

## Short-term Effects of Hypertonic Shock on Na<sup>+</sup>, K<sup>+</sup>-ATPase Responses in Gills and Kidneys of the Spotted Green Pufferfish, *Tetraodon nigroviridis*

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**Chia-Hao Lin and Tsung-Han Lee (2016)** Freshwater (FW) spotted green pufferfish (*Tetraodon nigroviridis*) could be directly transferred to seawater (SW). This indicated that the spotted green pufferfish possesses an efficient osmoregulatory mechanism to overcome salinity challenges. Although previous studies explored osmoregulation in the spotted green pufferfish in FW and SW acclimation, to our knowledge, no study has addressed short-term time-course changes to elucidate the responsible mechanism. In the present study, spotted green pufferfish were transferred directly from FW to SW. We explored time-course changes in plasma osmolality, and Na<sup>+</sup> and Cl<sup>-</sup> concentrations, as well as Na<sup>+</sup>, K<sup>+</sup>-ATPase (NKA) responses (activity and protein expression) in gills and kidneys in a short-term (96 h) time-course experiment using the spotted green pufferfish. Plasma osmolality and Cl<sup>-</sup> levels were upregulated within 3 h post-transfer, and were constant thereafter. Plasma Na<sup>+</sup> concentration responded with a rapid increase 6 h post-transfer, and returned to the original level at 48 h post-transfer. Gills and kidneys are vital osmoregulatory organs, and NKA expressed in these organs provides the driving force for osmoregulation in euryhaline teleosts. Herein, both branchial and renal NKA responses were modulated soon after SW transfer. Branchial NKA responses including activity and protein abundance of  $\alpha$ -subunit were significantly stimulated at 3 h post-transfer; thenceforth were maintained in a steady state. In contrast, the activity and protein expression of renal NKA were downregulated at 3 and 12 h post-transfer, respectively. The physiological profiles found in this study illustrated how spotted green pufferfish cope with direct transferred from FW to SW.

**Key words:** Euryhaline teleost, Ionocyte, Na<sup>+</sup>, K<sup>+</sup>-ATPase, Osmoregulation, Pufferfish.

### BACKGROUND

Physiological and biochemical activity is affected by the osmolality of body fluid in vertebrates, and therefore, the maintenance of osmolality homeostasis is an important issue. Unlike terrestrial vertebrates, teleosts live in aquatic environments where their bodies are surrounded by water. Body fluids of teleosts are passively affected by external aquatic environments. The estuary teleosts continually experience the challenge of variable osmolalities and ion concentrations in their aquatic environment. Therefore, they must maintain the osmolality of their body fluids in the tolerable

range. Gills and kidneys are important organs in the osmoregulation in teleosts. The gill is exposed to the external environment and is thus the primary osmoregulatory organ in teleosts. Whether gills must cope with ion uptake or excretion depends on the external osmolality, such as hypotonic fresh water (FW) and hypertonic seawater (SW) (Takei et al. 2014). On the other hand, kidneys eliminate excess water in FW and excrete solutes in SW (Takei et al. 2014). Hence, the osmoregulatory action of the gill and kidney is flexible when facing a salinity challenge.

Na<sup>+</sup>, K<sup>+</sup>-ATPase (NKA) is a membrane-bound P-type ATPase consisting of  $\alpha$ -subunit and

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$\beta$ -subunits. The  $\alpha$ -subunit of NKA is a catalytic subunit that can bind and hydrolyze ATP. The  $\beta$ -subunit is a glycosylated and accessory subunit and thought to anchor and stabilize the  $\alpha$ -subunit in the cell membrane. NKA pumps 3 Na<sup>+</sup> out and 2 K<sup>+</sup> into the cell and provides an electrochemical gradient to drive other transport pathways in osmoregulatory organs (Blanco and Mercer 1998). Changes in NKA activity occur in the osmoregulatory organs of animals confronted with changes in environmental salinities (Marshall 2002; Burg et al. 2007). Ionocytes are major sites for osmoregulation in gills, and dominant signals of NKA can be detected in ionocytes (Hwang and Lee 2007). In teleost kidneys, NKA expression signals were predominantly identified in the collecting ducts, distal convoluted tubules, and proximal convoluted tubules. These segments may play a role in excretion and retention of water and solutes (Miyazaki et al. 2002; Lin et al. 2004a; Tang et al. 2010; Duffy et al. 2011). Changes in mRNA, protein, and activity of branchial NKA in euryhaline teleosts are thought to be vital responses for successful adaption to environmental salinity challenges in euryhaline teleosts (Hwang and Lee 2007). Several studies revealed that the mRNA, protein, and activity of NKA in the kidney were regulated in euryhaline teleosts during salinity adaption (Tang et al. 2010; Tang et al. 2012), and these modulations were related to osmoregulation. Different affinities of branchial NKA for Na<sup>+</sup> and K<sup>+</sup> were reported in FW- and SW-acclimated milkfish (*Chanos chanos*), tilapia (*Oreochromis mossambicus*), and pufferfish (*Tetraodon nigroviridis*) (Lin and Lee 2005). Such changes in the affinity of NKA may improve the osmoregulatory capacity of tilapia, pufferfish, and milkfish for acclimation to varied environmental salinities. Furthermore, mRNA expressions of FXYD proteins, the regulatory protein of NKA, could be detected in gills and kidneys of teleosts (Tipsmark 2008; Wang et al. 2008). Wang et al. (2008) determined that the salinity-dependent expression of branchial FXYD mRNA and protein, and branchial FXYD colocalized, and interacted with NKA in the spotted green pufferfish. The interaction between FXYD and NKA may regulate NKA activity in gills of euryhaline teleosts upon salinity challenge. (Wang et al. 2008)

Studies revealed that the short-term NKA responses in fish gills are critical for osmoregulation and survival following a salinity challenge. In milkfish, branchial NKA activity and expression was up-regulated within 3 h

during hypotonic acclimation and the fish could successfully adapt to FW from SW (Lin et al. 2006). SW tilapia also can successfully adapt to FW by downregulating the protein abundance and activity of NKA within 12 and 3 h, respectively (Lin et al. 2004b). In contrast, when FW tilapia was directly transferred from FW to SW, they died within 5 h (Wang et al. 2009). The FW tilapia was not able to modulate NKA expression and activity following the abrupt SW challenge, which eventually resulted in the disturbance of plasma osmolality (up to 530 mOsm.kg<sup>-1</sup>) (Wang et al. 2009). Therefore, short-term regulation in branchial NKA responses is vital for salinity adaption of euryhaline teleosts. In addition, renal NKA responses have been shown to be related to salinity adaption (see above), but short-term NKA responses are still poorly understood in euryhaline teleosts.

The spotted green pufferfish is a euryhaline teleost and natively inhabits estuaries of Southeast Asia (Rainboth 1996). In estuaries, the salinity is not constant, and hence pufferfish face the risk of abrupt salinity changes. In our previous study, the spotted green pufferfish was treated with FW, BW (Brackish water, 15‰), and SW (35‰) for two weeks, respectively. The NKA responses (*i.e.*, activity and protein abundance of  $\alpha$ -subunit) were highest in the gills of SW-acclimated pufferfish. Differently, the NKA responses were highest in the kidney of FW-acclimated pufferfish (Lin et al. 2004a). Differences in NKA responses between gills and kidneys reflected physiological demands for salinity adaption of the pufferfish. With excellent osmoregulatory ability, the green spotted pufferfish can be directly transferred from FW to SW, or vice versa. Nevertheless, no study has explored the short-term effect of salinity challenges on NKA responses in gills and kidneys of the spotted green pufferfish. Therefore, we investigated the short-term effect of hypertonic shock on adaptive responses in gills and kidneys of the spotted green pufferfish. The plasma osmolality, Na<sup>+</sup> and Cl<sup>-</sup> levels, and NKA responses (protein expression and activity) were explored. This information will extend our knowledge of the short-term osmoregulatory mechanisms in euryhaline teleosts.

## MATERIALS AND METHODS

### Fish and experimental environments

Green spotted pufferfish were obtained from

a local aquarium. They were  $6.2 \pm 0.5$  cm in total length. SW ( $\text{Na}^+$ , 582.86 mM;  $\text{K}^+$ , 10.74 mM;  $\text{Ca}^{2+}$ , 15.75 mM;  $\text{Mg}^{2+}$ , 32.92 mM;  $\text{Cl}^-$ , 520.84 mM) used in the present study consisted of local tap water and the addition of the proper amounts of the synthetic sea salt, Instant Ocean (Aquarium Systems Co., USA). The green spotted pufferfish were reared in FW ( $\text{Na}^+$ , 2.6 mM;  $\text{K}^+$ , 0.04 mM;  $\text{Ca}^{2+}$ , 0.58 mM;  $\text{Mg}^{2+}$ , 0.16 mM;  $\text{Cl}^-$ , 0.18 mM) at  $27 \pm 1^\circ\text{C}$  with a daily photoperiod having 12 h of light for at least two weeks before the experiments. The water was continuously circulated through fabric-floss filters. Fish were fed a daily diet of commercial, sterilized-sludge worm.

### Seawater acclimation

Because the green spotted pufferfish reared in FW could be directly transferred and acclimated to SW (Lin et al. 2004a), we sampled pufferfish raised in FW after direct transfer to SW at 0, 3, 6, 12, 24, 48, and 96 h. Fish in the control group were moved from FW to FW and sampled with a time-course identical to the transfer group. Tissues and blood were sampled for biochemical analyses. Fish were anesthetized with ethylene glycol monophenyl ether (0.3 ml.l<sup>-1</sup>; Merck) before sampling.

### Plasma analyses

Collecting blood from caudal veins of green spotted pufferfish is difficult and unstable because of the small size of the fish. Therefore, instead of collecting blood from the caudal veins, gill arteries were dissected and blood was collected by a heparinized capillary (1 mm in diameter). After centrifugation at 13,000 g for 5 min, the plasma was stored at  $4^\circ\text{C}$  until analyses. Plasma osmolality was assessed with a WESCOR 5520 VAPRO osmometer (Logan, Utah, USA).  $\text{Na}^+$  was measured with a Hitachi Z-8000 polarized Zeeman atomic absorption spectrophotometer (Tokyo, Japan).  $\text{Cl}^-$  was evaluated using the ferricyanide method (Franson 1985). Photometric analysis was carried out using a Hitachi U-2001 spectrophotometer (Tokyo, Japan).

### $\text{Na}^+/\text{K}^+$ -ATPase (NKA) antibody

A mouse monoclonal antibody ( $\alpha 5$ ) against the  $\alpha$ -subunit of the avian NKA (Takeyasu et al. 1988) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA) and

used in the present study.

### Immunoblotting

Tissue scrapings were suspended in a mixture of homogenization medium with proteinase inhibitor (100:1). Homogenization was performed with a motorized Teflon pestle at 600 rpm for 30 s. The homogenate was then centrifuged at 13,000 g,  $4^\circ\text{C}$  for 20 min. The supernatants were used for determination of protein and immunoblotting. The immunoblotting was performed by referring to Lin et al. (2004a). Briefly, aliquots containing 100  $\mu\text{g}$  of tissue homogenates ( $\sim 10 \mu\text{g}/\mu\text{l}$ ) and pre-stained molecular weight standards (Invitrogen) were heated at  $100^\circ\text{C}$  for 5 min and fractionated by electrophoresis on SDS-containing 7.5% polyacrylamide gels. Separated proteins were transferred from unstained gels to PVDF (PolyScreen, NEN) using a tank transfer system (Bio-Rad, Mini Protean 3). Blots were preincubated for 1 h in PBST buffer (137 mM NaCl, 3 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM  $\text{KH}_2\text{PO}_4$ , 0.2% (v/v) Tween 20, pH 7.4) containing 5% (w/v) nonfat dried milk to minimize non-specific binding, and then incubated at  $4^\circ\text{C}$  with primary antibody ( $\alpha 5$ ) diluted in PBST (1:5,000) overnight. The blot was washed in PBST, followed by a 1-h incubation with AP-conjugated secondary antibody (Jackson) diluted 2,500x in PBST. Blots were visualized after incubation with a NBT/BCIP kit (Chemecon). Immunoblots were scanned, images were imported in TIFF format into a commercial software package (Kodak Digital Science 1D, 1995), and results were converted to numerical values to compare the relative intensities of the immunoreactive bands.

### Specific Activity of NKA

Gill and kidney NKA activity was determined as described by Hwang et al. (1989). Aliquots of the suspension of gill and kidney homogenates, prepared as described above, were used for determination of protein and enzyme activities. NKA activity was assayed by adding the supernatant to the reaction mixture (100 mM imidazole-HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, 7.5 mM  $\text{MgCl}_2$ , 5 mM  $\text{Na}_2\text{ATP}$ ). The reaction was run at  $37^\circ\text{C}$  for 30 min and then stopped by addition of 200  $\mu\text{l}$  of ice-cold 30% trichloroacetic acid. The inorganic phosphate concentration was measured according to Peterson's method (1978). The enzyme activity of NKA was defined as the difference between the inorganic phosphates liberated in the presence

and absence of 3.75 mM ouabain in the reaction mixture. Each sample was assayed in triplicate.

**Statistical analysis**

Values were compared using a one-way analysis of variance (ANOVA) followed by Dunnett's pair-wise method. The significance level was  $P < 0.05$ . Values were expressed as mean  $\pm$  S.E.M (standard error of the mean).

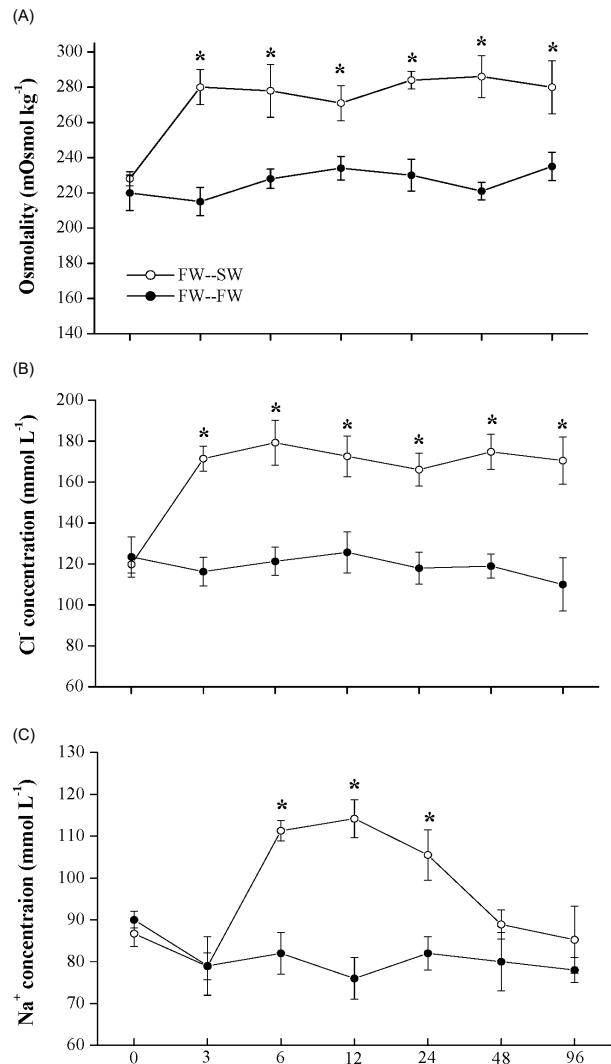
**RESULTS**

**Plasma analysis**

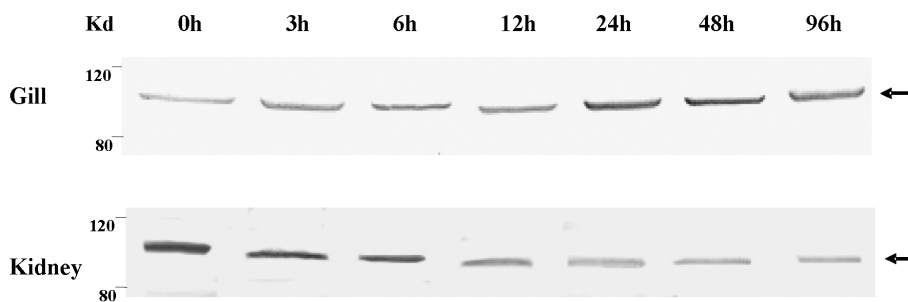
Significant increases of plasma osmolality and  $\text{Cl}^-$  were found in the experimental group 3 h after transfer and onwards (Figs. 1A and B). Compared to initial data, plasma  $\text{Na}^+$  increased to a level significantly higher at 6 h post-transfer, and returned to the initial level by 48 h post-transfer (Fig. 1C). No change in plasma osmolality,  $\text{Na}^+$  or  $\text{Cl}^-$  was found for fish in the control group (Figs. 1A-C).

**$\text{Na}^+/\text{K}^+$ -ATPase (NKA) responses**

In green spotted pufferfish transferred directly from FW to SW, hypertonic shock induced significantly higher responses of gill NKA. Relative protein abundance of the NKA  $\alpha$ -subunit increased significantly (approximately 1.5-2 fold) within 3 h (Figs. 2 and 3A), and a 2-3 fold elevation of NKA activity was found at the same time (Fig. 3B). In contrast, expression of kidney NKA in pufferfish transferred directly from FW to SW exhibited the opposite pattern. In the experimental group, relative abundance of NKA  $\alpha$ -protein declined significantly within 12 h post-transfer, and became



**Fig. 1.** Time-course changes of plasma osmolality,  $\text{Cl}^-$ , and  $\text{Na}^+$  in the green spotted pufferfish transferred directly from FW to SW. The asterisks indicate a significant difference ( $P < 0.05$ ) using Dunnett's multiple-comparison test following a one-way ANOVA. Values are means  $\pm$  SEM ( $n = 5$ ). Significant increase in osmolality and  $\text{Cl}^-$  occurred within 3 h, and in  $\text{Na}^+$ , within 6 h post-transfer.



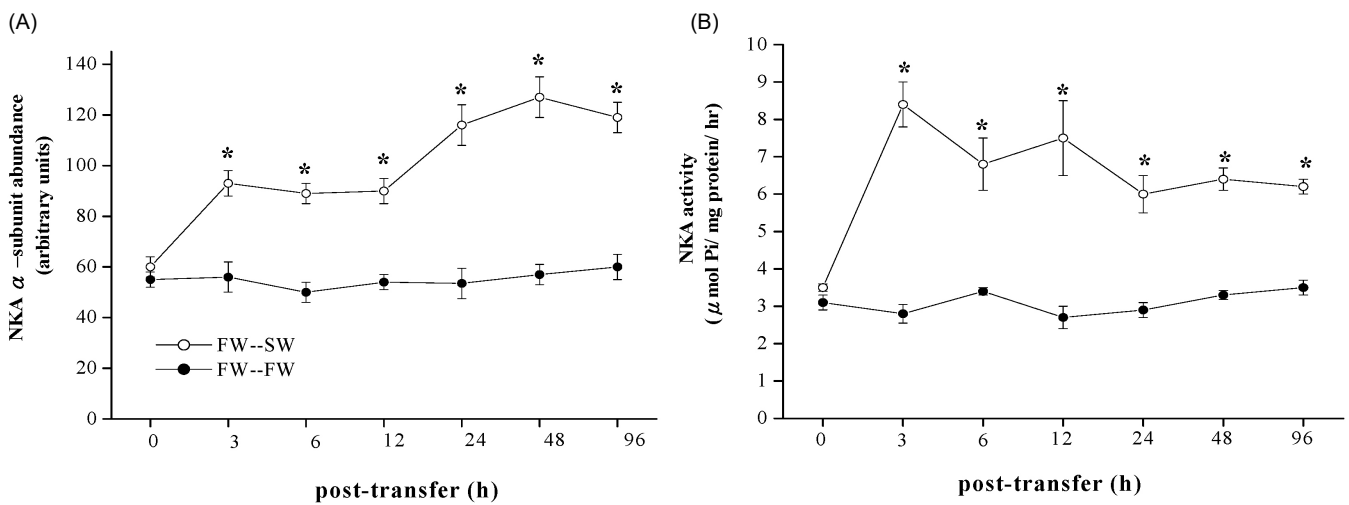
**Fig. 2.** Representative immunoblots of the  $\text{Na}^+/\text{K}^+$ -ATPase (NKA)  $\alpha$ -subunit of the green spotted pufferfish gill and kidney sampled during a time-course following transfer from FW to SW. Single immunoreactive bands were observed with  $\alpha 5$  monoclonal antibody corresponding to a molecular mass of approximately 100 kDa. Arrows indicate immunoreactive bands at approximately 100 kDa.

almost half of the initial value in FW 24 h post-transfer (Figs. 2 and 4A). Kidney NKA activity dropped significantly 3 h after transfer, and reached a level of approximately one half the level in the control group at 12 h following transfer and onwards (Fig. 4B).

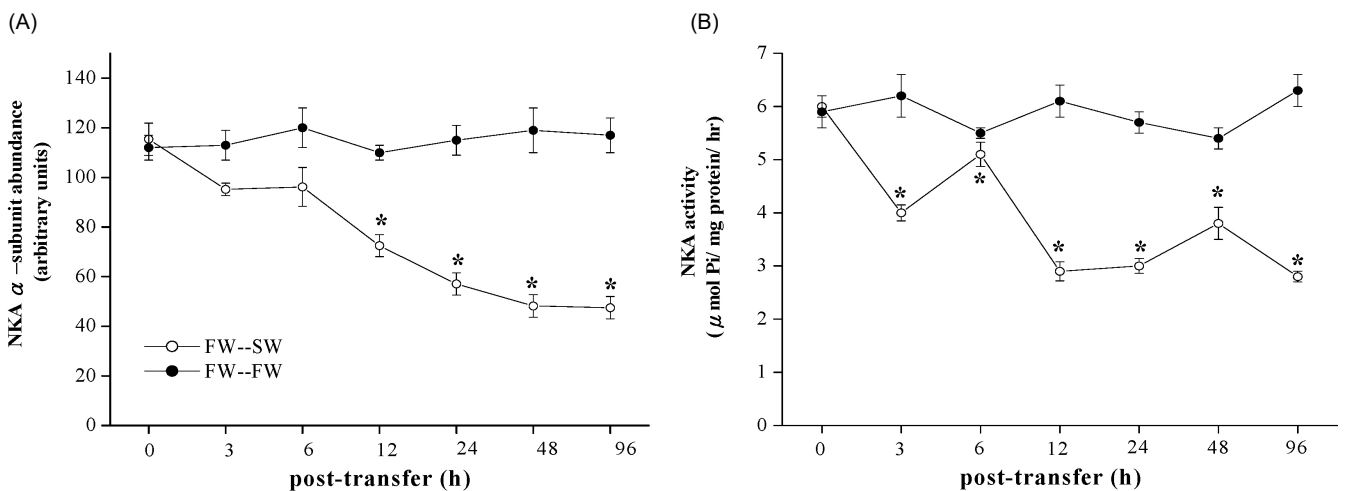
SW. During SW acclimation, the pufferfish faces the risk of dehydration and ion load. Burg et al. (2007) explained that cells had several responses, such as decreases in cell volume, DNA breaks, protein oxidation, and short-term cell cycle arrest following exposure to hypertonic stress. Furthermore, severe hypertonicity causes changes in cell volume and leads to irreversible harm or death of the organ and the nervous system (Ayus et al. 1996; Stiefel and Petzold 2007). We found that plasma osmolality of pufferfish was upregulated approximately from 220 to 280 mOsmol kg<sup>-1</sup> within

### DISCUSSION

The green spotted pufferfish is a euryhaline teleost that can be directly transferred from FW to



**Fig. 3.** Effects of direct transfer from FW to SW on protein abundance (A) and activity (B) of Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) in gill epithelia of the green spotted pufferfish. The asterisks indicate a significant difference ( $P < 0.05$ ) using Dunnett's multiple-comparison test following a one-way ANOVA. Values are means  $\pm$  SEM ( $n = 5$ ). NKA protein and activity increase significantly within 3 h post-transfer.



**Fig. 4.** Effects of direct transfer from FW to SW on protein abundance (A) and activity (B) of Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) in kidneys of the green spotted pufferfish. The asterisks indicate significant differences ( $P < 0.05$ ) using Dunnett's multiple-comparison test following a one-way ANOVA. Values are means  $\pm$  SEM ( $n = 5$ ). NKA protein decreases gradually within 6 h post-transfer and significantly decreases after 12 h post-transfer. NKA activity decreases significantly within 3 h post-transfer returns to the initial level at 6 h, and decreases significantly after 12 h post-transfer.

3 h, after levels were kept in a stable range. Similar responses were also observed in other euryhaline teleosts when exposed to higher salinities. The osmolality of SW milkfish dropped from ~340 to 310 mOsmol kg<sup>-1</sup> within 3 h after transfer to FW. Thereafter, osmolality was maintained in a stable range (Lin et al. 2006). SW tilapia can also modulate plasma osmolality soon after FW transfer (Lin et al. 2004b). However, when FW tilapia were directly transferred to SW, osmolality was seriously disturbed (approximately from 300 to 530 mOsmol kg<sup>-1</sup>) so that the homeostasis of fish could not be maintained. Finally, tilapia died at 5 h after SW transfer (Wang et al. 2009). Hence, the maintenance of osmolality during hyper- or hypotonic acclimation is critical for the euryhaline teleosts.

In the present study, we found that plasma Na<sup>+</sup> and Cl<sup>-</sup> concentrations of the spotted green pufferfish were significantly elevated within 6 and 3 h, respectively, after SW transfer. There was no difference in plasma Na<sup>+</sup> levels between long-term SW- and FW-acclimated pufferfish. On the other hand, plasma Cl<sup>-</sup> levels were lower in FW- than SW-acclimated pufferfish (about 110 vs. 150 mM on average; Lin et al. 2004a). The plasma Cl<sup>-</sup> concentrations of pufferfish were significantly elevated within 3 h after transfer to SW. Meanwhile, Na<sup>+</sup> levels were elevated after 6 h and then restored to initial (FW) level after 24 h. These results revealed that SW transfer resulted in raises of the plasma Na<sup>+</sup> and Cl<sup>-</sup> concentration of FW pufferfish quickly. Na<sup>+</sup> and Cl<sup>-</sup> are the major electrolytes of osmolality in body fluid (Terry 1994). Therefore, the trend in changes in plasma osmolality corresponded to the trend in changes in plasma Na<sup>+</sup> and Cl<sup>-</sup> concentrations in the present study. In tilapia and milkfish, the time-course changes of plasma osmolality were also tightly correlated to the alterations of Na<sup>+</sup> and Cl<sup>-</sup> levels during SW or FW acclimation (Lin et al. 2004b; Lin et al. 2006; Wang et al. 2009).

The changes in NKA protein abundance and activity in gills and kidneys were thought to be critical for euryhaline teleosts during salinity acclimation (Hwang and Lee 2007; Tang et al. 2010; Tang et al. 2012). In the present study, we found that the branchial NKA activity of the spotted green pufferfish was stimulated at 3 h following transfer to SW and then maintained at a constant value until 96 h. There are many euryhaline teleosts with their natural habitats in FW that also exhibit higher branchial NKA activity in SW (Lee et al. 2000; Uchida et al. 2000;

Marsigliante et al. 2000; Bystriansky et al. 2006; Nilsen et al. 2007; Kang et al. 2008; Yang et al. 2009). Increased NKA activity was thought to enhance the ability of ion excretion in SW. The NKA creates a transmembrane Na<sup>+</sup> gradient by pumping Na<sup>+</sup> out of the cell. This Na<sup>+</sup> gradient drives the basolateral Na<sup>+</sup>, K<sup>+</sup>, 2Cl<sup>-</sup> cotransporter (NKCC) in ionocytes, and facilitates Cl<sup>-</sup> transport into ionocytes. Thereafter, apical chloride channel (CFTR) in ionocytes emits Cl<sup>-</sup> (Hwang et al. 2011). In ionocytes of SW spotted green pufferfish, NKCC and CFTR were demonstrated to localize in basolateral and apical membranes, respectively, by immunocytochemistry (Tang et al. 2011). However, no transcellular transporter was proposed to remove Na<sup>+</sup> from ionocytes in SW. It was suggested that NKA pumps Na<sup>+</sup> across the basolateral membrane of ionocytes, and then Na<sup>+</sup> flows out through paracellular pathways between the ionocytes and adjacent accessory cells (Marshall 2002; Evans et al. 2005; Bagherie-Lachidan et al. 2008). The trend of NKA  $\alpha$ -subunit protein abundance in this study was similar to NKA activity during SW acclimation. NKA  $\alpha$ -subunit pumps sodium out and potassium into the cell by hydrolyzing ATP (Blanco and Mercer 1998). Previous studies showed that the higher NKA activity accompanied an increase in the abundance of NKA  $\alpha$ -subunit protein in gills of euryhaline teleosts, e.g., tilapia (Lee et al. 2000), Atlantic salmon (*Salmo salar*) (D'Cotta et al. 2000), milkfish (Lin et al. 2006) and medaka (*Oryzias latipes*) (Kang et al. 2008). Thus, the increased protein expression of NKA in the spotted green pufferfish may contribute to the increase in NKA activity. In the present result, plasma Na<sup>+</sup> concentration restored to the initial level at 48 h after SW transfer. Branchial NKA expression and activity were stimulated after SW transfer. In addition, the action of NKA is active transport and pumps 3Na<sup>+</sup> out. NKA was suggested to excrete Na<sup>+</sup> from ionocytes through paracellular pathways (Marshall 2002; Evans et al. 2005; Bagherie-Lachidan et al. 2008; Shen et al. 2011). Hence, the lasting upregulation of branchial NKA expression and activity may contribute to restore the Na<sup>+</sup> level after transfer for 48 h. In contrast, plasma Cl<sup>-</sup> level was still high until 96h. In SW-acclimated green spotted pufferfish, apical CFTR was suggested to emit Cl<sup>-</sup> and CFTR protein expression was stimulated (Tang and Lee 2007). Therefore, increased CFTR expression may be able to maintain the Cl<sup>-</sup> level in a stable range. Nevertheless, the NKA protein expression was stronger than CFTR in SW-

acclimated spotted green pufferfish (Tang and Lee 2007). Besides, active transport of NKA is more efficient than CFTR. These differences may lead to inconsistent change in plasma  $\text{Na}^+$  and  $\text{Cl}^-$  level after SW transfer. Nevertheless, further investigation is necessary in future.

Our results revealed that renal NKA responses (activity and protein) of the pufferfish decreased after 3 and 12 h, respectively. Thereafter, NKA responses were maintained in a stable state. Our data agreed with Lin et al. (2004a) who reported that renal NKA protein and activity were higher in FW- and SW-acclimated pufferfish than in BW-acclimated individuals. Moreover, significant increases in renal NKA activity were reported in other FW-acclimated euryhaline species, such as mummichog killifish (*Fundulus heteroclitus*) (Epstein et al. 1969), thicklip gray mullet (*Chelon labrosus*) (Lasserre 1971; Gallis and Bourdichon 1976), European sea bass (*Dicentrarchus labrax*) (Nebel et al. 2005), thinlip mullet (*Liza ramada*) (Gallis and Bourdichon 1976), striped bass (*Morone saxatilis*) (Madsen et al. 1994), black seabream (*Spondyliosoma cantharus*) (Kelly et al. 1999), starred sturgeon (*Acipenser stellatus*) (Krayushkina et al. 2006), milkfish (Tang et al. 2010), and Japanese eel (*Anguilla japonica*) (Tang et al. 2012). In FW, teleosts face the risk of water load and ion loss because of hypotonic environments. Kidneys of teleosts perform the excretion of excess water and reabsorption of solutes in FW. Hence, 95% of the filtered water is excreted to produce diluted urine in the kidneys of FW teleosts (Miyazaki et al. 2002). Depending on results of immunohistochemistry, NKA and the chloride channel (omCIC-K) had a similar distribution in the distal nephron segment of tilapia (Miyazaki et al. 2002). In addition, the transepithelial ion transport in the lumen of the distal tubule with NKCC antagonist treatment was dominantly inhibited in FW trout (Miyazaki et al. 2002; Nishimura et al. 1983). Overall, it was suggested that NKA provided the driving force for the chloride channel and NKCC to execute  $\text{Cl}^-$  reabsorption in the kidney of FW teleosts (Miyazaki et al. 2002). On the other hand, the renal NKA activity was also lower in SW- than in FW-acclimated pufferfish (*Tetraodon biocellatus*). The decline in NKA-rich distal tubules in SW-acclimated fish may have resulted in the downregulation of NKA activity (Duffy et al. 2011). According to these studies, the decrease in renal NKA activity in SW-acclimated pufferfish might reflect the role of NKA in ion reabsorption in the present study. Herein, the NKA protein expression

of kidneys was also downregulated after SW acclimation. However, decreased NKA protein expression was observed within 12 h, later than downregulation of NKA activity (within 3 h). In gills of the Atlantic cod, European eel and striped bass, NKA activity increased prior to protein expression of the catalytic subunit. This suggested that NKA activity was modulated by phosphorylation via protein kinase A (PKA) and cAMP (Crombie et al. 1996, Marsigliante et al. 1997; Tipsmark and Madsen 2001; Tipsmark et al. 2004). In the present study, protein properties of renal NKA might also be modified by PKA and cAMP within 12 h after SW acclimation, and increased renal NKA expression was devoted to the enhancement of NKA activity after 12 h.

There are 3 isoforms ( $\alpha 1$ -3) of NKA  $\alpha$ -subunit in fish, and the tissue expression pattern of these isoforms was different (Serluca et al. 2001; Feng et al. 2002; Richards et al. 2003; Gharbi et al. 2005; Armesto et al. 2014).  $\alpha 1$  is the most dominant isoform in kidneys and/or gills, and its expression salinity-dependent (Feng et al. 2002; Richards et al. 2003; McCormick et al. 2009; Armesto et al. 2014). Liao et al. (2009) further indicated there were 5 paralogues of  $\alpha 1$  subunit specifically expressed in different subtypes of ionocytes in stenohaline zebrafish, and these paralogues may be related to different ionregulation. In euryhaline teleost, two  $\alpha 1$  paralogues ( $\alpha 1a$  and  $\alpha 1b$ ) were identified in gills and/or ionocytes (Richards et al. 2003, Mackie et al. 2005, Bystriansky et al. 2006, Madsen et al. 2009, McCormick et al. 2009; Tipsmark et al. 2011; Urbina et al. 2013). Furthermore, FW and SW treatment differentially induced branchial  $\alpha 1a$  and  $\alpha 1b$  expression in euryhaline teleosts (Richards et al. 2003, Mackie et al. 2005, Bystriansky et al. 2006, Madsen et al. 2009, McCormick et al. 2009; Tipsmark et al. 2011; Urbina et al. 2013).  $\alpha 1a$  and  $\alpha 1b$  are thought as a FW and SW subtype of  $\alpha 1$  in euryhaline teleosts, respectively. In the present study, the results of NKA  $\alpha$ -subunit protein expression and activity reflected the sum of total NKA. Further studies are required to elucidate whether the switch of  $\alpha 1$  paralogous expression are involved in NKA protein expression and activity between FW- and SW-acclimated spotted green pufferfish in future.

## CONCLUSIONS

Plasma osmolality,  $\text{Na}^+$  and  $\text{Cl}^-$  levels in the spotted green pufferfish changed rapidly and were

maintained efficiently at stable levels following transfer into SW. One of the major regulatory mechanisms for the modulation of NKA activity and protein expression occurred in the osmoregulatory organs, the gills and kidneys, over a short time period (within 3 h or 12 h, respectively) following transfer to SW. The changes in NKA protein expression and activity provided the driving force for secondary transporters to maintain the homeostasis of osmolality, as well as Na<sup>+</sup> and Cl<sup>-</sup> concentrations. Our results illustrated how the green spotted pufferfish can counteract the abrupt hypertonic challenge in estuaries.

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