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Short-term Effects of Hypertonic Shock on Na^+, K^+-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^+ and Cl^- concentrations, as well as Na^+, K^+-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^- levels were upregulated within 3 h post-transfer, and were constant thereafter. Plasma Na^+ 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 α-subunit were significantly stimulated at 3 h post-transfer; thenceforth were maintained in at 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^+, K^+-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^+, K^+-ATPase (NKA) is a membrane-bound P-type ATPase consisting of α-subunit and

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β-subunits. The α-subunit of NKA is a catalytic subunit that can bind and hydrolyze ATP. The β-subunit is a glycosylated and accessory subunit and thought to anchor and stabilize the α-subunit in the cell membrane. NKA pumps 3 Na^+ out and 2 K^+ 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^+ and K^+ 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 $mOsm.kg^{-1}$) (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 α-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^+ and Cl^- 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 ± 0.5 cm in total length. SW (Na^+, 582.86 mM; K^+, 10.74 mM; Ca^{2+}, 15.75 mM; Mg^{2+}, 32.92 mM; 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 (Na^+, 2.6 mM; K^+, 0.04 mM; Ca^{2+}, 0.58 mM; Mg^{2+}, 0.16 mM; Cl^-, 0.18 mM) at 27 ± 1°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 $ml.l^{-1}$; 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°C until analyses. Plasma osmolality was assessed with a WESCOR 5520 VAPRO osmometer (Logan, Utah, USA). Na^+ was measured with a Hitachi Z-8000 polarized Zeeman atomic absorption spectrophotometer (Tokyo, Japan). Cl^- was evaluated using the ferricyanide method (Franson 1985). Photometric analysis was carried out using a Hitachi U-2001 spectrophotometer (Tokyo, Japan).

Na^+/K^+-ATPase (NKA) antibody

A mouse monoclonal antibody (α5) against the α-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°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 μg of tissue homogenates (~10 μg/μl) and pre-stained molecular weight standards (Invitrogen) were heated at 100°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 Na_2HPO_4, 2 mM KH_2PO_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°C with primary antibody (α5) 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 $MgCl_2$, 5 mM Na_2ATP). The reaction was run at 37°C for 30 min and then stopped by addition of 200 μl 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 ± S.E.M (standard error of the mean).

RESULTS

Plasma analysis

Significant increases of plasma osmolality and Cl^- were found in the experimental group 3 h after transfer and onwards (Figs. 1A and B). Compared to initial data, plasma 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, Na^+ or Cl^- was found for fish in the control group (Figs. 1A-C).

Na^+/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 α-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 α-protein declined significantly within 12 h post-transfer, and became

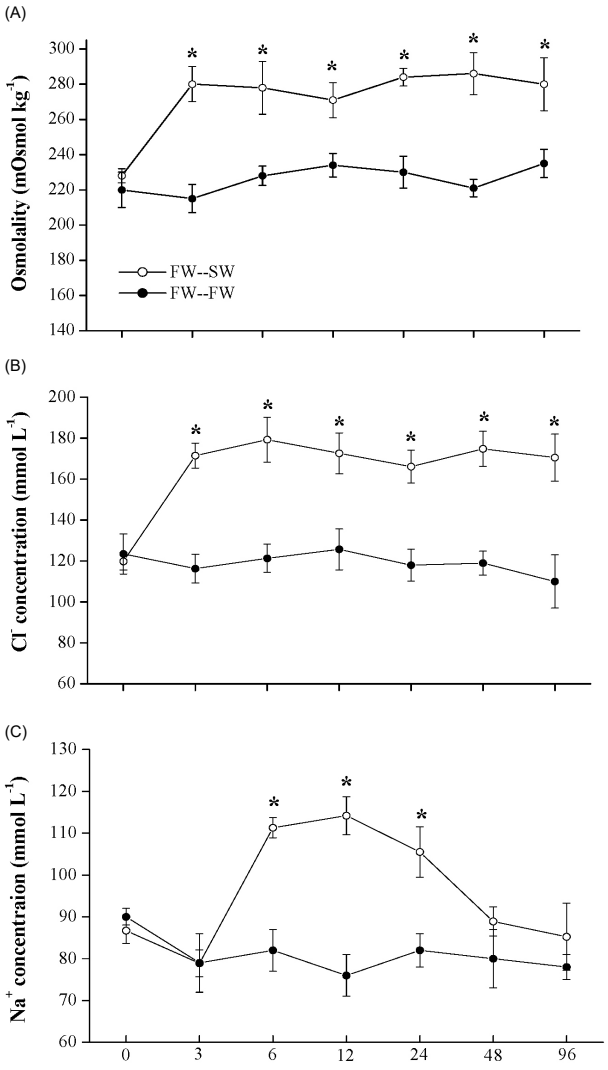

Fig. 1. Time-course changes of plasma osmolality, Cl^-, and Na^+ in the green spotted pufferfish transferred directly from FW to SW. The asterisks indicate a significant difference ($P < 0.05$) using Dunnett's multiple-comparison test following a one-way ANOVA. Values are means ± SEM ($n = 5$). Significant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

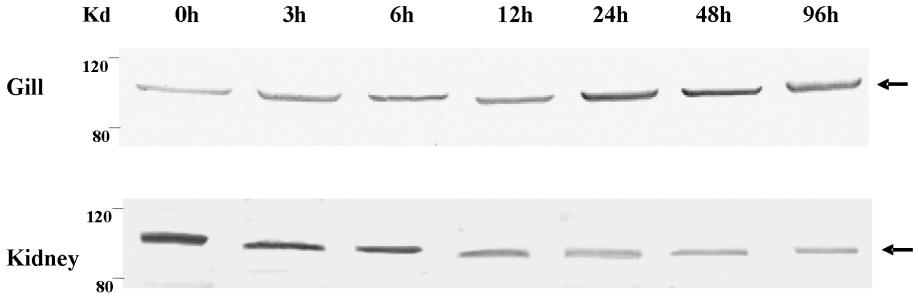

Fig. 2. Representative immunoblots of the Na^+/K^+-ATPase (NKA) α-subunit of the green spotted pufferfish gill and kidney sampled during a time-course following transfer from FW to SW. Single immunoreactive bands were observed with α5 monoclonal antibody corresponding to a molecular mass of approximately 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).

DISCUSSION

The green spotted pufferfish is a euryhaline teleost that can be directly transferred from FW to 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^{-1} within

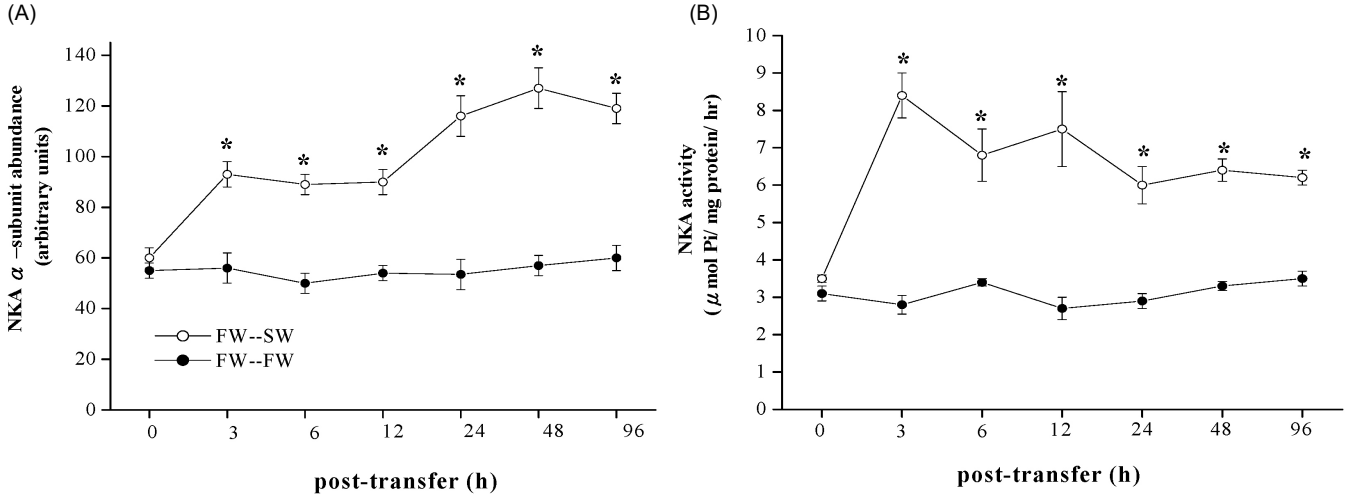

Fig. 3. Effects of direct transfer from FW to SW on protein abundance (A) and activity (B) of Na^+/K^+-ATPase (NKA) in gill epithelia of the green spotted pufferfish. The asterisks indicate a significant difference ($P < 0.05$) using Dunnett's multiple-comparison test following a one-way ANOVA. Values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

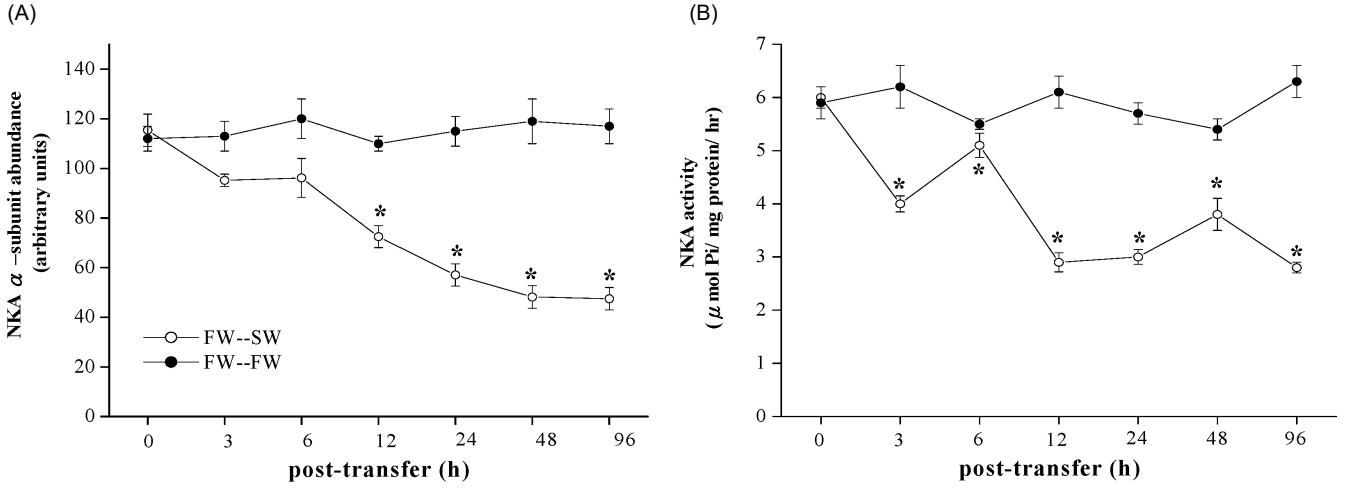

Fig. 4. Effects of direct transfer from FW to SW on protein abundance (A) and activity (B) of Na^+/K^+-ATPase (NKA) in kidneys of the green spotted pufferfish. The asterisks indicate significant differences ($P < 0.05$) using Dunnett's multiple-comparison test following a one-way ANOVA. Values are means ± 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^{-1} 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^{-1}) 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 euryhalineteleosts.

In the present study, we found that plasma Na^+ and Cl^- 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^+ levels between long-term SW- and FW-acclimated pufferfish. On the other hand, plasma Cl^- levels were lower in FW- than SW-acclimated pufferfish (about 110 vs. 150 mM on average; Lin et al. 2004a). The plasma Cl^- concentrations of pufferfish were significantly elevated within 3 h after transfer to SW. Meanwhile, Na^+ 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^+ and Cl^- concentration of FW pufferfish quickly. Na^+ and Cl^- 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^+ and Cl^- 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^+ and Cl^- 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^+ gradient by pumping Na^+ out of the cell. This Na^+ gradient drives the basolateral Na^+, K^+, $2Cl^-$ cotransporter (NKCC) in ionocytes, and facilitates Cl^- transport into ionocytes. Thereafter, apical chloride channel (CFTR) in ionocytes emits Cl^- (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^+ from ionocytes in SW. It was suggested that NKA pumps Na^+ across the basolateral membrane of ionocytes, and then Na^+ 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 α-subunit protein abundance in this study was similar to NKA activity during SW acclimation. NKA α-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 α-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^+ 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 $3Na^+$ out. NKA was suggested to excrete Na^+ 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^+ level after transfer for 48 h. In contrast, plasma Cl^- level was still high until 96h. In SW-acclimated green spotted pufferfish, apical CFTR was suggested to emit Cl^- and CFTR protein expression was stimulated (Tang and Lee 2007). Therefore, increased CFTR expression may be able to maintain the Cl^- 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 Na^+ and 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 (omClC-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 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 (α1-3) of NKA α-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). α1 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 α1 subunit specifically expressed in different subtypes of ionocytes in stenohaline zebrafish, and these paralogues may be related to different ionregulation. In euryhaine teleost, two α1 paralogues (α1a and α1b) 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 α1a and α1b 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). α1a and α1b are thought as a FW and SW subtype of α1 in euryhaline teleosts, respectively. In the present study, the results of NKA α-subunit protein expression and activity reflected the sum of total NKA. Further studies are required to elucidate whether the switch of α1 paralogous expression are involved in NKA protein expression and activity between FW- and SW-acclimated spotted green pufferfish in future.

CONCLUSIONS

Plasma osmolality, Na^+ and 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^+ and Cl^- concentrations. Our results illustrated how the green spotted pufferfish can counteract the abrupt hypertonic challenge in estuaries.

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REFERENCES

Armesto P, Campinho MA, Rodríguez-Rúa A, Cousin X, Power DM, Manchado M, Infante C. 2014. Molecular characterization and transcriptional regulation of the Na^+/K^+ ATPase α subunit isoforms during development and salinity challenge in a teleost fish, the Senegalese sole (*Solea senegalensis*). Comp Biochem Physiol B Biochem Mol Biol **175:**23-38. doi:10.1016/j.cbpb.2014.06.004.

Ayus JC, Armstrong DL, Arieff AI. 1996. Effects of hypernatraemia in the central nervous system and its therapy in rats and rabbits. J Physiol **492:**243-255.

Bagherie-Lachidan M, Wright SI, Kelly SP. 2008. Claudin-3 tight junction proteins in *Tetraodon nigroviridis*: cloning, tissue-specific expression, and a role in hydromineral balance. Am J Physiol Regul Integr Comp Physiol **294(5):**R1638-47. doi:10.1152/ajpregu.00039.2008.

Blanco G, Mercer RW. 1998. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. Am J Physiol **275:**F633-F650.

Burg MB, Ferraris JD, Dmitrieva NI. 2007. Cellular response to hyperosmotic stresses. Physiol Rev **87:**1441-74.

Bystriansky JS, Richards JG, Schulte PM, Ballantyne JS. 2006. Reciprocal expression of gill Na^+/K^+-ATPase alpha-subunit isoforms α1a and α1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. J Exp Biol **209:**1848-58.

Crombi HJ, Bell MV, Tytler P. 1996. Inhibition of sodium-plus-potassium stimulated adenosine triphosphatase (Na^+-K^+-ATPasae) by protein kinase C activators in the gills of Atlantic cod (*Gadus morhua*). Comp Biochem Physiol B **113:**765-772.

D'Cotta H, Valotaire C, Le Gac F, Prunet P. 2000. Synthesis of gill Na^+-K^+-ATPase in Atlantic salmon smolts: differences in α-mRNA and α-protein levels. Am J Physiol **278:**R101-R110.

Duffy NM, Bui P, Bagherie-Lachidan M, Kelly SP. 2011. Epithelial remodeling and claudin mRNA abundance in the gill and kidney of puffer fish (*Tetraodon biocellatus*) acclimated to altered environmental ion levels. J Comp Physiol B **181(2):**219-38. doi:10.1007/s00360-010-0517-3.

Epstein FH, Manitius A, Weinstein E, Katz AI, Pickford GE. 1969. Sodium- and potassium-activated adenosine triphosphatase in kidneys of *Fundulus heteroclitus* adapted to fresh and salt water. Yale J Biol Med **41:**388-393.

Evans DH, Piermarini PM, Choe KP. 2005. The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol Rev **85:**97-177.

Feng SH, Leu JH, Yang CH, Fang MJ, Huang CJ, Hwang PP. 2002. Gene expression of Na^+-K^+-ATPase α 1 and α 3 subunits in gills of the teleost *Oreochromis mossambicu*s, adapted to different environmental salinities. Mar Biotechnol (NY) **4(4):**379-91.

Franson MAH. 1985. Standard Methods for the Examination of Water and Waste Water, 16th ed. American Public Health Association, Washington, DC.

Gallis JL, Bourdichon M. 1976. Changes of (Na^+-K^+) dependent ATPase activity in gills and kidneys of two mullets *Chelon labrosus* (Risso) and *Liza ramada* (Risso) during fresh water adaptation. Biochimie **58:**625-627.

Gharbi K, Ferguson MM, Danzmann RG. 2005. Characterization of Na, K-ATPase genes in Atlantic salmon (*Salmo salar*) and comparative genomic organization with rainbow trout (*Oncorhynchus mykiss*). Mol Genet Genomics **273:**474-483.

Hwang PP, Sun CM, Wu SM. 1989. Changes of plasma osmolality, chloride concentration and gill Na-K-ATPase activity in tilapia *Oreochromis mossambicus* during seawater acclimation. Mar Biol **100:**295-299.

Hwang PP, Lee TH. 2007. New insights into fish ion regulation and mitochondrionrich cells. Comp Biochem Physiol A **148:**479-497.

Hwang PP, Lee TH, Lin LY. 2011. Ion regulation in fish gills: Recent progress in the cellular and molecular mechanisms. Am J Physiol Regul Integr Comp Physiol **301:**R28-R47. doi:10.1152/ajpregu.00047.2011.

Kang CK, Tsai SC, Lee TH, Hwang PP. 2008. Differential expression of branchial Na^+/K^+-ATPase of two medaka species, *Oryzias latipes* and *Oryzias dancena*, with different salinity tolerances acclimated to fresh water, brackish water and seawater. Comp Biochem Physiol A Mol Integr Physiol **151(4):**566-575. doi:10.1016/j.cbpa.2008.07.020.

Kelly SP, Chow INK, Woo NYS. 1999. Haloplasticity of black seabream (*Mylio macrocephalus*): hypersaline to freshwater acclimation. J Exp Zool **283:**226-241.

Krayushkina LS, Semenova OG, Vyushina AV. 2006. Level of serum cortisol and Na^+/K^+ ATP-ase activity of gills and kidneys in different acipenserids. J Appl Ichthyol **22:**182-187.

Lasserre P. 1971. Increase of Na^+, K^+-dependent ATPase activity in gills and kidneys of two euryhaline marine teleost, *Crenimugil labrosus* (Risso, 1826) and *Dicentrarchus labrax* (Linnaeus, 1758), during adaptation to fresh water. Life Sci **10:**113-119.

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

Liao BK, Chen RD, Hwang PP. 2009. Expression regulation of Na^+-K^+-ATPase α1-subunit subtypes in zebrafish gill

ionocytes. Am J Physiol Regul Integr Comp Physiol **296:**R1897-R1906. doi:10.1152/ajpregu.00029.2009.

Lin CH, Hwang CL, Yang CH, Lee TH, Hwang PP. 2004b. Time course changes in the expression of Na, K-ATPase and morphometry of mitochondrion-rich cells in gills of euryhaline tilapia (*Oreochromis mossambicus*) during freshwater acclimation. J Exp Zool **301A:**85-96.

Lin CH, Lee TH. 2005. Sodium or potassium ions activate different kinetics of gill Na, K-ATPase in three seawater- and freshwater-acclimated euryhaline teleosts. J Exp Zool **303A:**57-65.

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

Lin YM, Chen CN, Yoshinaga T, Tsai SC, Shen ID, Lee TH. 2006. Short-term effects of hyposmotic shock on Na^+/K^+-ATPase expression in gills of the euryhaline milkfish, *Chanos chanos*. Comp Biochem Physiol A **143:**406-415.

Mackie P, Wright PA, Glebe BD, Ballantyne JS. 2005. Osmoregulation and gene expression of Na^+/K^+-ATPase in families of Atlantic salmon (*Salmo salar*) smolts. Can J Fish Aquat Sci **62:**2661-2672.

Madsen S, McCormick S, Young G, Endersen J, Nishioka R, Bern H. 1994. Physiology of seawater acclimation in the striped bass, *Morone saxatilis* (Walbaum). Fish Physiol Biochem **13:**1-11.

Madsen SS, Kiilerich P, Tipsmark CK. 2009. Multiplicity of expression of Na^+/K^+-ATPase α-subunit isoforms in the gill of Atlantic salmon (*Salmo salar*): cellular localisation and absolute quantification in response to salinity change. J Exp Biol **212:**78-88. doi:10.1242/jeb.024612.

Marshall WS. 2002. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. J Exp Zool **293:**264-283.

Marsigliante S, Muscella A, Vinson GP, Storelli C. 1997. Angiotensin II receptors in the gill of seawater- and freshwater-adapted eel. J Mol Endocrinol **18:**67-76.

Marsigliante S, Muscella A, Vilella S, Storelli C. 2000. Dexamethasone modulates the activity of the eel branchial Na^+/K^+ ATPase in both chloride and pavement cells. Life Sci **66:**1663-1673.

McCormick SD, Regish AM, Christensen AK. 2009. Distinct freshwater and seawater isoforms of Na^+/K^+-ATPase in gill chloride cells of Atlantic salmon. J Exp Biol **212:**3994-4001. doi:10.1242/jeb.037275.

Miyazaki H, Kaneko T, Uchida S, Sasaki S, Takei Y. 2002. Kidney specific chloride channel, OmClC-K, predominantly expressed in the diluting segment of freshwater-adapted tilapia kidney. Proc Natl Acad Sci USA **99:**15782-15787.

Nebel C, Romestand B, Negre-Sadargues G, Grousset E, Aujoulat F, Bacal J, Bonhomme F, Charmantier G. 2005. Differential freshwater adaptation in juvenile sea-bass *Dicentrarchus labrax*: involvement of gills and urinary system. J Exp Biol **208:**3859-3871.

Nilsen TO, Ebbesson LOE, Madsen SS, McCormick SD, Andresson E, Björnsson BT, Prunet P, Stefansson SO. 2007. Differential expression of gill Na^+, K^+- ATPase α- and β-subunits, Na^+,K^+,$2Cl^-$ cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. J Exp Biol **210:**2885-2896.

Nishimura H, Imai M, Ogawa M. 1983. Sodium chloride and water transport in the renal distal tubule of the rainbow trout. Am J Physiol **244(3):**F247-54.

Peterson GL. 1978. A simplified method for analysis of inorganic phosphate in the presence of interfering substances. Anal Biochem **84:**164-172.

Rainboth WJ. 1996. Fishes of the Cambodian Mekong. FAO Species Identification Field Guide for Fishery Purposes. FAO, Rome, 265 pp.

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

Serluca FC, Sidow A, Mably JD, Fishman MC. 2001. Partitioning of tissue expression accompanies multiple duplications of the Na^+/K^+ ATPase α subunit gene. Genome Res **11:**1625-1631.

Shen WP, Horng JL, Lin LY. 2011. Functional plasticity of mitochondrion-rich cells in the skin of euryhaline medaka larvae (*Oryzias latipes*) subjected to salinity changes. Am J Physiol Regul Integr Comp Physiol **300(4):**R858-68. doi:10.1152/ajpregu.00705.2010.

Stiefel D, Petzold A . 2007. H_2O coma. Neurocrit Care **6:**67-71.

Takei Y, Hiroi J, Takahashi H, Sakamoto T. 2014. Diverse mechanisms for body fluid regulation in teleost fishes. Am J Physiol RegulIntegr Comp Physiol **307(7):**R778-92. doi:10.1152/ajpregu.00104.2014.

Takeyasu K, Tamkum MM, Renaud KJ, Fambrough DM. 1988. Ouabain-sensitive (Na^+, K^+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the α-subunit of an avian sodium pump. J Biol Chem **263:**4347-4354.

Tang CH, Lee TH. 2007. The effect of environmental salinity on the protein expression of Na^+/K^+-ATPase, Na^+/K^+/$2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator, anion exchanger 1, and chloride channel 3 in gills of a euryhaline teleost, *Tetraodon nigroviridis*. Comp Biochem Physiol A Mol Integr Physiol **147(2):**521-8.

Tang CH, Wu WY, Tsai SC, Yoshinaga T, Lee TH. 2010. Elevated Na^+/K^+-ATPase responses and its potential role in triggering ion reabsorption in kidneys for homeostasis of marine euryhaline milkfish (*Chanos chanos*) when acclimated to hypotonic fresh water. J Comp Physiol B **180(6):**813-24. doi:10.1007/s00360-010-0458-x.

Tang CH, Hwang LY, Shen ID, Chiu YH, Lee TH. 2011. Immuno-localization of chloride transporters to gill epithelia of euryhalineteleosts with opposite salinity-induced Na^+/K^+-ATPase responses. Fish Physiol Biochem **37(4):**709-24. doi:10.1007/s10695-011-9471-6.

Tang CH, Lai DY, Lee TH. 2012. Effects of salinity acclimation on Na^+/K^+-ATPase responses and FXYD11 expression in the gills and kidneys of the Japanese eel (*Anguilla japonica*). Comp Biochem Physiol A Mol Integr Physiol **163(3-4):**302-10. doi:10.1016/j.cbpa.2012.07.017.

Terry J. 1994. The major electrolytes: sodium, potassium, and chloride. J Intraven Nurs **17(5):**240-7.

Tipsmark CK, Madsen SS. 2001. Rapid modulation of Na^+/K^+-ATPase activity in osmoregulatory tissues of a salmonid fish. J Exp Biol **204:**701-709.

Tipsmark CK, Madsen SS, Borski RJ. 2004. Effect of salinity on expression of branchial ion transporters in striped bass (*Morone saxatilis*). J Exp Zool **301A:**979-991.

Tipsmark CK. 2008. Identification of FXYD protein genes in a teleost: tissue-specific expression and response to salinity change. Am J Physiol Regul Integr Comp Physiol **294(4):**R1367-78. doi:10.1152/ajpregu.00454.2007.

Tipsmark CK, Breves JP, Seale AP, Lerner DT, Hirano T, Grau

EG. 2011. Switching of Na^+,K^+-ATPase isoforms by salinity and prolactin in the gill of a cichlid fish. J Endocrinol **209(2):**237-44. doi:10.1530/JOE-10-0495.

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

Urbina MA, Schulte PM, Bystriansky JS, Glover CN. 2013. Differential expression of Na^+/K^+-ATPase α1 isoforms during seawater acclimation in the amphidromous galaxiid fish *Galaxias maculatus*. J Comp Physiol B **183(3):**345-57. doi:10.1007/s00360-012-0719-y.

Wang PJ, Lin CH, Hwang HH, Lee TH. 2008. Branchial FXYD protein expression in response to salinity change and its interaction with Na^+/K^+-ATPase of the euryhaline teleost *Tetraodon nigroviridis*. J Exp Biol **211:**3750-8. doi:10.1242/jeb.018440.

Wang PJ, Lin CH, Hwang LY, Huang CL, Lee TH, Hwang PP. 2009. Differential responses in gills of euryhaline tilapia, *Oreochromis mossambicus*, to various hyperosmotic shocks. Comp Biochem Physiol A **152(4):**544-51. doi:10.1016/j.cbpa.2008.12.012.

Yang WK, Hseu JR, Tang CH, Chung MJ, Wu SM, and Lee TH. 2009. Na^+/K^+-ATPase expression in gills of the euryhaline sailfin molly, *Poecilia latipinna*, is altered in response to salinity challenge. J Exp Mar Biol Ecol **375:**41-50.