iwama GK (2000) Stress hormones and the cellular stress response in salmonids. Fish Physiol Biochem 23:327–336

An KW, Kim NN, Choi CY (2008) Cloning and expression of aquaporin 1 and arginine vasotocin receptor mRNA from the black porgy, *Acanthopagrus schlegeli*: effect of freshwater acclimation. Fish Physiol Biochem 34:185–194

Bystriansky JS, Richards JG, Schulte PM, Ballantyne JS (2006) Reciprocal expression of gill Na^+/K^+-ATPase α-subunit isoforms α1a and α1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. J Exp Biol 209:1848–1858

Chambers AF, Matrisian LM (1997) Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 89:1260–1270

Chang YJ, Min BH, Choi CY (2007) Black porgy (*Acanthopagrus schlegeli*) prolactin cDNA sequence: mRNA expression and blood physiological responses during freshwater acclimation. Comp Biochem Physiol 147B:122–128

Choi CY, An KW (2008) Cloning and expression of Na^+/K^+-ATPase and osmotic stress transcription factor 1 mRNA in black porgy, *Acanthopagrus schlegeli* during osmotic stress. Comp Biochem Physiol 149B:91–100

Clarke WC, Bern HA (1980) Comparative endocrinology of prolactin. In: Li CH (ed) Hormonal proteins and peptides, vol VIII. Academic Press, New York, NY, pp 105–197

Evans DH (1993) Osmotic and ionic regulation. In: Evans DH (ed) The physiology of fishes. CRC Press, Boca Raton, FL, pp 141–315

Evans DH, Piermarini PM, Potts WTW (1999) Ionic transport in the fish gill epithelium. J Exp Zool 283:641–652

Giffard-Mena I, Lorin-Nebel C, Charmantier G, Castille R, Boulo V (2008) Adaptation of the sea bass (*Dicentrarchus labrax*) to fresh water: role of aquaporins and Na^+/K^+-ATPases. Comp Biochem Physiol 150A:332–338

Hootman SR, Philpott CW (1979) Ultracytochemical localization of Na^+, K^+-activated ATPase in chloride cells from the gills of a euryhaline teleost. Anat Rec 193:99–130

Lee TH, Hwang PP, Shieh YE, Lin CH (2000) The relationship between 'deep-hole' mitochondria-rich cells and salinity adaptation in the euryhaline teleost, *Oreochromis mossambicus*. Fish Physiol Biochem 23:133–140

Lee TH, Feng SH, Lin CH, Hwang YH, Huang CL, Hwang PP (2003) Ambient salinity modulates the expression of sodium pumps in branchial mitochondria-rich cells of Mozambique tilapia, *Oreochromis mossambicus*. Zool Sci 20:29–36

Lin YM, Chen CN, Lee TH (2003) The expression of gill Na^+/K^+-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and fresh water. Comp Biochem Physiol 135A:489–497

Lin CH, Tsai RS, Lee TH (2004) Expression and distribution of Na^+/K^+-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. Comp Biochem Physiol 138A:287–295

Madsen SS, Kiilerich P, Tipsmark CK (2009) Multiplicity of expression of Na^+, K^+-ATPase α-subunit isoforms in the gill of Atlantic salmon (*Salmo salar*): cellular localisation and absolute quantification in response to salinity change. J Exp Biol 212:78–88

Mancera JM, Carrión RL, Martín del Río MP (2002) Osmoregulatory action of PRL, GH, and cortisol in the gilthead seabream (*Sparus aurata* L.). Gen Comp Endocrinol 129:95–103

Marshall WS (2002) Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. J Exp Zool 293:264–823

Marshall WS, Bryson SE (1998) Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. Comp Biochem Physiol 119A:97–106

McCormick SD (1995) Hormonal control of gill Na^+/K^+-ATPase and chloride cell function. In: Wood CM, Shuttleworth TJ (eds) Cellular and molecular approaches to fish ionic regulation, vol 14. Academic Press, San Diego, pp 285–315

Richards JG, Semple JW, Bystriansky JS, Schulte PM (2003) Na^+/K^+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. J Exp Biol 206:4475–4486

Sakamoto T, Shepherd BS, Madsen SS, Nishioka RS, Siharath K, Richman NH 3rd, Bern HA, Grau G (1997) Osmoregulatory action of growth hormone and prolactin in an advanced teleost. Gen Comp Endocrinol 106:95–101

Sakamoto T, Uchida K, Yokota S (2001) Regulation of the ion-transporting mitochondrion-rich cell during adaptation of teleost fishes to different salinities. Zool Sci 18:1163–1174

Uchida K, Kaneko T, Yamauchi K, Hirano T (1996) Morphometrical analysis of chloride cell activity in the gill filaments and lamellae and changes in Na^+, K^+-ATPase activity during seawater adaptation in chum salmon fry. J Exp Zool 276:193–200

Uchida K, Kaneko T, Miyazaki H, Hasegawa S, Hirano T (2000) Excellent salinity tolerance of mozambique tilapia (*Oreochromis mossambicus*): elevated chloride cell activity in the branchial and opercular epithelia of the fish adapted to concentrated seawater. Zool Sci 17:149–160

Ura K, Soyano K, Omoto N, Adachi S, Yamauchi K (1996) Localization of Na^+/K^+-ATPase in tissues of rabbit and teleost using an antiserum directed against a partial sequence of the α-subunit. Zool Sci 13:219–227

Wang PJ, Lin CH, Hwang LY, Huang CL, Lee TH, Hwang PP (2009) Differential responses in gills of euryhaline tilapia, *Oreochromis mossambicus*, to various hyperosmotic shocks. Comp Biochem Physiol A 152:544–551

Wilson JM, Laurent P (2002) Fish gill morphology: inside out. J Exp Zool 293:192–213