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Salinity-dependent expression of the branchial $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase in the sailfin molly correlates with hypoosmoregulatory endurance

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Abstract

In the branchial mitochondrion-rich (MR) cells of euryhaline teleosts, the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) is an important membrane protein that maintains the internal Cl^- concentration, and the branchial Na^+/K^+-ATPase (NKA) is crucial for providing the driving force for many other ion-transporting systems. Hence this study used the sailfin molly (*Poecilia latipinna*), an introduced aquarium fish in Taiwan, to reveal that the potential roles of NKCC and NKA in sailfin molly were correlated to fish survival rates upon salinity challenge. Higher levels of branchial NKCC were found in seawater- (SW-) acclimated sailfin molly compared to freshwater- (FW-) acclimated individuals. Transfer of the sailfin molly from SW to FW revealed that the expression of the NKCC and NKA proteins in the gills was retained over seven days in order to maintain hypoosmoregulatory endurance. Meanwhile, their survival rates after transfer to SW varied with the duration of FW-exposure and decreased significantly when the SW-acclimated individuals were acclimated to FW for 21 days. Double immunofluorescence staining showed that in SW-acclimated sailfin molly, NKCC signals were expressed on the basolateral membrane of MR cells, whereas in FW-acclimated molly, they were expressed on the apical membrane. This study illustrated the salinity-dependent expression of NKCC and NKA in branchial MR cells to the post-transfer survival rates and evaluated the critical roles of NKCC and NKA in the hypoosmoregulatory endurance of the sailfin molly.

Keywords: Salinity tolerance; $Na^+/K^+/2Cl^-$ cotransporter; Na^+/K^+-ATPase; Gill; Osmoregulation; Sailfin molly.

Introduction

Euryhaline teleosts that inhabit either fresh water (FW) or seawater (SW) have to regulate their internal water and ion concentrations in order to maintain a blood and tissue fluid osmolality within the range of physiological homeostasis (Evans et al., 2005; Kaneko et al., 2008). In fish, the gill is the major organ responsible for osmoregulation and ionoregulation (Hirose et al., 2003; Evans, 2008). In the gill epithelium, the mitochondrion-rich cells (MR cells; i.e., chloride cells) are thought to be the "ionocytes" responsible for ion uptake in FW and ion secretion in SW (Hirose et al., 2003; Hwang and Lee, 2007). These MR cells are characterized by the presence of a rich population of mitochondria and an extensive tubular system in the cytoplasm. The tubular system is continuous with the basolateral membrane and provides a large surface area for the expression of ion transporting proteins such as the Na^+/K^+-ATPase (NKA, sodium-potassium pump, or sodium pump), a key enzyme for ion transport (Marshall, 2002; Evans et al., 2005). NKA is a ubiquitous membrane-spanning enzyme that actively transports Na^+ and K^+ out of and into animal cells, respectively. It is a P-type ATPase consisting of an $(\alpha\beta)_2$ protein complex. The molecular weights of the catalytic α-subunit and the smaller glycosylated β-subunit are about 100 and 55 kDa, respectively (Scheiner-Bobis, 2002). NKA is crucial for maintaining intracellular homeostasis because it provides a driving force for many other ion-transporting systems (Hirose et al., 2003; Hwang and Lee, 2007). Most euryhaline teleosts exhibit adaptive changes in branchial NKA activity following salinity changes (Marshall, 2002; Hwang and Lee, 2007). Most of the NKA detected in fish gills by immunostaining is expressed in MR cells. Therefore, in fish gills, the NKA immunoreactive (NKA-IR) cells are thought to be MR cells (Hirose et al., 2003; Hwang and Lee, 2007).

The functional and structural differentiation of the branchial MR cells is considerably influenced by environmental salinity (Evans, 2008). In the current model of branchial MR cells in SW-acclimated teleosts, salt secretion is mediated by NKA, $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR; the Cl^- channel) for Cl^- and "leaky" tight junction for Na^+ (Marshall, 2002; Evans, 2008). Among them, NKCC is a member of the cation-chloride cotransporter (CCC) family (i.e., the solute carrier family 12, SLC12) (Marshall and Bryson, 1998; Hebert et al., 2004; Gamba, 2005), and is crucial for maintaining plasma osmolality in euryhaline teleosts in SW (Marshall, 2002; Hirose et al., 2003). In the gills of euryhaline teleosts, studies on the CCC family mainly focused on NKCC (ion secretion) and Na^+/Cl^- cotransporter (NCC; ion absorption) groups at present (Cutler and Cramb, 2002; 2008; Hiroi et al., 2008; Wang et al., 2009). NKCC, which transports Na^+, K^+, and Cl^- into animal cells simultaneously, maintains internal Cl^- concentration and regulates cell volume (Russell, 2000; Hebert et al., 2004; Gamba, 2005). In the gills of SW-acclimated teleosts, the abundance of NKCC protein was significantly increased (Pelis et al., 2001; Tipsmark et al., 2002, 2004; Wu et al., 2003; Scott et al., 2004; Wilson et al., 2004, 2007; Lorin-Nebel et al., 2006; Tse et al., 2006; Hiroi and McCormick, 2007; Nilsen et al., 2007; Tang and Lee, 2007; Chew et al., 2009; Peh et al., 2009; Flemmer et al., 2010; Kang et al., 2010). However, there was no direct evidence for a relationship between branchial NKCC expression and survival of a salinity challenge in euryhaline teleosts. On the other hand, the NCC was mainly expressed in fish gills (Hiroi et al. 2008), kidneys, and intestines (Cutler and Cramb, 2008). In hypoosmotic environments, branchial NCC of the Mozambique tilapia (*Oreochromis mossambicus*) was highly expressed in the gill (Hiroi et al. 2008; Inokuchi et al, 2008, 2009; Breves et al., 2010),

and branchial NCC-immunoreactive signals were found in the apical regions of MR cells (Hiroi et al. 2008). Therefore, NCC was considered to be involved in ion absorption in hypoosmotic environments (Hiroi et al. 2008; Inokuchi et al, 2008, 2009).

A monoclonal anti-human NKCC antibody T4 (Lytle et al., 1995), considered that could recognize NKCC and NCC (Lytle et al., 1995; Cutler and Cramb, 2002; Inokuchi et al., 2008; 2009), has been widely used in many euryhaline teleosts as a marker to determine NKCC expression and distribution and to distinguish SW- and FW-MR cells (Marshall, 2002; Evans et al., 2005; Hwang and Lee, 2007). In teleosts, immunohistochemical staining with the T4 antibody on gills revealed that secretory and absorptive isoforms were localized to the basolateral membrane of MR cells in SW-acclimated fish and the apical regions of MR cells in FW-acclimated individuals, respectively (Wu et al., 2003; Hiroi and McCormick, 2007; Katoh et al., 2008; Inokuchi et al., 2008; 2009). Therefore, the existence or lack of branchial secretory isoforms (basolateral signals) was used to confirm the presence or absence of branchial hypoosmoregulatory mechanism of sailfin molly in this study.

The sailfin molly (*Poecilia latipinna*), named for the large dorsal fins of the males, is an introduced livebearing aquarium species in Taiwan (Froese and Pauly, 2010; Shao, 2010). They are natively distributed in low elevations from North Carolina, USA, to Veracruz, Mexico (Nordlie et al., 1992; Ptacek and Breden, 1998; Froese and Pauly, 2010) and have been introduced to many countries (Froese and Pauly, 2010). The natural habitats of the sailfin molly are lakes, ponds, streams, salt marshes, estuaries, and coastal waters (Nordlie et al., 1992; Froese and Pauly, 2010). In Taiwan, the euryhaline sailfin molly is mainly distributed in the lower reaches (in FW) and river mouths (in brackish water, BW) over the southwestern part of the island (Shao, 2010).

Although negative ecological effects of the sailfin molly were not reported in Taiwan, they still had the potential risks of their ecological impacts in other countries (Englund, 1999; Koehn and MacKenzie, 2004). The sailfin molly is able to survive in environments of wide salinities (0-80 ‰; Nordlie et al., 1992; Gonzalez et al., 2005), and proved to be an efficient osmoregulator upon salinity challenge (Gonzalez et al., 2005; Yang et al., 2009). Previous studies reported that in the sailfin molly, both branchial NKA expression and plasma osmolality were elevated with increasing salinities (Gonzalez et al., 2005; Yang et al., 2009).

Our preliminary results showed that SW-acclimated sailfin molly survived well after a direct transfer to FW; however, only half of the fish survived when FW-acclimated individuals were transferred directly to SW. Furthermore, when SW-acclimated sailfin molly was exposed to FW for different periods of time (FW-shower) followed by a direct transfer back to SW (salinity challenge), we observed that the longer the duration of the FW-shower, the higher the mortality of fish upon salinity challenge. These preliminary data implied that the decreasing survival rates in the FW-shower sailfin molly were due to decreased ionoregulatory capacity upon hyperosmotic challenge. Hence, in this euryhaline species, a study of the retention of branchial NKCC and NKA expression (i.e., the current model of branchial SW-type MR cells/hypoosmoregulatory mechanism) when exposed to hypoosmotic environments (e.g., FW) that correlates protein expression with fish survival rates upon hyperosmotic (e.g., SW) challenge will clarify the mechanisms of hypoosmoregulatory endurance.

In the present study, we investigated the expression and localization of ion transporters of the model of SW-type branchial MR cells including NKA and NKCC in experiments of salinity acclimation (FW, BW, or SW). In addition, the survival rates and corresponding expression of branchial NKCC and NKA in sailfin molly

after FW-shower (transfer from SW to FW for different periods of time, followed by transfer back to SW) were evaluated to reveal the potential role of NKCC and NKA in fish survival upon salinity challenge. Physiological parameters (plasma osmolality and Cl^- concentrations), biochemical expression (protein expression levels and cellular localization of NKCC and NKA α-subunit and NKA activity), and survival rates after transfer were analyzed in order to determine the hypoosmoregulatory mechanisms of the sailfin molly.

Materials and methods

Fish and experimental environments

Adult sailfin molly (*P. latipinna*), 41.2 ± 3.4 mm in standard length, were captured in Linyuan, Kaohsiung, Taiwan (120.38 °E 22.49 °N) and transported to the laboratory. SW (35‰) and BW (15‰) were prepared from aerated dechlorinated tap FW by adding standardized amounts of the synthetic sea salt "Instant Ocean" (Aquarium Systems, Mentor, OH, USA). The fish were acclimated to FW ($[Na^+]$ 0.22 ± 0.01 mM; $[K^+]$ 0.04 ± 0.01 mM; $[Ca^{2+}]$ 0.68 ± 0.01 mM; $[Mg^{2+}]$ 0.28 ± 0.01 mM; $[Cl^-]$ 0.14 ± 0.01 mM), BW ($[Na^+]$ 156.11 ± 4.50 mM; $[K^+]$ 5.72 ± 0.06 mM; $[Ca^{2+}]$ 9.29 ± 0.28 mM; $[Mg^{2+}]$ 30.34 ± 1.29 mM; $[Cl^-]$ 270.60 ± 12.07 mM), or SW ($[Na^+]$ 482.9 7± 12.73 mM; $[K^+]$ 11.38 ± 0.07 mM; $[Ca^{2+}]$ 15.34 ± 0.16 mM; $[Mg^{2+}]$ 67.87 ± 1.45 mM; $[Cl^-]$ 572.89 ± 24.24 mM) at 28 ± 1 °C with a daily 14 h:10 h L:D photoperiod in the laboratory. The water was continuously circulated through fabric-floss filters and partially refreshed every two weeks. Fish were fed a daily diet of commercial pellets *ad libitum*. In the following experiments, fish were not fed for at least 24 h and were anesthetized with MS-222 (100-200 mg/L) before sampling.

Experimental design

Acclimation experiments

Sailfin molly was acclimated to FW, BW, or SW for at least four weeks before making physiological or biochemical measurements.

Survival rates for different salinity-transfer regimes

The fish were transferred directly from the initial saline environment to another saline environment, and their survival rate after transfer was determined. The numbers of surviving fish were recorded at different time-points for 96 h after the transfer and then the survival rates of the fish were calculated. Meanwhile, the survival rate of the control group, FW-acclimated fish transferred directly to FW, was compared to that of the other transfer groups. Fish were fed as described above. Dead fish were immediately removed from the experimental tanks to maintain the water quality.

FW-shower experiments

SW-acclimated fish were transferred directly to FW (FW shower) and the changing patterns of the plasma osmolality, plasma Cl^- concentrations, protein expression levels and distribution of branchial NKCC and NKA, and NKA activity were analyzed at days 3, 7, 14, and 21 post transfer.

The effects of FW-shower on survival rates upon SW challenge

To determine the effects of FW exposure period on sailfin molly survival rates upon SW challenge, SW-acclimated fish were exposed to FW for 3, 7, 14, or 21 days (FW-shower), followed by transfer back to SW (SW challenge). Meanwhile, the survival rate of the control group, FW-shower fish which were transferred directly to FW, was compared to that of the corresponding study group. To confirm the observed effects were due to the salinity challenge and control for the stress of the transfer process, SW-acclimated fish were transferred directly to FW, and *vice versa*. The survival rates of SW challenge groups exposed to different FW-shower regimes and the control group were recorded and compared. Fish were fed as described above. Dead fish were immediately removed from experimental tanks to maintain the water quality.

Plasma analyses

Plasma from sailfin molly acclimated to FW, BW, or SW was collected according to Yang et al. (2009). In brief, after centrifugation at 1,000 *g* at 4 °C for 20 min, the plasma was stored at -20 °C until analysis. Plasma osmolality was measured with the Wescor 5520 vapro osmometer (Logan, UT, USA). Plasma Cl^- concentrations were determined using the ferricyanide method (Franson, 1985) using a Hitachi U-2001 spectrophotometer (Tokyo, Japan) at 460 nm.

Sample preparation

The gills were dissected, rinsed in phosphate buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), blotted dry, and stored immediately in a microcentrifuge tube at -80 °C until homogenizing for immunoblotting and NKA activity assay. For cryosectioning and immunofluorescent staining, the dissected gills were immediately fixed in methanol and dimethyl sulfoxide (DMSO) (vol/vol: 4: 1) at -20 °C.

Antibodies/antisera

Four primary antibodies/antisera were used in the present study: (1) NKCC: a mouse monoclonal antibody (T4, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) raised against the c-terminus of human NKCC for immunoblotting (dilution 1:500) and immunofluorescent staining (dilution 1:50); (2) NKA: a mouse monoclonal antibody (α5, Developmental Studies Hybridoma Bank) raised against the α-subunit of the avian

NKA for immunoblotting (dilution 1:2500); (3) NKA: a rabbit polyclonal antiserum (#11), which was kindly provided by Prof. Pung-Pung Hwang (Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan) raised against 565 amino acids of α-subunit of NKA (Hwang et al., 1998) for immunofluorescent staining (dilution 1:100); (4) β-actin: a mouse monoclonal antibody (#8226, Abcam, Cambridge, UK) raised against residues 1-100 of human β-actin was applied as the loading control for immunoblotting (dilution 1:5000), as in previous studies on teleosts (Wang et al., 2008; Kang et al., 2010). The secondary antibodies were (i) horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (#31430, Pierce, Rockford, IL, USA; dilution 1:5000) for immunoblotting; (ii) Alexa-Fluor-488 conjugated goat anti-rabbit IgG or Alexa-Fluor-546-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA; dilution 1:50 or 1:200, respectively) for immunofluorescent staining. Preliminary experiments of negative controls (sections stained with only primary or secondary antibodies) were performed to confirm our results (data not shown).

Cryosectioning and immunofluorescent staining

After fixation at -20 °C for 3 h, gills of sailfin molly were washed with PBS and then infiltrated with optimal cutting temperature (O.C.T.) compound (Sakura, Tissue-Tek, Torrance, CA, USA) overnight at 4 °C. Then, the tissue was mounted in O.C.T. compound for cryosectioning. Cross sections of gills were cut at 5 μm thick using a Cryostat Microtome (Microm HM 505E, Walldorf, Germany) at -25 °C. The sections were placed on 0.01% poly-L-lysine (Sigma, St. Louis, MO, USA) coated slides, and kept in slide boxes at -20 °C until immunofluorescent staining.

Cryosections of the gills were rinsed with PBS and then incubated in 5% bovine serum albumin (BSA; Sigma) and 2% Tween 20 (Merck, Hohenbrunn, Germany) in PBS for 30 min. For double staining, the cryosections were washed with PBS and incubated with the polyclonal anti-NKA (#11) diluted in PBS overnight at 4 °C. After incubation, the cryosections were washed several times with PBS, exposed to the secondary antibody (Alexa-flour 546 goat anti-rabbit antibody) at room temperature for 2 h, and washed with PBS again. After the first staining, the cryosections were incubated with PBS-diluted monoclonal anti-NKCC (T4) and incubated for 2 h at room temperature. After incubation, the cryosections were washed with PBS several times, and exposed to secondary antibody (Alexa-flour 488 goat anti-mouse antibody) at room temperature for 2 h, and washed again with PBS several times. Finally, the sections were covered with a slip using clearmount™ mounting solution (Zymed, South San Francisco, CA, USA) and imaged using a fluorescent microscope (BX50, Olympus, Tokyo, Japan). The micrographs were taken with a digital camera (Coolpix 5000, Nikon, Tokyo, Japan).

Preparation of gill homogenates

Frozen gills were rapidly thawed and homogenized in 700 µL SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.1% sodium deoxycholate, pH 7.5) containing proteinase inhibitors (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 mL aprotinin) (vol/vol: 50:1). Homogenization was performed in a 2-mL microtube with a Polytron PT1200E (Kinematica, Lucerne, Switzerland) at maximum speed for 30 sec on ice. The homogenate was then centrifuged at 5,000 *g*, 4 °C for 5 min. The supernatants were

used for protein concentration measurements, enzyme activity assays, or immunoblotting. Protein concentrations were determined with the BCA Protein Assay (Pierce) using BSA (Pierce) as a standard. The supernatants were processed immediately for determination of NKA activity or stored at -80 °C for immunoblotting.

Immunoblotting

For immunoblotting of branchial NKCC, aliquots containing 20 µg of branchial supernatants were added to sample buffer and heated at 60 ºC for 15 min followed by electrophoresis on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The pre-stained protein molecular weight marker was purchased from Fermentas (SM0671, Hanover, MD, USA). Separated proteins were transferred from unstained gels to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting using a tank transfer system (Mini Protean 3, Bio-Rad, Hercules, CA, USA). Blots were preincubated for 2 h in PBST (phosphate buffered-saline with 0.05% Tween 20) buffer containing 5% (wt/vol) nonfat dried milk to minimize non-specific binding. Then the blots were cut into upper (for NKCC) and lower (for β-actin) sections prior to antibody incubation. The blots were incubated overnight at 4 °C with the primary antibodies (T4 or β-actin) diluted in 1% BSA and 0.05% sodium azide in PBST. The blots were subsequently washed in PBST three times, followed by a 1-h incubation with HRP-conjugated secondary antibody diluted in PBST. Then the blots were incubated with the SuperSignal West Pico Detection Kit (#34082, Pierce) and signals were transferred to Kodak BioMax light film (#178 8207, Eastman Kodak, Rochester, NY, USA). The immunoblotting protocol for the negative control, which was used

to confirm that the immunoreactivity was due to the presence of NKCC rather than non-specific binding, was identical to that described above except that the pre-immune serum of mouse (BALB/c strain) was substituted for the primary antibody (T4).

For immunoblotting of branchial NKA α-subunit, sample buffer was mixed with aliquots containing 25 μg of branchial supernatant. The subsequent protocol was identical to that for branchial NKCC immunoblotting.

Immunoblots were scanned and imported as TIFF files. Immunoreactive bands were analyzed using MCID software version 7.0 (Imaging Research, Ontario, Canada). The results were converted to numerical values in order to compare the relative protein abundance of the immunoreactive bands.

Assay of NKA activity

Enzyme activity was measured using the NADH-linked method (McCormick, 1993) modified from Kang et al. (2008). The reaction was started by mixing 10 μL sample supernatant and 200 μL reaction mixture (0.38 mM ATP, 1.50 mM phosphoenolpyruvate, 0.24 mM NADH, 2.48 U/mL lactate dehydrogenase, 2.70 U/mL pyruvate kinase, 47.3 mM NaCl, 2.63 mM $MgCl_2 \cdot 6H_2O$, 10.5 mM KCl, and 50 mM imidazole, pH 7.5) with or without 0.75 mM ouabain per well; each sample was assayed in triplicate. The 96-well plate was read every 15 sec for up to 10 min in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 340 nm and 28 °C. The linear rate from 2 to 8 min was determined for each pair of triplicate wells. The standard curve was determined using 10 μL ADP per well at concentrations of 0 to 30 nmol at 340 nm at 28 °C, and the plate was read for at least 5 min after adding 200 μL reaction mixture (without 0.75 mM ouabain). The slope of the

standard curve should be from -0.012 to -0.015. The NKA activity was calculated as the difference in slope of ATP hydrolysis (NADH reduction) in the presence and absence of ouabain, and the activity was expressed as μmol ADP per mg protein per hour.

Statistical analysis

Values were expressed as means ± the standard error of the mean (SEM), unless stated otherwise. Survival experiments were analyzed using the chi-square test, and the other experiments were compared using one-way analysis of variance (ANOVA; Tukey's pairwise method). The significance level was set at $P<0.05$.

Results

Acclimation experiments

The plasma [Cl^-] in the sailfin molly increased with environmental salinities, which was similar to the pattern of plasma osmolality (Fig. 1). Immunoblotting of NKCC in gills from sailfin molly acclimated to environments with different salinities (FW, BW, and SW) resulted in the major immunoreactive bands (range form 130 to 170 kDa) compared with the results from negative controls (Figs. 2a, b). Quantification of the immunoreactive bands revealed that the branchial NKCC protein abundance of SW-acclimated sailfin molly (SWA) was significantly higher than that of FW- or BW-acclimated sailfin molly (FWA or BWA): about 18.7- and 2.2-fold, respectively (Fig. 2c). The results of double immunofluorescent staining revealed that NKCC signals (green) were colocalized to NKA-IR cells (red) in all groups (Fig. 3). In FW-acclimated sailfin molly, signals were only found in the apical membrane of NKA-IR cells (arrow; Fig. 3c), whereas in BW-acclimated sailfin molly, signals were found in the basolateral (arrowhead) or apical membrane of NKA-IR cells (Fig. 3f). In SW-acclimated sailfin molly, however, signals were localized to the basolateral membrane of NKA-IR cells (Fig. 3i). NKA-IR cells without NKCC signal were found in both BW- and FW-acclimated sailfin molly (asterisks; Figs. 3c and f).

The survival rates for different salinity-transfer regimes

The survival rate was approximately 100% when FW-acclimated sailfin molly were transferred directly to BW or *vice versa* (Fig. 4; FA/B; BA/F), which was similar to the control group in which FW-acclimated sailfin

molly were transferred to FW (FA/F). When the SW-acclimated sailfin molly were transferred directly to FW, all the individuals survived (SA/F). In contrast, when the FW-acclimated sailfin molly were transferred directly to SW, only half of the fish survived (chi-square test, P <0.001; FA/S). However, when the FW-acclimated sailfin molly were pre-acclimated to BW for 12 and 24 h before a direct transfer to SW, the survival rates increased significantly to 83% and 100%, respectively (FA/B12/S and FA/B24/S).

FW-shower experiments

Three days after transfer from SW to FW, the plasma osmolality and chloride concentrations in SW-acclimated sailfin molly declined significantly (Fig. 5). The abundance of branchial NKCC protein reduced gradually and was significantly decreased by seven days post-transfer (Fig. 6). NKA α-subunit protein abundance and NKA activity in the gills were significantly decreased after 14 days post-transfer (Figs. 7 and 8). The control group, SW-acclimated sailfin molly which were transferred to SW, showed no significant change in the above parameters at all time-points (Figs. 5-8). Double immunofluorescent staining of cryosections of gills revealed that basolaterally distributed NKCC signals were colocalized to NKA-IR cells at the third and seventh days post transfer (Fig. 9c, f). In the 7-days-post-transfer fish, a few apical signals were found in some NKA-IR cells (Fig. 9d-f). However, at the 14th day after transfer, all signals were found in the apical region of NKA-IR cells (Fig. 9g-i). Furthermore, some NKA-IR cells without NKCC signal (asterisks) were found in both the seven days- and 14 days-FW-shower fish (Fig. 9f, i).

The effects of FW-shower on survival rates upon SW challenge

When SW-acclimated sailfin molly were exposed to FW (i.e., FW-shower) for durations identical to that in the previous experiment and subsequently transferred back to SW (SW challenge), their survival rates varied with the duration (3, 7, 14, and 21 days) of the FW-shower (Fig. 10). In the 3 and 7 day FW-shower groups (SA/F3/S and SA/F7/S), no mortality was found. In the 14 days FW-shower group (SA/F14/S), the survival rate was reduced to about 80%, but there was no statistical difference between the study and control groups (chi-square test, P=0.06). However, the survival rates of the 21 day FW-shower group (SA/F21/S) and the FW-acclimated sailfin molly transferred directly to SW (FA/S) decreased significantly compared to the control groups (chi-square test, P <0.001).

Discussion

In this study, we explained the relationship between the expression of branchial NKCC and NKA and the survival rates of sailfin molly upon salinity challenge after chronic (acclimation) and acute (FW-shower) experiments by reduced expression of branchial NKCC and NKA and the disappearance of branchial secretory type MR cells. Moreover, our results revealed a correlation between the gradual reductions in expression of branchial NKCC and NKA and decreasing survival rates after hyperosmotic challenge in sailfin molly.

In the acclimation experiments, branchial NKCC protein expression was higher in SW-acclimated sailfin molly (SWA) than FW-acclimated fish (FWA; Fig. 2c), which was similar to the pattern of NKA expression (Yang et al., 2009). These results conformed to the current model of Cl^- excretion (Marshall, 2002; Hirose et al., 2003; Evans, 2008), and were similar to studies of other SW-acclimated euryhaline teleosts. Furthermore, when transferred from SW to FW, by seven days post transfer, the level of branchial NKCC protein expression decreased to the levels measured in BW sailfin molly and the lowest expression levels, similar to those seen in FW fish, were found in the 14- and 21-day post-transfer groups (Figs. 2 and 6). The changing branchial NKCC expression profiles in sailfin molly transferred from SW to FW were similar to those observed in other species, which indicated that the degradation of NKCC protein in hypoosmotic environments took about one week (Tipsmark et al., 2002, 2004, 2008a, b; Lorin-Nebel et al., 2006).

Although higher levels of branchial NKA α-subunit protein and NKA activity were found in SW-acclimated sailfin molly (Yang et al., 2009), which is similar to the pattern for branchial NKCC (this study), NKA activity and NKA α-subunit protein abundance were not significantly decreased until 14 days after the fish

were transferred from SW to FW, which was later than the significant changes in the expression of NKCC (Figs. 6-8). Compared to changes in NKCC expression, slower degradation of NKA α-subunit protein expression (Tipsmark et al., 2008a), NKA activity (Tipsmark et al., 2008b), or both (Tipsmark et al., 2002, 2004) were also reported in other euryhaline teleosts. On the other hand, in some euryhaline teleosts, the abundance of mRNA or protein or activity of branchial NKA altered quickly after transfer to hypoosmotic environments (about three days post-transfer) (Uchida et al., 1997; Laiz-Carrión et al., 2005; Arjona et al., 2007; Tipsmark et al., 2008b; Tomy et al., 2009). The different profiles of change in branchial NKA activity in different euryhaline teleosts usually correlated with their natural habitats (Hwang and Lee, 2007). Freire et al. (2008) also considered that osmoregulatory capabilities may be correlated with a species' evolutionary history or natural habitat. Therefore, the time course of the pattern of NKA expression following environmental challenge might vary with teleostean species.

Changes were also observed in the double immunofluorescent staining of NKCC and NKA in gills of sailfin molly (Figs. 3 and 9). In the present study, the NKA-IR cells were mainly distributed in the interlamellar region and the afferent artery region of the filaments (data not shown), which conformed to our previous study (Yang et al., 2009). Previous immunostaining studies using the T4 antibody on gills showed that in SW-acclimated euryhaline teleosts the NKCC signals were localized to the basolateral membrane of NKA-IR cells (i.e., MR cells). However, the studies in FW-acclimated euryhaline teleosts exhibited three patterns of immunostaining: (1) no positive signal (Hiroi and McCormick, 2007; Chew et al., 2009); (2) immunoreactive signals localized to the basolateral membrane of MR cells (McComick et al., 2003; Prodocimo and Freire, 2006;

Tse et al., 2006; Hiroi and McCormick, 2007; Sardella and Kültz, 2009); and (3) immunoreactivity in the apical region of MR cells (Wu et al., 2003; Hiroi et al., 2005b; Lorin-Nebel et al., 2006; Inokuchi et al., 2008; Katoh et al., 2008; Ouattara et al., 2009; Choi et al., 2010; Flemmer et al., 2010; Kang et al., 2010).

In this study, the NKCC signals (green) in the gills of SW-acclimated sailfin molly (SWA; Fig. 3g-i) were colocalized to the basolateral membrane of NKA-IR cells (red), similar to findings reported for other SW-acclimated teleosts. However, in FW-acclimated sailfin mollies, the signals were only found in the apical membrane of NKA-IR cells, similar to the findings of the third group described above, and some NKA-IR cells were not exhibited any apical immunoreactive signal (FWA; Fig. 3a-c). NKA-IR cells without NKCC immunoreactivity might occur because the apical region of that cell appeared in another section, the NKA-IR cells were immature (Hiroi et al., 2005a), or these may be other types of MR cells that do not express either apical- or basolateral-NKCC (Hiroi et al., 2008; Inokuchi et al., 2008). The ion-transport function (absorption or secretion) of an MR cell can be indicated by the localization patterns of osmoregulation-related proteins at the apical and basolateral membranes (Hiroi et al., 2008; Inokuchi et al., 2008). Therefore, according to previous studies of branchial NKCC in euryhaline teleosts, in the sailfin molly, the basolateral-NKCC signals should be NKCC1a, which was the secretory isoform highly expressed in fish gills (Cutler and Cramb, 2002; Mackie et al., 2007; Hiroi et al., 2008; Inokuchi et al., 2008; Kang et al., 2010). On the other hand, in the sailfin molly, like the other euryhaline teleosts, the apical-immunoreactive signals may be NCC, that was mainly expressed in fish gills in hypoosmotic environments (Hiroi et al. 2008) rather than NKCC2, which was another NKCC isoform and expressed prominently in non-gill tissues of other teleosts (Cutler and Cramb, 2008; Hiroi et al., 2008; Kang

et al., 2010). Therefore, the NKA-IR cells of BW-acclimated sailfin molly (BWA; Fig. 3d-f) with apical-, basolateral-, or no-NKCC signals, which combine the characteristics of SW- and FW-type MR cells, suggest that the gills of BW-acclimated fish were able to perform absorption and secretion simultaneously. Hence, the BW-acclimated sailfin molly may be equipped with both hypo- and hyperosmoregulation mechanisms, like Mozambique tilapia (Inokuchi et al., 2008).

After FW-shower, the changing profiles of branchial NKA-IR cells corresponded to the relative levels of branchial NKCC protein expression (Figs. 7 and 9). The type of branchial NKA-IR cells in SW-acclimated sailfin molly changed from SW-type to BW-type (by the seventh day) and then to FW-type (by the 14th day) (Figs. 3 and 9). Change in morphology, type, or localization of branchial MR cells or NKA-IR cells after abrupt hypoosmotic challenge were also reported in other euryhaline teleosts (Caberoy and Quinitio, 2000; Lin et al., 2004, 2006; Hiroi et al., 2005a, 2008; Lorin-Nebel et al., 2006). Among them, the BW-type of NKA-IR cells in Mozambique tilapia were found by three days after transfer from SW to FW (Hiroi et al., 2005a, 2008), and were not found in FW-acclimated fish (Wu et al., 2003; Inokuchi et al., 2008). Therefore, in sailfin molly, the BW-type of branchial NKA-IR cells may be a transitional type rather than the BW-dependent type. Furthermore, in the sailfin molly, the transitional type of branchial NKA-IR cells may provide the buffering effect in response to salinity challenge.

To compare the salinity tolerances of euryhaline teleosts, survival rates following direct or gradual transfer between different salinity environments were determined (Hiroi and McCormick, 2007; Kang et al., 2010). In this study, the sailfin molly survived well after transfer directly from BW to FW, and *vice versa* (BA/F and FA/B;

Fig. 4). When FW-acclimated sailfin molly were transferred to SW, the survival rates were improved by a pre-acclimation in BW (FA/B12/S and FA/B24/S; Fig. 4). Therefore, the BW-type MR cells might facilitate their euryhalinity during acclimation to different salinity environments. Similar results were reported in other euryhaline teleosts such as Mozambique tilapia (Hwang, 1987), Japanese medaka (*Oryzias latipes*; Inoue and Takei, 2003), and brook trout (*Salvelinus fontinalis*; Hiroi and McCormick, 2007). Furthermore, Nordlie et al. (1992) reported that salinity tolerance was better in BW-inhabiting sailfin molly than that in FW-inhabiting fish population. These results suggested that, when exposed to SW, pre-acclimation to BW usually increases the salinity tolerance of FW-acclimated euryhaline teleosts, including sailfin molly. However, the SW-acclimated sailfin molly survived successfully after transfer to FW (SA/F; Fig. 4). The different survival rates found under the various regimes might occur because of the innate osmoregulatory ability determined by their natural habitats (FW and BW; Nordlie et al., 1992) rather than the stressful environment (SW; Yang et al., 2009) or due to the reduced hypoosmoregulatory mechanism of SW-acclimated sailfin molly.

After FW-shower, the reduced hypoosmoregulatory mechanism of SW-acclimated sailfin molly was signified by decreased expression of NKCC and NKA, the representative ion transporters of current SW-model MR cells, and was verified by FW-shower effects on survival rates upon SW challenge (Kang et al., 2010). In this study, the decreased survival rates were correlated with the reduced expression of branchial NKCC and NKA and the disappearance of basolateral NKCC1a-like-signals on NKA-IR cells (Figs. 6-10). Similar results were reported in other euryhaline teleost such as brackish medaka (*Oryzias dancena*; Kang et al., 2010). However, significant decrease in the survival rates occurred only when the levels of branchial NKCC protein,

NKA α-subunit protein, and NKA activity were significantly reduced. These results revealed that the hypoosmoregulatory endurance of SW-acclimated sailfin molly after exposure to FW was maintained for at least 21 days. Taken together, our data showed that decreased expression of branchial NKCC and NKA α-subunit proteins, as well as NKA activity and changing patterns of NKCC immunoreactivity in NKA-IR cells correlated with reduction in the hypoosmoregulatory endurance of the sailfin molly (Figs. 6-10).

The branchial osmoregulatory mechanisms of the sailfin molly were responsible for the maintenance of their homeostasis and survival upon salinity challenge. The gills of euryhaline teleosts normally absorb and secrete Cl^- in order to maintain constant plasma Cl^- concentrations in various saline environments (Evans et al., 2005). Euryhaline teleosts have excellent osmoregulatory abilities and can maintain their plasma osmolalities within narrow physiological ranges in both hypoosmotic and hyperosmotic environments (Marshall and Grosell, 2006; Kaneko et al., 2008). The sailfin molly is an efficient osmoregulator that is able to maintain physiological parameters, such as plasma Cl^- concentrations (Fig. 1), plasma Na^+ concentrations, plasma osmolality, and muscle water content (Gonzalez et al., 2005; Yang et al, 2009) in environments of different salinities. Furthermore, their physiological responses changed quickly. The plasma osmolality and Cl^- concentrations of SW-acclimated sailfin molly were efficiently regulated and maintained within a tolerated range within three days after transfer to FW (Fig. 5). These results indicated that the osmoregulatory ability of the sailfin molly was activated in order to maintain appropriate plasma parameters. Although the experimental designs were different, Gonzalez et al. (2005) reported a similar pattern of change in plasma Cl^- concentrations when sailfin molly were transferred from SW to hypersaline water. Previous studies showed that changing patterns in plasma

osmolality in euryhaline teleosts were correlated with their primary habitats and salinity tolerance (Kato et al., 2005; Bystriansky et al., 2006). Other studies also revealed that when exposed to hypoosmotic environments, the plasma osmolality and/or Cl^- concentrations of euryhaline teleosts decreased significantly in a very short period of time (about one day post-transfer) and then maintained these lower levels (Claiborne et al., 1994; Jensen et al., 1998; Seale et al., 2002; Lin et al., 2004, 2006; Laiz-Carrión et al., 2005; Motohashi et al., 2009; Kato et al., 2010). In euryhaline teleosts, the crisis and regulatory phases accompany abrupt transfer to hypoosmotic environments and express a rapid drop in plasma osmolality and Cl^- concentrations in adapting to hypoosmotic environments (Lin et al., 2004). Therefore, through effective osmoregulatory mechanisms, the sailfin molly, like the other euryhaline species, is able to overcome changes in external salinity and reach a new steady state of blood ion concentrations.

In conclusion, this study has identified the salinity-dependent expression and localization of NKCC in branchial MR cells in the euryhaline sailfin molly. Expression of the NKCC protein was increased with environmental salinity. Meanwhile, the NKCC-signals were localized to the apical region of FW NKA-IR cells and the basolateral membrane of SW NKA-IR cells. In addition, the transitional type (BW-type) of branchial NKA-IR cells may enhance their salinity tolerance due to the presence of different types of MR cells that can buffer the effect of salinity challenge. We also performed the time-course transfer experiments in order to illustrate the critical roles of NKCC and NKA in the hypoosmoregulatory endurance of the sailfin molly. As indicated by the changing profiles of NKCC and NKA expression and localization and the survival rates, the hypoosmoregulatory capability reduced gradually until 21 days after FW-shower. These results illustrated that

the sailfin molly was proved to be an efficient osmoregulator with gill NKCC and NKA expression altering in response to acute or chronic salinity challenge in order to maintain ion and water homeostasis in environments of different salinities.

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Figure Legends

Fig. 1. The effect of salinity on plasma osmolality (crosshatched bars; modified from Yang et al., 2009) and Cl^- concentrations (white bars; N=16) in the sailfin molly. Dissimilar letters or symbols indicate significant differences among groups (mean ± SEM, one way ANOVA with Tukey's comparison, $P<0.05$). The data of plasma osmolality was modified from Yang et al. (2009). FWA, freshwater-acclimated sailfin molly; BWA, brackish water-acclimated sailfin molly; SWA, seawater-acclimated sailfin molly.

Fig. 2. Representative immunoblot and negative control of sailfin molly gills probed with (a) pre-immune serum of mouse and (b) a monoclonal antibody (T4) to NKCC, respectively. The immunoreactive bands indicate a molecular mass from 130 to 170 kDa, centered at 150 kDa (bracket). (c) The relative intensity of immunoreactive bands of branchial NKCC from different groups. Dissimilar letters indicate significant differences among groups (N=5, mean ± SEM, one way ANOVA with Tukey's comparison, $P<0.05$). M, marker (kDa); FWA, freshwater-acclimated sailfin molly; BWA, brackish water-acclimated sailfin molly; SWA, seawater-acclimated sailfin molly.

Fig. 3. Immunofluorescent staining of frozen cross-sections of the gill filaments of freshwater-acclimated sailfin molly (FWA; Figs. a-c), brackish water-acclimated sailfin molly (BWA; Figs. d-f), and seawater-acclimated sailfin molly (SWA; Figs. g-i). Gill filament cryosections were double-stained with anti-NKCC antibody (green; Figs. a, d, and g) and anti-NKA antibody (red; Figs. b, e, and h). The merged images (Figs. c, f, and i) showed that NKCC signals were found in the basolateral membrane of the NKA-immunoreactive (IR) cells (arrowheads) of SW-acclimated sailfin molly. In contrast, the

signals of FW fish were found in the apical membrane of their NKA-IR cells (arrows). In BW fish, more signals were found in the basolateral membrane of NKA-IR cells (arrowheads) and less signals were localized to the apical membrane of NKA-IR cells (arrows). Some NKA-IR cells without NKCC signal (asterisks) were found in both BW- and FW-acclimated fish. Scale bar, 25 µm.

Fig. 4. The survival rate (%) of sailfin molly in different salinity-transfer regimes. Dissimilar letters indicate significant differences between two groups (N=30-60, chi-square test; a vs. b and b vs. c, P <0.05; a vs. c, P <0.001). FWA, freshwater-acclimated sailfin molly; BWA, brackish water-acclimated sailfin molly; SWA, seawater-acclimated sailfin molly; FW, fresh water; BW, brackish water; SW, seawater.

Fig. 5. The time course of changes in osmolality (a; N=5) and [Cl^-] (b; N=8) in the plasma of seawater-acclimated sailfin molly (SWA) transferred directly to seawater (SW; control group) or fresh water (FW; study group). Dissimilar letters indicate significant differences among the test time-points in the study group (one way ANOVA with Tukey's comparison, P <0.05). Values are mean ± SEM. Significant decreases in plasma osmolality and [Cl^-] occurred at the third day after transfer. No significant difference was found among the test time-points in the control group. FWA, freshwater-acclimated sailfin molly.

Fig. 6. The time course of changes in NKCC protein expression in the gills of seawater-acclimated sailfin molly (SWA) transferred directly to seawater (SW; control group) or fresh water (FW; study group) (N=5). (a, b) Representative immunoblots of branchial NKCC protein expression in the study group (a) and the control group (b). (c) Relative intensity of immunoreactive bands of branchial NKCC in different groups.

Significant reduction in protein abundance of branchial NKCC occurred at the seventh day after transfer. No significant difference was found among the test time-points in the control group. Dissimilar letters indicate significant differences among the test time-points in the study group (one way ANOVA with Tukey's comparison, P <0.05). Values are mean ± SEM. FWA, freshwater-acclimated sailfin molly.

Fig. 7. The time course of changes in NKA protein expression in the gills of seawater-acclimated sailfin molly (SWA) transferred directly to seawater (SW; control group) or fresh water (FW; study group) (N=5). (a, b) Representative immunoblots of branchial NKA α-subunit protein expression in the study group (a) and the control group (b). (c) Relative intensity of immunoreactive bands of branchial NKA of different groups. Significant reduction in protein abundance of branchial NKA occurred at the 14th day after transfer. No significant difference was found among the test time-points in the control group. Dissimilar letters indicate significant differences among the test time-points in the study group (one way ANOVA with Tukey's comparison, P <0.05). Values are mean ± SEM. FWA, freshwater-acclimated sailfin molly.

Fig. 8. Effects of direct transfer of seawater-acclimated sailfin molly (SWA) from seawater (SW) to fresh water (FW) (study group) and from SW to SW (control group) on gill NKA activity (N=5). Significant reduction in branchial NKA activity occurred at the 14th day after transfer. No significant difference was found among the test time-points in the control group. Dissimilar letters indicate significant differences among the test time-points in the study group (one way ANOVA with Tukey's comparison, P <0.05). Values are mean ± SEM. FWA, freshwater-acclimated sailfin molly.

Fig. 9. Immunofluorescent staining of frozen cross-sections of the gill filaments of seawater-acclimated sailfin

molly at the third (a-c), seventh (d-f), and 14th days (g-i) after direct transfer from seawater (SW) to fresh water (FW). Gill filaments were double stained with anti-NKCC (green; a, d, and g) and anti-NKA (red; b, e, and h) antibodies. The merged images (c, f, and i) showed that at the third day after transfer, NKCC signals were found in the basolateral membrane of the NKA-immunoreactive (IR) cells (arrowheads), whereas at the 14th day after transfer, NKCC signals occurred in the apical membrane of NKA-IR cells (arrows). At the seventh day after transfer, NKCC signals were found in either basolateral or apical membrane of different NKA-IR cells. Furthermore, some NKA-IR cells without NKCC signal (asterisks) were found in both the seven days- and 14 days-FW-shower fish. Scale bar, 25 μm.

Fig. 10. The effects of different regimes of fresh water (FW) exposure on the survival rates (%) of seawater-acclimated sailfin molly (SWA) when they were transferred back to seawater (SW) directly after the FW-shower. Reduction in survival rate upon SW challenge were found in the 14 days- and 21 days-FW-shower group, respectively. The asterisks indicate significant differences compared to their corresponding control group (N=30, chi-square test, ***P <0.001). FWA, freshwater-acclimated sailfin molly.

Fig. 1.

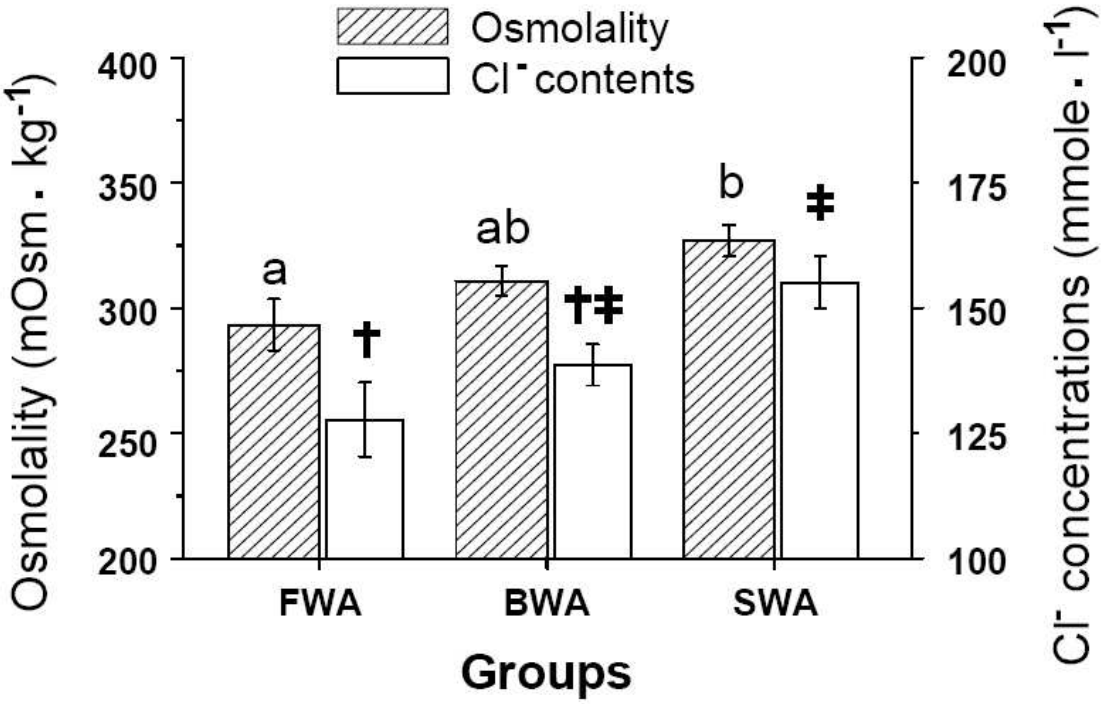


Fig. 2.

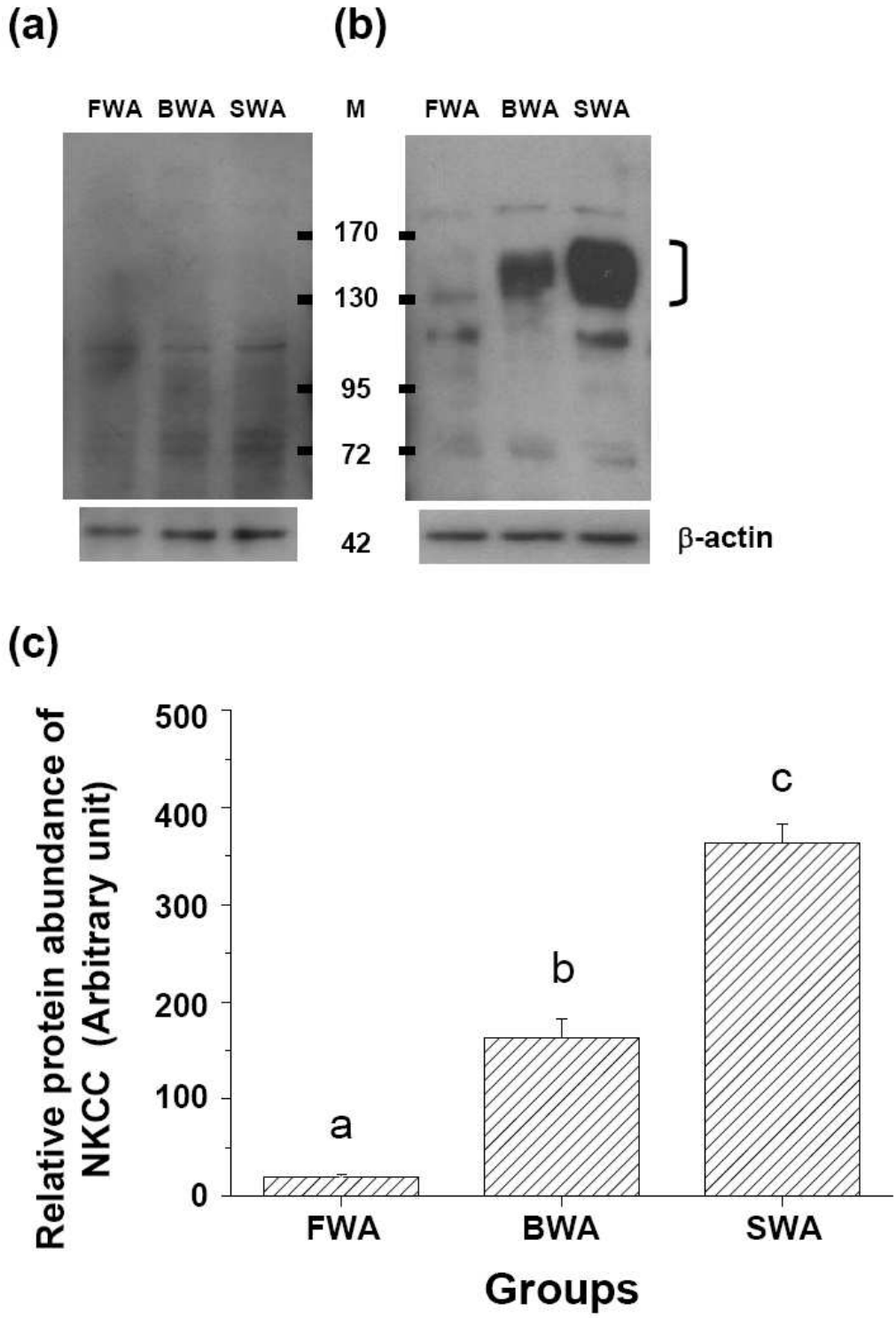
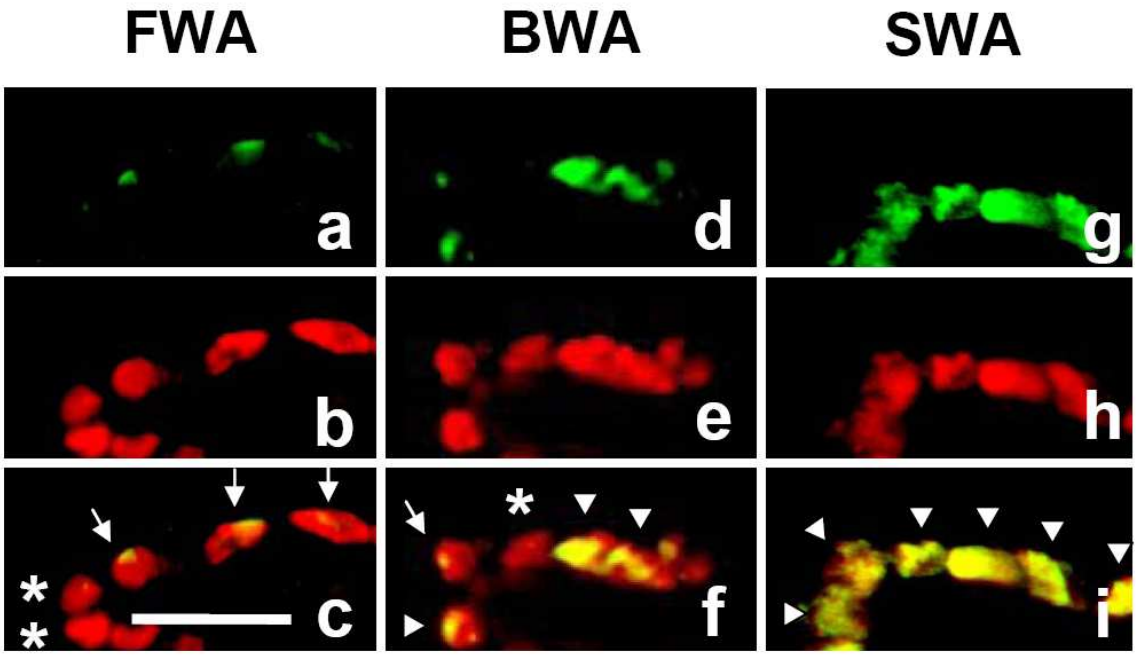

(a)
(b)
FWA BWA SWA
M
FWA BWA SWA
170
130
95
72
42
β-actin
(c)
Relative protein abundance of NKCC (Arbitrary unit)
500
400
300
200
100
0
a
b
c
FWA
BWA
SWA
Groups

Fig. 3.



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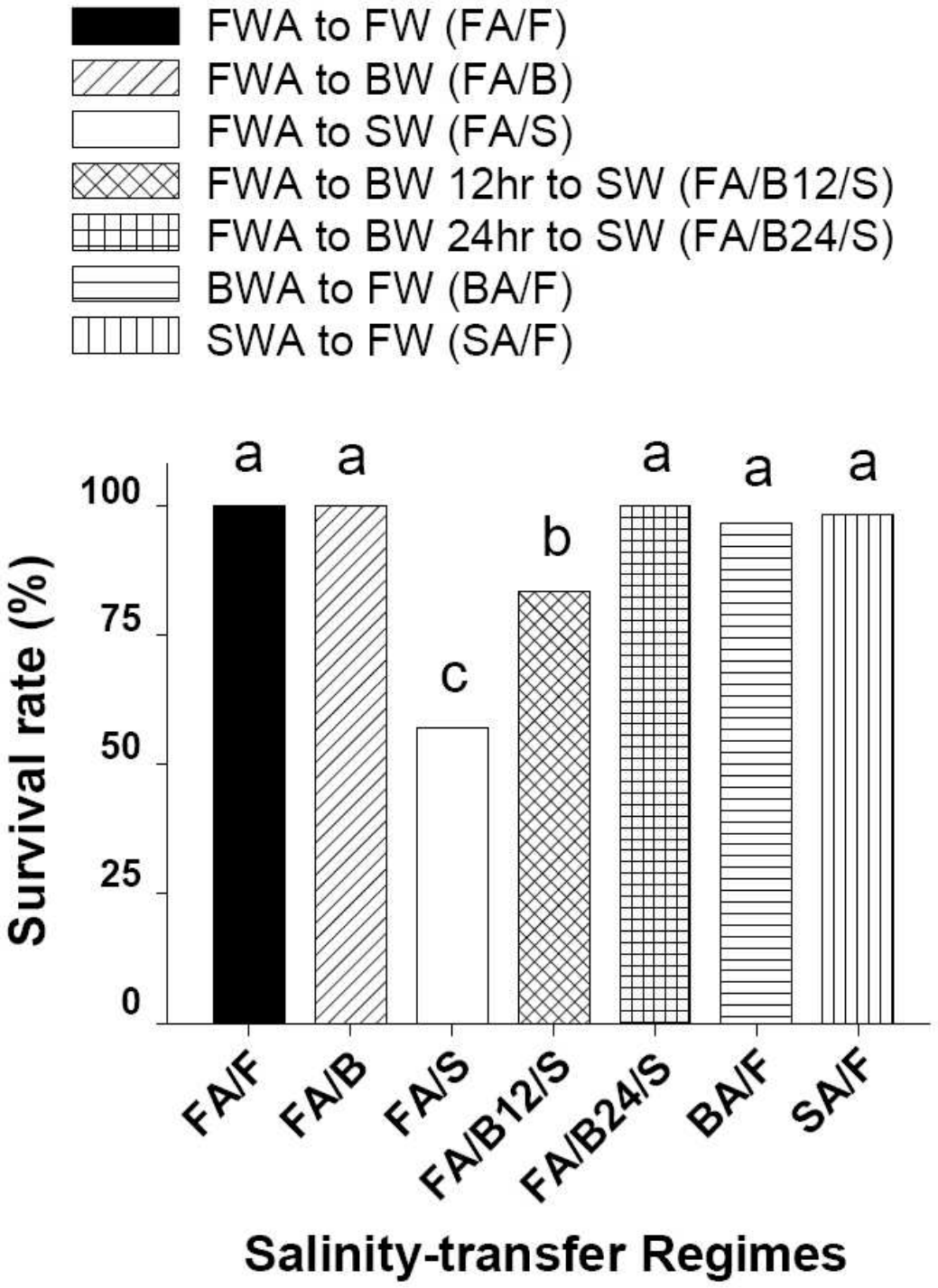

FWA to FW (FA/F)
FWA to BW (FA/B)
FWA to SW (FA/S)
FWA to BW 12hr to SW (FA/B12/S)
FWA to BW 24hr to SW (FA/B24/S)
BWA to FW (BA/F)
SWA to FW (SA/F)
a
a
c
b
a
a
a
100
75
50
25
0
Survival rate (%)
FA/F
FA/B
FA/S
FA/B12/S
FA/B24/S
BA/F
SA/F
Salinity-transfer Regimes

Fig. 5.

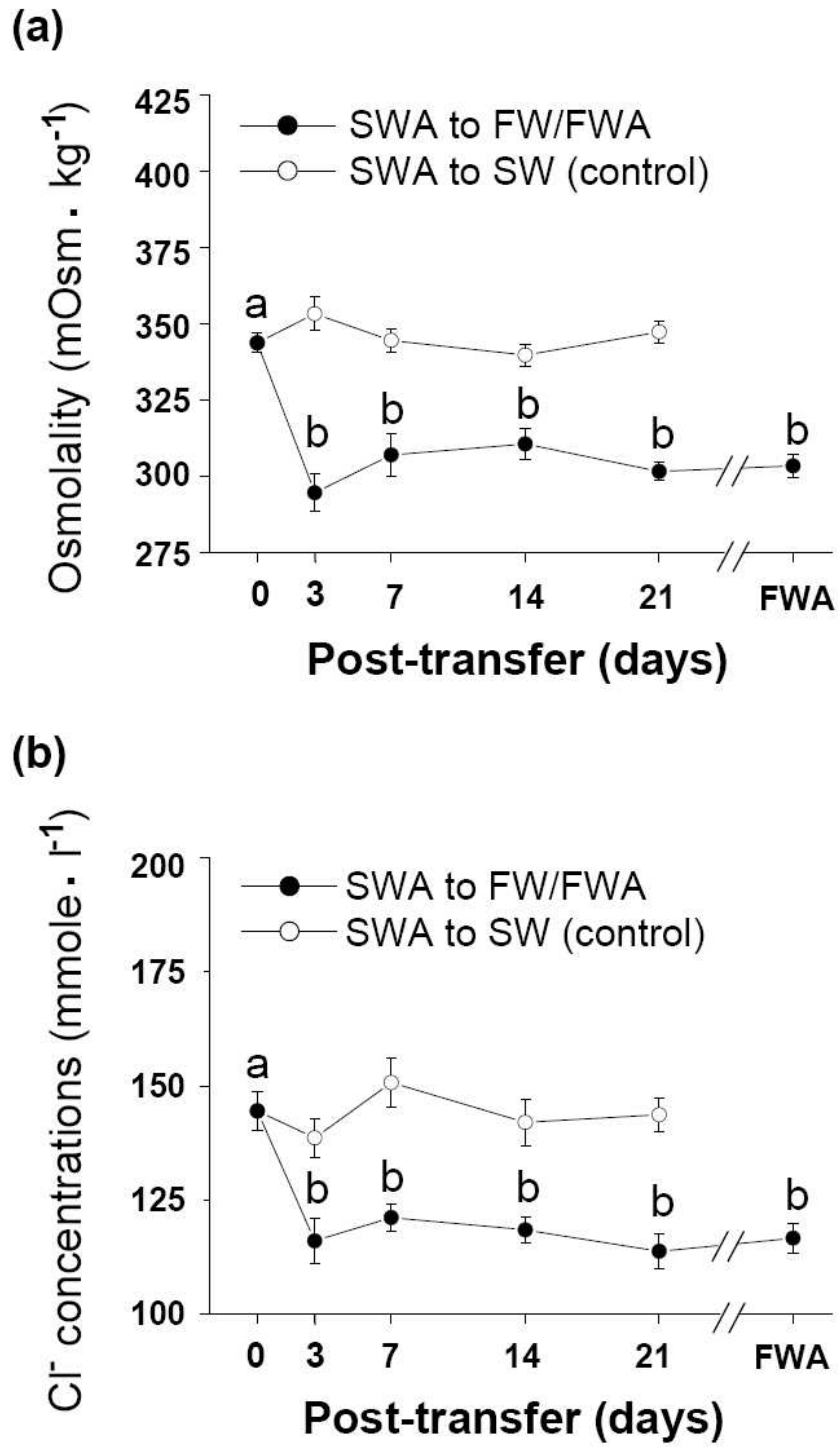

(a)
Osmolality (mOsm . kg-1)
425
400
375
350
325
300
275
SWA to FW/FWA
SWA to SW (control)
a
b
b
b
b
b
0
3
7
14
21
FWA
Post-transfer (days)
(b)
Cl- concentrations (mmole . l-1)
200
175
150
125
100
SWA to FW/FWA
SWA to SW (control)
a
b
b
b
b
b
0
3
7
14
21
FWA
Post-transfer (days)

Fig. 6.

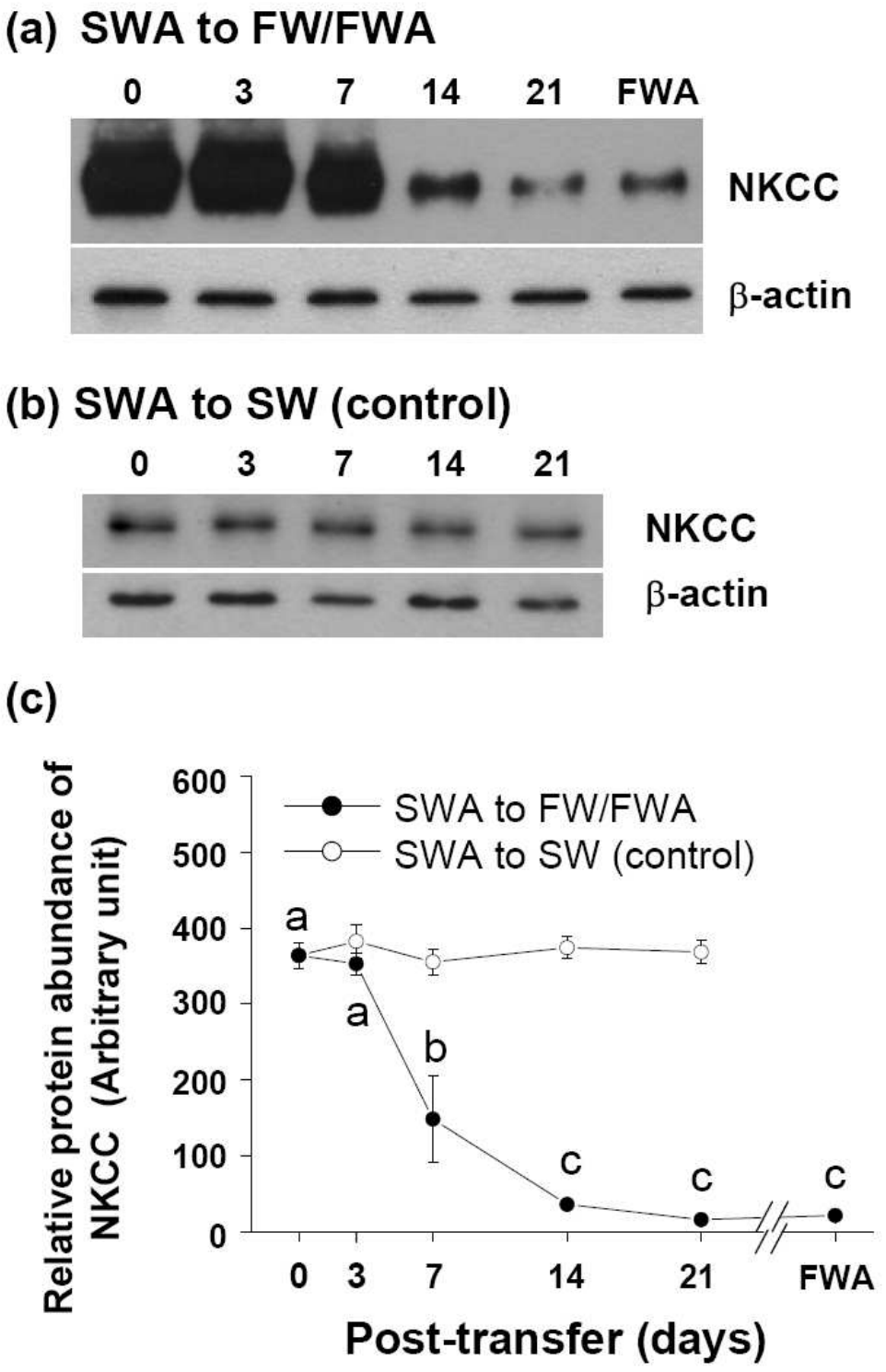

(a) SWA to FW/FWA
0
3
7
14
21
FWA
NKCC
β-actin
(b) SWA to SW (control)
0
3
7
14
21
NKCC
β-actin
(c)
Relative protein abundance of NKCC (Arbitrary unit)
600
500
400
300
200
100
0
SWA to FW/FWA
SWA to SW (control)
a
a
b
c
c
c
0
3
7
14
21
FWA
Post-transfer (days)

Fig. 7.

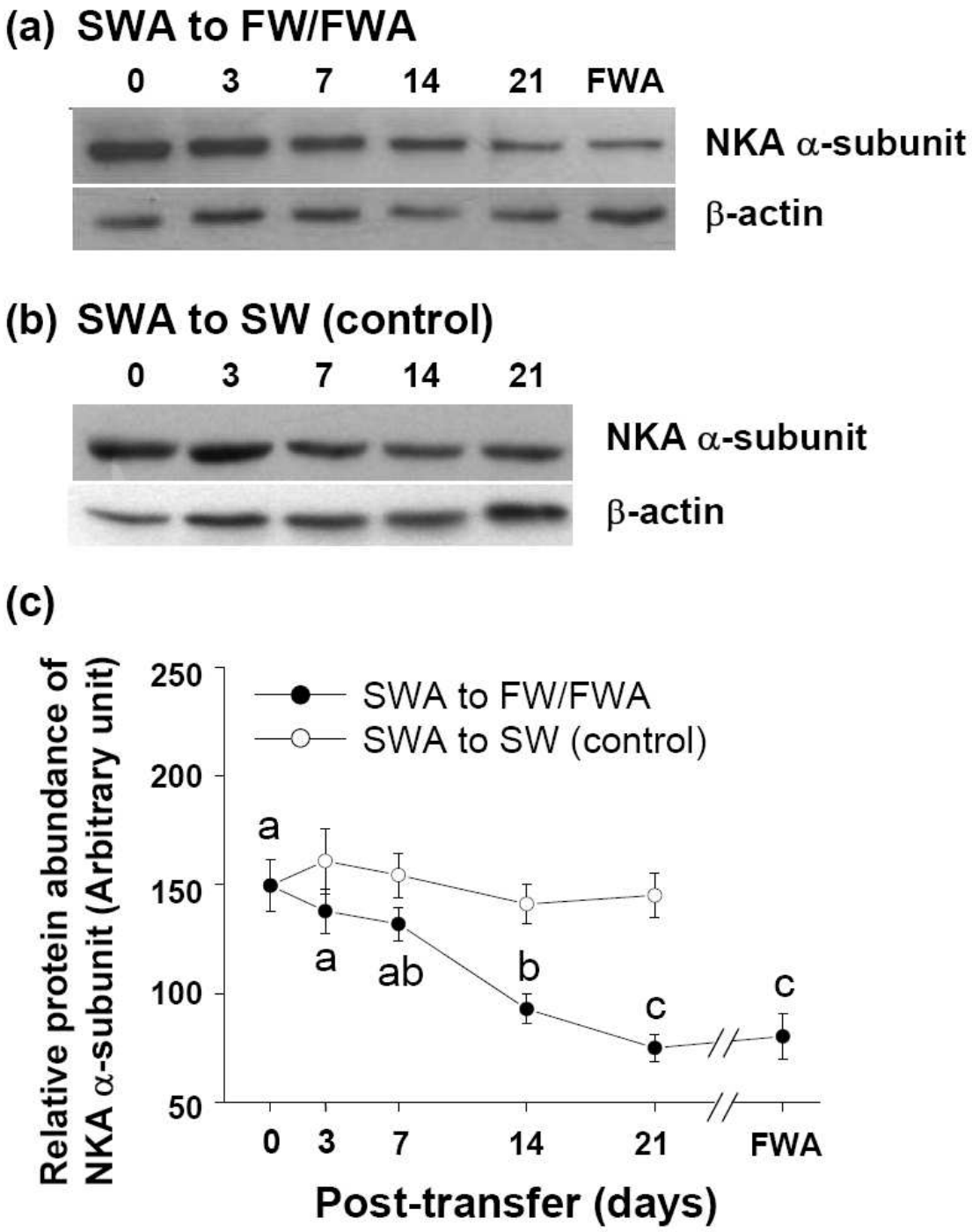

(a) SWA to FW/FWA
0
3
7
14
21
FWA
NKA α-subunit
β-actin
(b) SWA to SW (control)
0
3
7
14
21
NKA α-subunit
β-actin
(c)
Relative protein abundance of NKA α-subunit (Arbitrary unit)
250
200
150
100
50
SWA to FW/FWA
SWA to SW (control)
a
a
ab
b
c
c
0
3
7
14
21
FWA
Post-transfer (days)

Fig. 8.

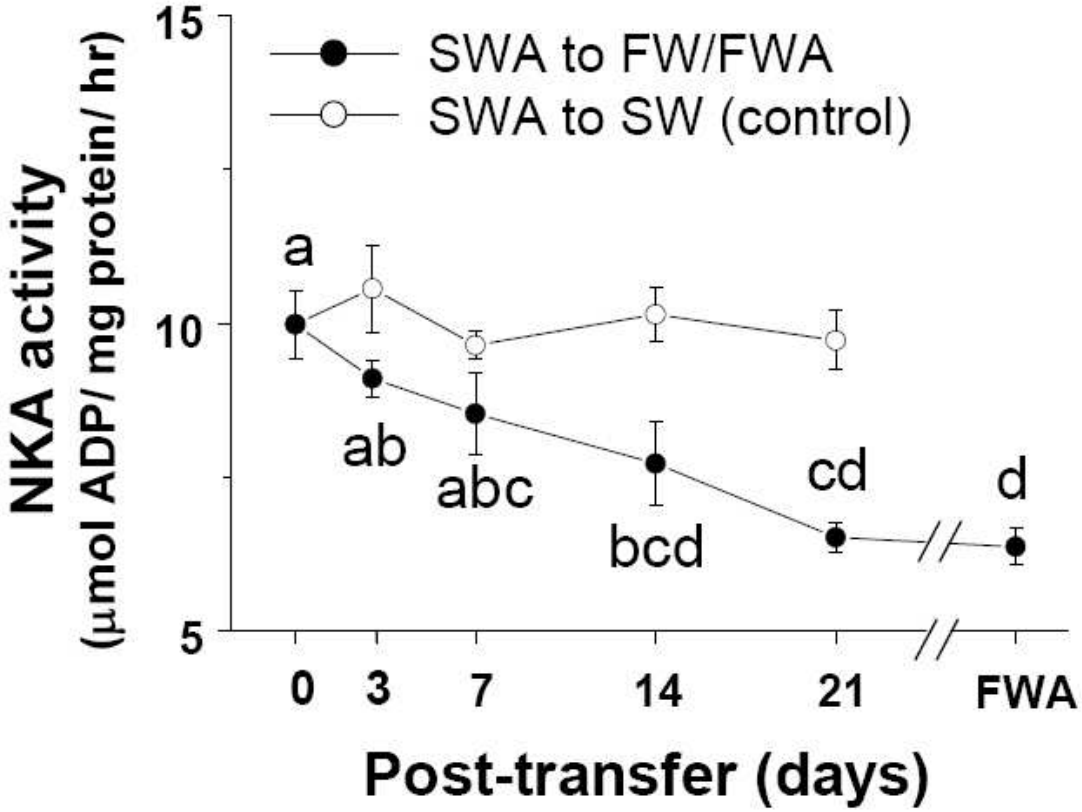

NKA activity
(μmol ADP/ mg protein/ hr)
15
10
5
SWA to FW/FWA
SWA to SW (control)
a
ab
abc
bcd
cd
d
0
3
7
14
21
FWA
Post-transfer (days)

Fig. 9.

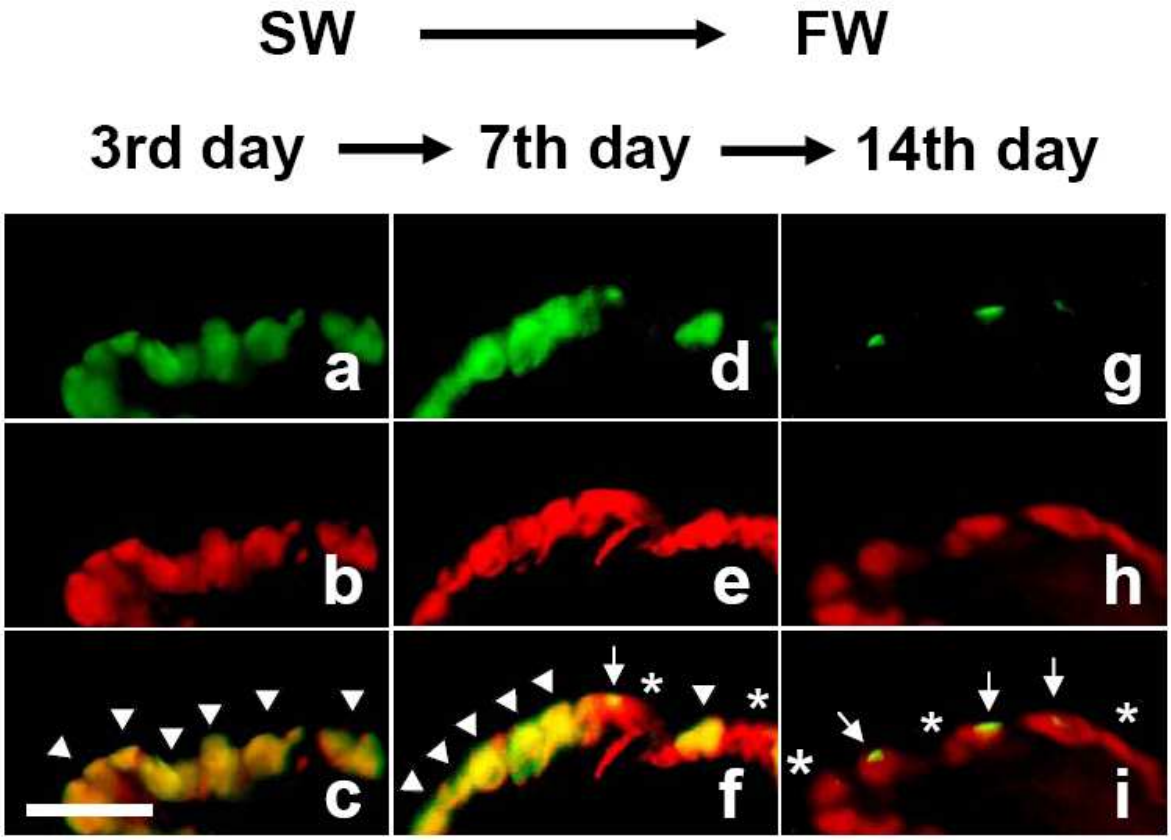
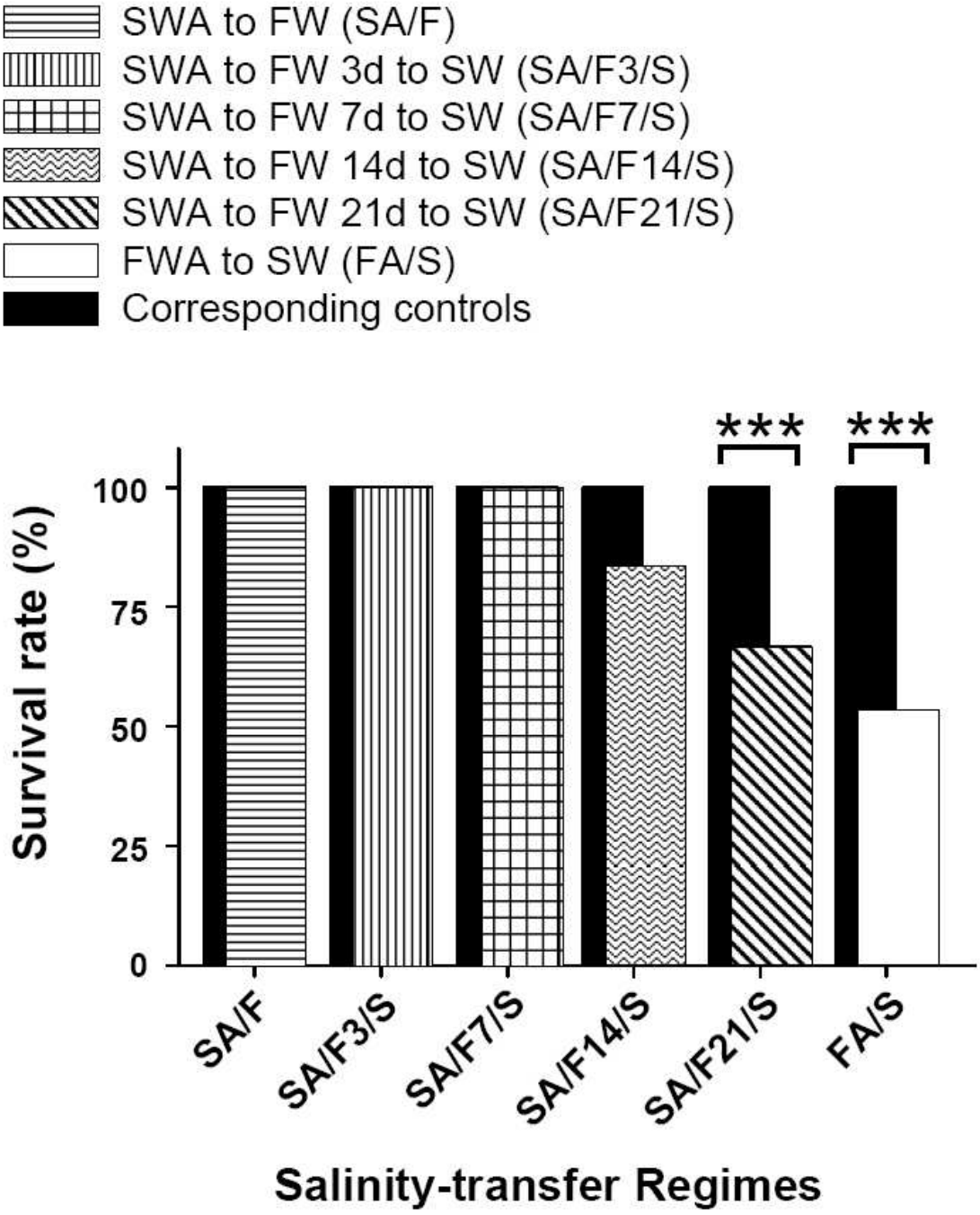


Fig. 10.


SWA to FW (SA/F)
SWA to FW 3d to SW (SA/F3/S)
SWA to FW 7d to SW (SA/F7/S)
SWA to FW 14d to SW (SA/F14/S)
SWA to FW 21d to SW (SA/F21/S)
FWA to SW (FA/S)
Corresponding controls
***
***
Survival rate (%)
100
75
50
25
0
SA/F
SA/F3/S
SA/F7/S
SA/F14/S
SA/F21/S
FA/S
Salinity-transfer Regimes