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ORIGINAL PAPER

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 7 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 correlation between the gradual reductions in expression of branchial NKCC and NKA (i.e., the hypoosmoregulatory endurance) and decreasing survival rates after hyperosmotic challenge in sailfin molly.

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

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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). 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 (Evans et al. 2005; Hwang and Lee 2007). 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; 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).

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), 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). 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 (defined as 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 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/branchial hypoosmoregulatory mechanism) when exposed to hypoosmotic environments (e.g., FW) that the mechanism of hypoosmoregulatory endurance (i.e., the retention of hypoosmoregulatory capability) will be clarify by a correlation between the gradual reductions of protein expression and decreasing survival rates upon hyperosmotic challenge (e.g., SW).

Although the respective profiles of gill NKCC, gill NKA, or salinity tolerance were reported in the previous studies, few studies focused simultaneously on the three indicators for osmoregulation. Moreover, we verified the correlation among them by the multi-transfer survival experiments. 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 the small tributary (nearby river mouths; salinity: 15–29‰) of Gaoping River, 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 (Osmolality: 3 mOsm · kg^{-1}), BW (473 mOsm · kg^{-1}), or SW (1010 mOsm · kg^{-1}) at 28 ± 1°C with a daily 14 h:10 h L:D photoperiod in the laboratory. The details of the water parameters were identical to our previous study (Kang et al. 2010). The water was continuously circulated through fabric-floss filters and partially renewed every 2 weeks. Dead fish were immediately removed from the experimental tanks to maintain the water quality. 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. The animal protocols and procedures followed the rules of the Institutional Animal Care and Use Committee of National Chung-Hsing University.

Experimental design

Acclimation experiments

Sailfin molly was acclimated to FW, BW, or SW for at least 4 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 one, 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.

FW-shower experiments

SW-acclimated fish were transferred directly to FW during different periods except long-term FW-acclimation (i.e., 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 of 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.

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 types of primary antibody/antiserum 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 and immunofluorescent staining (dilution 1:500 and 1:50, respectively); (2) NKA: a mouse monoclonal antibody (α5, Developmental Studies Hybridoma Bank) raised against the α-subunit of the avian NKA for immunoblotting (dilution 1:2,500); (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 1:5,000), as in previous studies on teleosts (Wang et al. 2008; Kang et al. 2010). The secondary antibodies were (1) horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (#31430, Pierce, Rockford, IL, USA; dilution 1:5,000) for immunoblotting; (2) Alexa-Fluor-488 conjugated goat anti-mouse IgG or Alexa-Fluor-546-conjugated goat anti-rabbit 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

The methods used were modified from the previous study (Kang et al. 2010). In brief, after fixation and infiltrated with optimal cutting temperature (OCT) compound (Sakura, Tissue-Tek, Torrance, CA, USA), the tissue was cryosectioned (5 μm thick) using a Cryostat Microtome (Microm HM 505E, Walldorf, Germany) at −25°C. Cryosections of the gills were incubated sequentially in blocking buffer, the polyclonal anti-NKA (#11), the related secondary antibody (Alexa-flour 546 goat anti-rabbit antibody), the monoclonal anti-NKCC (T4), and the related secondary antibody (Alexa-flour 488 goat anti-mouse antibody). Finally, immunostained sections were observed 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

The methods used were modified from previous studies (Kang et al. 2008, 2010). After homogenizing and centrifuging, the gill supernatants were used for protein concentration measurements, enzyme activity assays, or immunoblotting. Protein concentrations were determined with the BCA Protein Assay (Pierce) using BSA (bovine serum albumin; Pierce) as a standard. The supernatants were processed immediately for determination of NKA activity or stored at −80°C for immunoblotting.

Immunoblotting

The protocols used were modified from the previous studies (Yang et al. 2009; Kang et al. 2010). In brief, sample buffer was mixed with aliquots containing 20 or 25 μg of branchial supernatant for immunoblotting of branchial NKCC or NKA α-subunit, respectively. After electrophoresis and transformation, blots (PVDF membranes; Millipore, Bedford, MA, USA) were preincubated in PBST (PBS 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 or NKA α-subunit) and lower (for β-actin) sections prior to antibody incubation. The blots were incubated in the primary antibodies (T4, α5, or β-actin) diluted in 1% BSA and 0.05% sodium azide in PBST, followed by the incubation with HRP-conjugated secondary antibody diluted in PBST. Then, the blots were developed using the SuperSignal West Pico Detection Kit

(#34082, Pierce). The immunoblotting protocol for the negative control of NKCC, 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). 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 (Kang et al. 2008). 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 mean ± 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 from 130 to 170 kDa) compared with the results from negative controls (Fig. 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, f).

The survival rates for different salinity-transfer regimes

The survival rate was approximately 100% when FW-acclimated sailfin molly was 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 was transferred to FW (FA/F). When the SW-acclimated sailfin molly was transferred directly to FW, all the individuals survived (SA/F). In contrast, when the FW-acclimated sailfin molly was 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 was 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 7 days post-transfer (Fig. 6). NKA α-subunit protein abundance and NKA activity in the

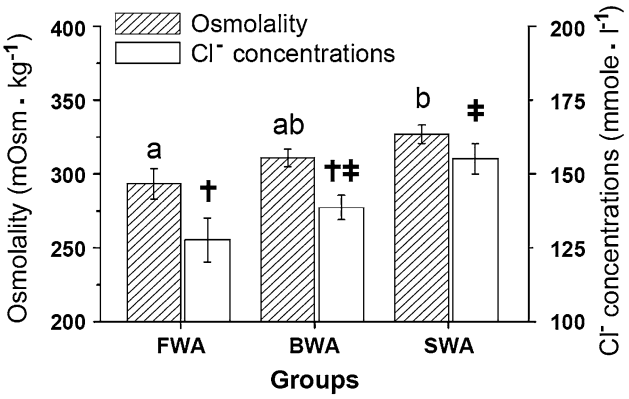

Fig. 1 The effect of salinity on plasma osmolality (*crosshatched bars*; modified from Yang et al. 2009) and Cl^- concentrations (*white bars*; $N = 16$) in the sailfin molly. *Dissimilar letters* or *symbols* indicate significant differences among groups (mean ± SEM, one way ANOVA with Tukey's comparison,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

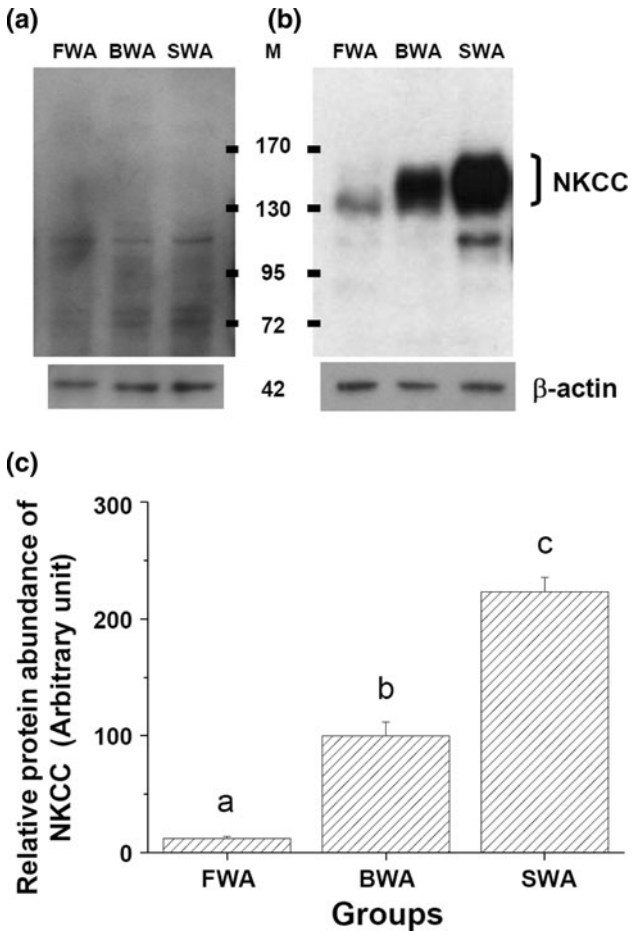

Fig. 2 Representative immunoblot and negative control of sailfin molly gills probed with **a** pre-immune serum of mouse and **b** a monoclonal antibody (T4) to NKCC, respectively. The immunoreactive bands indicate a molecular mass from 130 to 170 kDa, centered at 150 kDa (*bracket*). **c** The relative 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

gills were significantly decreased after 14 days post-transfer (Figs. 7, 8). The control group, SW-acclimated sailfin molly which was transferred to SW, showed no significant change in the above parameters at any time-points (Fig. 5, 6, 7, 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 day post transfer (Fig. 9c, f). In the 7-day-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 7- and 14-day-FW-shower fish (Fig. 9f, i).

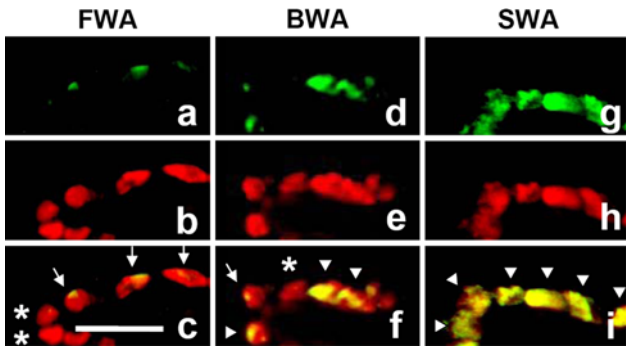

Fig. 3 Immunofluorescent staining of frozen cross-sections of the gill filaments of freshwater-acclimated sailfin molly (*FWA*; **a**–**c**), brackish water-acclimated sailfin molly (*BWA*; **d**–**f**), and seawater-acclimated sailfin molly (*SWA*; **g**–**i**). Gill filament cryosections were double stained with anti-NKCC antibody (*green*; **a**, **d**, **g**) and anti-NKA antibody (*red*; **b**, **e**, **h**). The merged images (**c**, **f**, **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

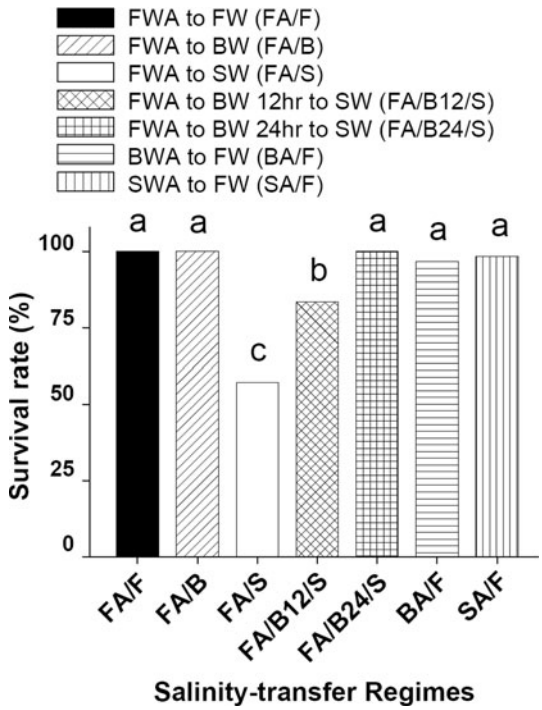

Fig. 4 The survival rate (%) of sailfin molly in different salinity-transfer regimes. *Dissimilar letters* indicate significant differences between two groups ($N = 30$–60, Chi-square test; *a* vs. *b* and *b* vs. *c*, $P < 0.05$; *a* vs. *c*, $P < 0.001$). *FWA* freshwater-acclimated sailfin molly,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

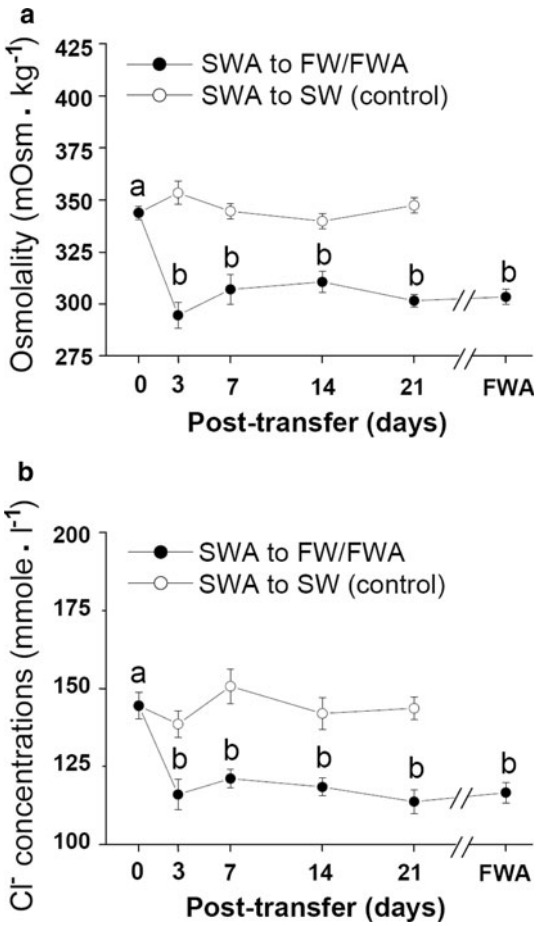

Fig. 5 The time course of changes in osmolality (**a**; $N = 5$) and [Cl^-] (**b**; $N = 8$) in the plasma of seawater-acclimated sailfin molly (*SWA*) transferred directly to seawater (*SW*; control group) or fresh water (*FW*; study group). *Dissimilar letters* indicate significant differences among 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

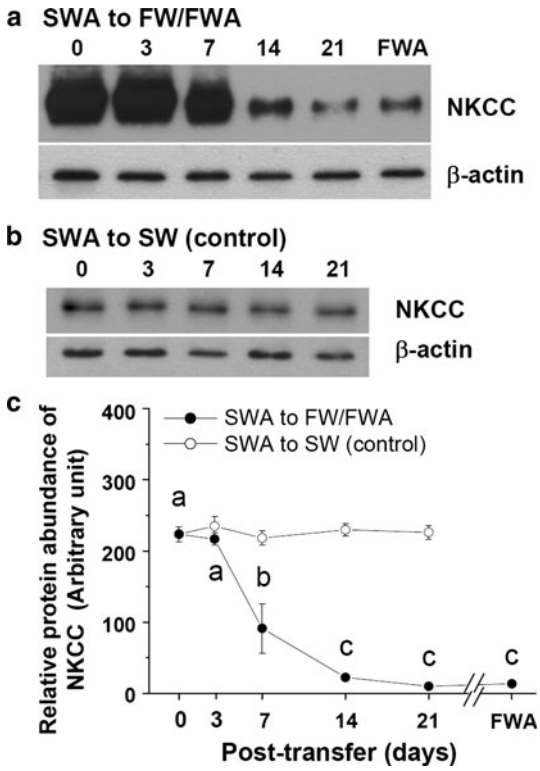

Fig. 6 The time course of changes in NKCC protein expression in the gills of seawater-acclimated sailfin molly (*SWA*) transferred directly to seawater (*SW*; control group) or fresh water (*FW*; study group) ($N = 5$). Representative immunoblots of branchial NKCC protein expression in the study group (**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

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

When SW-acclimated sailfin molly was 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-day-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.

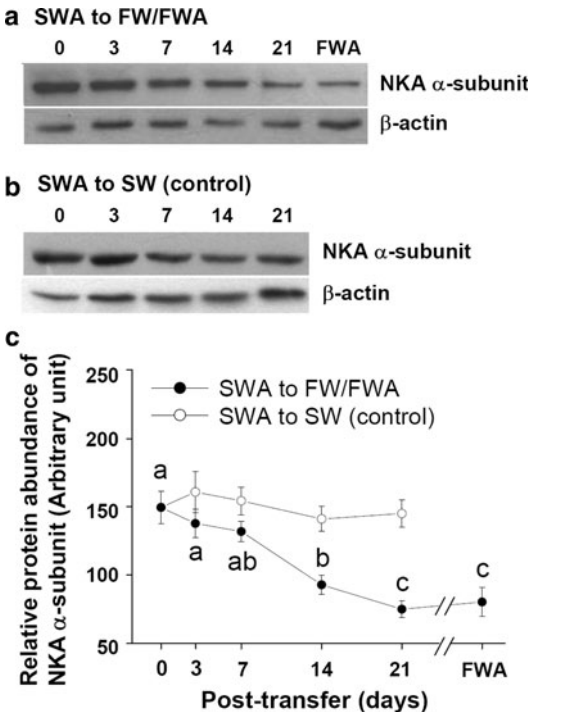

Fig. 7 The time course of changes in NKA protein expression in the gills of seawater-acclimated sailfin molly (*SWA*) transferred directly to seawater (*SW*; control group) or fresh water (*FW*; study group) ($N = 5$). Representative immunoblots of branchial NKA α-subunit protein expression in the study 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

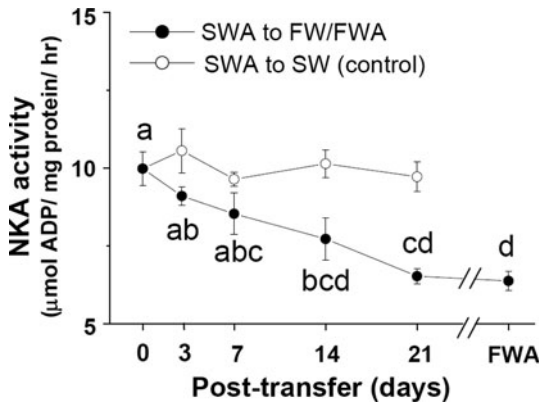

Fig. 8 Effects of direct transfer of seawater-acclimated sailfin molly (*SWA*) from seawater (*SW*) to fresh water (*FW*) (study group) and from SW to SW (control group) on gill NKA activity ($N = 5$). Significant reduction in branchial NKA activity occurred at the 14th day after transfer. No significant 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

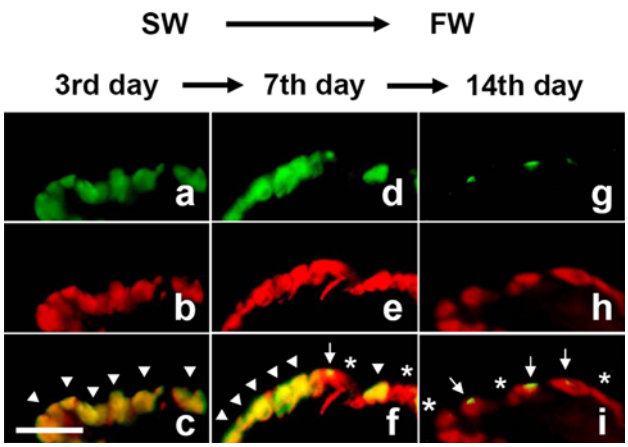

Fig. 9 Immunofluorescent staining of frozen cross-sections of the gill filaments of seawater-acclimated sailfin molly at the third (**a**–**c**), seventh (**d**–**f**), and 14th day (**g**–**i**) after direct transfer from seawater (*SW*) to fresh water (*FW*). Gill filaments were double stained with anti-NKCC (*green*; **a**, **d**, **g**) and anti-NKA (*red*; **b**, **e**, **h**) antibodies. The merged images (**c**, **f**, **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 7- and 14-day-FW-shower fish. *Scale bar* 25 μm

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 (Hirose et al. 2003; Evans 2008), and were similar to the studies of other SW-acclimated euryhaline teleosts. Furthermore, when transferred from SW to FW, by 7 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 (Fig. 2, 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 1 week (Tipsmark et al. 2008a, b; Kang et al. 2010).

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

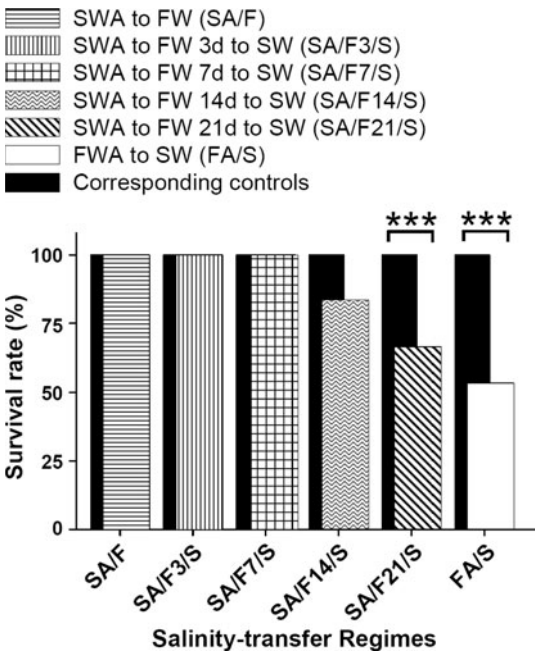

Fig. 10 The effects of different regimes of fresh water (*FW*) exposure on the survival rates (%) of seawater-acclimated sailfin molly (*SWA*) when they were transferred back to seawater (*SW*) directly after the FW-shower. Reduction in survival rate upon SW challenge was found in the 14- and 21-day-FW-shower 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

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, 7, 8). Compared to changes in the 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) was also reported in other euryhaline teleosts (although the experimental designs were different). 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 (within 3 days post-transfer) (Lin et al. 2004, 2006; Arjona et al. 2007). 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, 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 (Prodocimo and Freire 2006; Tse et al. 2006; Sardella and Kültz 2009); and (3) immunoreactivity in the apical region of MR cells (Wu et al. 2003; Katoh et al. 2008; 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). Except that the apical regions of the NKA-IR cells appeared in the other section, NKA-IR cells without NKCC immunoreactivity could be immature NKA-IR cells (Hiroi et al. 2005) or other types of MR cells that did 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 the 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; 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 by different cell types except that they were located in degenerating or non-functioning cells. 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, 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, 9). Changes in morphology, type, or localization of branchial MR cells or NKA-IR cells after abrupt hypoosmotic challenge were also reported in other euryhaline teleosts (Lin et al. 2004, 2006; Fielder et al. 2007). Among them, the BW-type of NKA-IR cells in Mozambique tilapia was found by 3 days after transfer from SW to FW (Hiroi et al. 2005, 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 was 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). Different survival rates found under 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 gradual reduced hypoosmoregulatory capability of SW-acclimated sailfin molly gills.

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, 7, 8, 9, 10). In other euryhaline teleost such as brackish medaka (*Oryzias dancena*; Kang et al. 2010), similar profiles of branchial NKCC expression and survival rates were reported. 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 capability of the sailfin molly (Figs. 6, 7, 8, 9, 10).

Although the hypoosmoregulatory mechanisms are complex, the branchial osmoregulatory mechanisms of the sailfin molly were crucial 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 3 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 was 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 1 day post-transfer) and then maintained these lower levels (Lin et al. 2004; 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 composite 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/indicators of branchial 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 illustrate that the sailfin molly is 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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