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Expression and distribution of Na, K-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge

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Abstract

Freshwater (FW) spotted green pufferfish (*Tetraodon nigroviridis*) were transferred directly from a local aquarium to fresh water (FW; 0‰), brackish water (BW; 15‰), and seawater (SW; 35‰) conditions in the laboratory and reared for at least two weeks. No mortality was found. To investigate the efficient mechanisms of osmoregulation in the euryhaline teleost, distribution and expression of Na,K-ATPase (NKA) in gill and kidney of the pufferfish were examined and the osmolality, [Na^+] and [Cl^-] of the blood were assayed. The lowest levels of both relative protein abundance and activity were found to be exhibited in the BW group, and higher levels in the SW group than FW group. In all salinities, branchial NKA immunoreactivity was found in epithelial cells of the interlamellar region of the filament and not on the lamellae. Relative abundance of kidney NKA α-subunit, as well as the NKA activity, was found to be higher in the FW pufferfish than fish in BW or SW. Renal NKA appeared in the epithelial cells of distal tubules, proximal tubules, and collecting tubules, but not in glomeruli, in fish groups of various salinities. Plasma osmolality and chloride levels were significantly lower in FW pufferfish than those in BW and SW, whereas plasma sodium did not differ among the groups. Although identical distributions of NKA were found in either gill or kidney of FW-, BW- or SW-acclimated spotted green pufferfish, differential NKA expression in fish of various salinity groups was associated with physiological homeostasis (stable blood osmolality), and illustrated the impressive osmoregulatory ability of this freshwater and estuarine species in response to salinity challenge.

Keywords: Na,K-ATPase; Pufferfish; Gills; Kidneys; Osmoregulation; Salinity; Euryhalinity

1. Introduction

Gills and kidneys are the most important organs responsible for ionoregulation in teleosts. In seawater (SW), teleosts are hypotonic to the medium and passively lose water and gain salt. To compensate, they drink seawater and actively excrete most monovalent ions via the gills, and small amounts of divalent ions through the kidneys. In contrast, in freshwater (FW) teleosts, which are hypertonic to the medium, the diffusional loss of ions and the osmotic influx of water are balanced by absorption of ions in the gills and excretion of large amounts of urine in the kidneys (Moyle and Cech, 2000).

Na,K-ATPase (NKA) is a ubiquitous membrane-bound enzyme that actively transports Na^+ out of and K^+ into animal cells. It is important not only for sustaining intracellular homeostasis, but also for providing a driving force for many transporting systems including fish gills and kidneys. Immunocytochemical studies demonstrated that NKA is located mainly in mitochondria-rich (MR) cells of gill epithelia (Wilson and Laurent, 2002) and epithelia of kidney tubules (Ura et al., 1996) in euryhaline teleosts. NKA is a P-type ATPase consisting of an $(\alpha\beta)_2$ protein complex and the catalytic α-subunit with a molecular mass of about 100 kDa, while the smaller glycosylated β-subunit exists with a molecular mass of approximately 55 kDa (Blanco and Mercer, 1998). Most euryhaline teleosts exhibit adaptive changes in NKA activity following salinity changes (Mar-

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shall, 2002). Renal NKA activity of euryhaline species is higher in FW-living specimens than in SW (Lasserre, 1971; Gallis and Bourdichon, 1976; Venturini et al., 1992; Madsen et al., 1994). On the other hand, branchial NKA is generally reported to be more activated in SW residing teleosts (reviewed by Sakamoto et al., 2001; Marshall, 2002) forming the well established "diadromid paradigm" (Marshall and Bryson, 1998; Lee et al., 2000, 2003). Yet, in several euryhaline species of non-estuarine marine teleosts such as flounder, sea bream, mullet (for reviews, see Marshall and Bryson, 1998), and milkfish (Lee et al., 2003), higher NKA activity was found in FW-adapted individuals than SW-adapted fish. These results are a deviation from and do not support the paradigm.

Salinity adaptation by euryhaline teleosts is a complex process involving a set of physiological responses in osmoregulatory organs (e.g., gill and kidney) to milieus with differing ionoregulatory requirements. The spotted green pufferfish (*Tetraodon nigroviridis*) is an advanced tetraodontid teleost whose native range covers the rivers and estuaries of Southeast Asia (Rainboth, 1996). Being a peripheral FW inhabitant (Helfman et al., 1997), this pufferfish demonstrates efficient osmoregulation in experimental conditions, as it can tolerate a direct transfer from FW to SW or vice versa. Since it is capable of great euryhalinity and is widely available, plus demands only trivial and inexpensive maintenance, the pufferfish makes a good experimental animal in the laboratory for studies of osmoregulation. Although residence in natural milieus with changing salinities is known, further investigation into the proficient mechanisms used by the puffer to maintain osmotic homeostasis is still warranted. The present set of experiments was conducted to characterize the adaptive response of NKA in gill and kidney of the spotted green puffer acclimated to salinities ranging from a hyperosmotic environment of 35‰(SW), down to a hyposmotic environment of 0‰(FW). Furthermore, distributions of NKA in gill and kidney of the spotted green pufferfish were examined to illustrate corresponding NKA expression. This study describes branchial and renal NKA expression of the spotted green pufferfish as a model for future physiological experimentation.

2. Materials and methods

2.1. Fish and experimental environments

The spotted green puffer (*T. nigroviridis*), 5.6 ± 0.4 g body mass, obtained from a local aquarium were 6.2 ± 0.5 cm in total length. Seawater (35‰ SW) and brackish water (15‰ BW) used were prepared from local tap water with proper amounts of synthetic sea salt added with Instant Ocean (Aquarium Systems, USA). The spotted green pufferfish were reared in SW ($[Na^+]$ 582.86 mM; $[K^+]$ 10.74 mM; $[Ca^{2+}]$ 15.75 mM; $[Mg^{2+}]$ 32.92 mM; $[Cl^-]$ 520.84 mM), BW ($[Na^+]$ 156.59 mM; $[K^+]$ 3.32 mM; $[Ca^{2+}]$ 5.25 mM; $[Mg^{2+}]$ 10.29 mM; $[Cl^-]$ 140.4 mM) and 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 12-h photoperiod for at least 2 weeks before experiments. The water was continuously circulated through fabric-floss filters. Fish were fed a daily diet of commercial sterilized sludge worm. The rate of diet mass per body mass was about 1/25.

2.2. Na,K-ATPase antibody

A mouse monoclonal antibody (α5) against the α-subunit of the avian sodium pump (Takeyasu et al., 1988) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA).

2.3. Immunohistochemistry

Tissues were dissected and fixed for 24 h in 10% formalin in 0.1 M phosphate buffer (pH 7.2) at 25 °C. Tissue samples were dehydrated through a graded ethanol series, embedded in paraffin, and serial sections of 7 μm were mounted on gelatin coated glass slides. The sections were stained immunohistochemically with the monoclonal antibody (α5) to Na,K-ATPase α-subunit and then stained with a commercial kit (PicTure™, Zymed, USA). Negative control experiments, in which normal goat serum was used instead of the primary antibody, were conducted (data not shown) to confirm the positive results.

2.4. Immunoblotting

The tissues scrapings were suspended in the mixture of homogenization medium and 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 procedures of immunobloting were run as described by Wu et al. (2003) with some modifications. Briefly, aliquots of gill homogenates and pre-stained molecular weight standards (Invitrogen, USA) 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, USA) using a tank transfer system (Bio-Rad, Mini Protean 3, USA). 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% (vol/vol) Tween 20, pH 7.4) containing 5% (wt/vol) nonfat dried milk to minimize non-specific binding, then incubated at 4 °C with primary antibody (α5) diluted in PBST (1:5000) overnight. The blot was washed in PBST, followed by a 1-h incubation with AP-conjugated secondary antibody (Jackson, USA) diluted 2500 × in PBST. Blots were visualized after incubation with an NBT/BCIP kit (Chemicon, UK). Immunoblots were

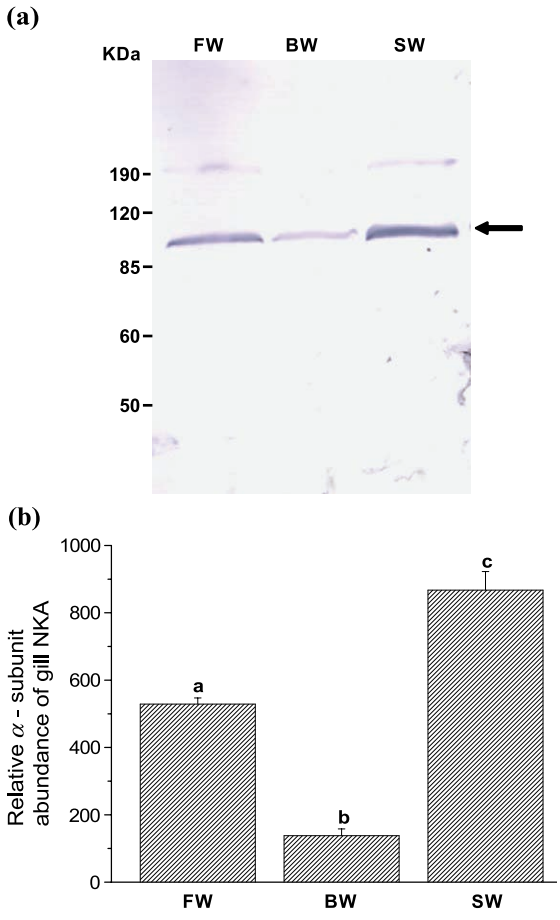

Fig. 1. Expression of Na, K-ATPase α-subunit in gill of spotted green pufferfish (*T. nigroviridis*) adapted to water of different salinities. (a) Immunoblots of Na, K-ATPase α-subunit probed with α5 antibody. Arrow indicates immunoreactive bands at 100 kDa. in gill of pufferfish adapted to different salinities. (b) Relative abundance of immunoreactive bands of Na, K-ATPase α-subunit in gill of different salinity groups; $n = 6$ for all groups. Expression of Na,K-ATPase α-subunit showed significant differences among groups; SW>FW>BW. Different letters indicate a significant difference ($p < 0.05$) using Tukey's multiple-comparison test following a one-way ANOVA. Values are means ± S.E.M.

scanned and images were imported as TIFF format into a commercial software package (Kodak Digital Science 1D, 1995) and the results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

2.5. Specific activity of NKA

Gill and kidney NKA activity was determined as described by Hwang et al. (1989). Aliquots of the suspension of gill 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 phosphate liberated in the presence and absence of 3.75 mM ouabain in the reaction mixture. Each sample was assayed in triplicate.

2.6. Plasma analysis

The blood of the spotted green pufferfish was collected from the caudal veins; we dissected gill arteries then collected blood from a heparinized capillary (1 mm in diameter). After centrifugation at 13,000 rpm for 5 min, the plasma was stored at 4 °C. Plasma osmolality was assessed with the WESCOR 5520 VAPRO Osmometer (USA). [Na^+] was measured with a Hitachi Z-8000 polarized Zeeman atomic absorption spectrophotometer (Japan). [Cl^-] was evaluated using the Ferricyanide method (Franson, 1985). Photometric analysis was carried out using a Hitachi U-2001 spectrophotometer (Japan).

2.7. Statistical analysis

Values were compared using a one-way analysis of variance (ANOVA) (Tukey's pair-wise method). Values were expressed as the means ± S.E.M.

3. Results

3.1. Branchial Na,K-ATPase expression

Immunoblotting of gill tissues from fish acclimated to different salinities (FW, BW and SW) resulted in a single immunoreactive band of about 100 kDa (Fig. 1a). Quantification of immunoreactive bands of each group show significant difference in each group. The SW-acclimated fish group was about 1.6-fold higher than FW-acclimated

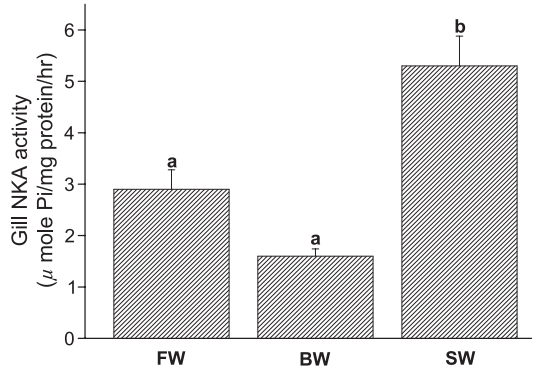

Fig. 2. Na,K-ATPase activity of gill in spotted green pufferfish (*T. nigroviridis*) adapted to different salinities; $n = 6$ for all groups. SW-adapted group was significantly higher than the other groups. Different letters indicate a significant difference ($p < 0.05$) using Tukey's multiple-comparison test following a one-way ANOVA. Values are means ± S.E.M.

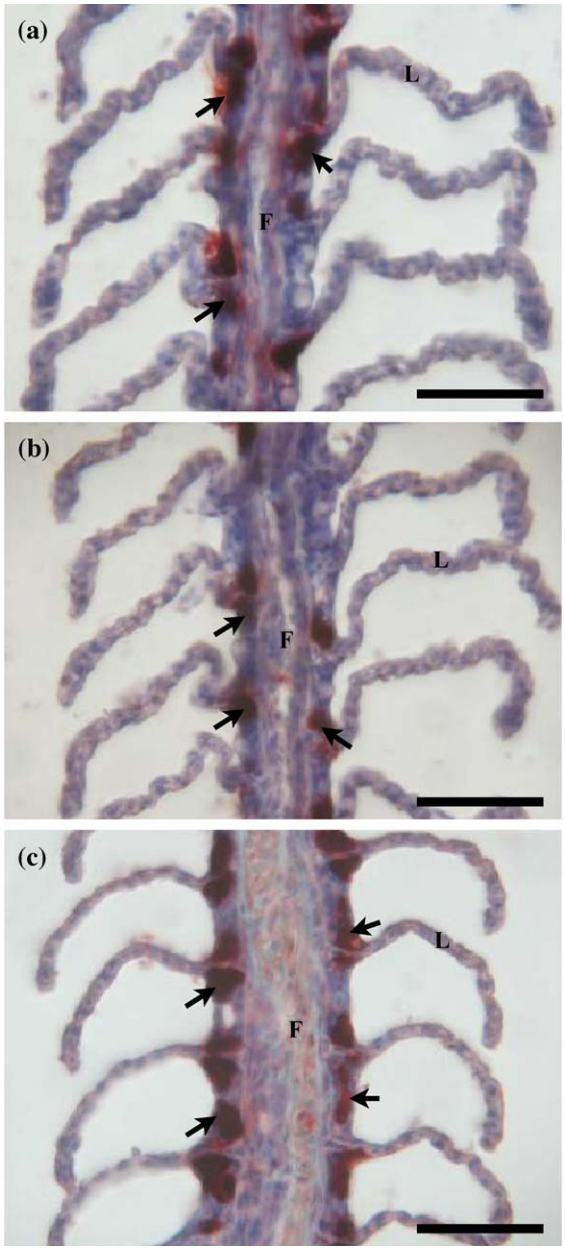

Fig. 3. Immunohistochemical localization of Na,K-ATPase immunoreactive (NKIR) cells recognized by α5 antibody in longitudinal sections of spotted green pufferfish (*T. nigroviridis*) gill adapted to different salinities. (a) SW-adapted fish. (b) BW-adapted fish. (c) FW-adapted fish. The objective lens was 40×. Arrows indicated NKIR cells. F; filament, L; lamellae. Bar 50 μm.

fish or 6.3-fold higher than the BW-acclimated fish (Fig. 1b). The specific activity of gill Na, K-ATPase (NKA) of fish acclimated to SW was significantly higher than that of fish acclimated to BW and FW (3.3- and 1.8-fold, Fig. 2). However, there was no significant difference between gill NKA activity of BW and FW-acclimated fish (Fig. 2).

3.2. Immunohistochemistry of gill NKA

No changes in the distribution of gill NKA-immunoreactive (NKIR) cells associated with salinity were visualized using immunolocalization of the α-subunit on paraffin sections (Fig. 3). Gill NKIR cells of SW, BW and FW-acclimated fish were distributed in filaments and the interlamellar region, yet none were found in lamellae (Fig. 3).

3.3. Kidney NKA expression

Similar to the immunoblotting result for gill NKA, kidney tissues from fish acclimated to different salinities (FW, BW and SW) also resulted in a single immunoreactive band of about 100 kDa (Fig. 4a). Quantification of immunoreactive bands of each group showed an opposite trend compared with the results for gill tissues. Kidney NKA protein of FW-acclimated fish proved to be higher

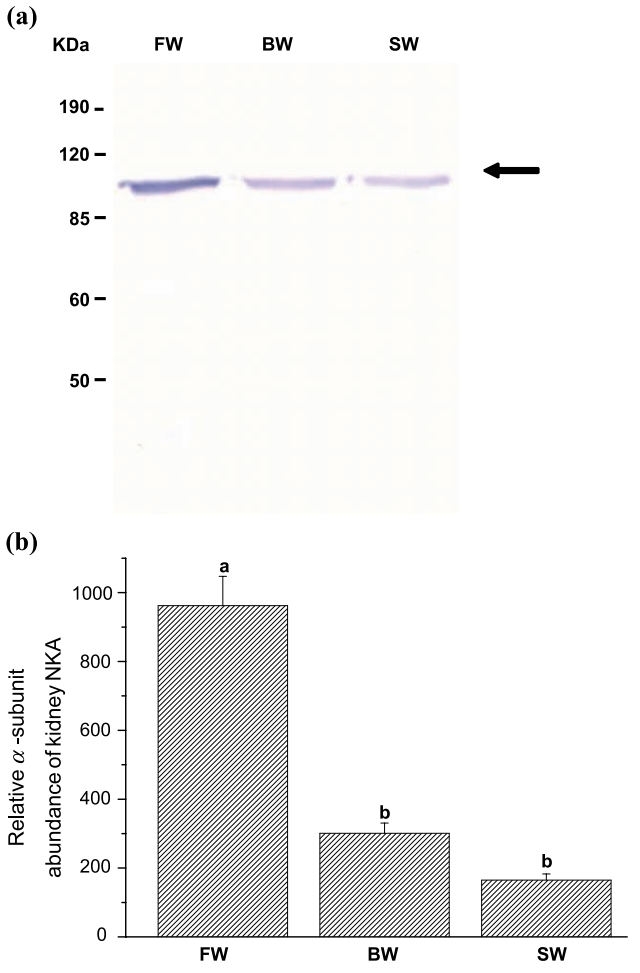

Fig. 4. Expression of Na, K-ATPase α-subunit in kidney of spotted green pufferfish (*T. nigroviridis*) adapted to water of different salinities. (a) Immunoblots of Na,K-ATPase α-subunit probed with α5 antibody. Arrow indicates immunoreactive bands at 100 kDa in kidney of pufferfish adapted to different salinities. (b) Relative abundance of immunoreactive bands of Na,K-ATPase α-subunit in kidneys of different salinity groups; $n = 6$ for all groups. Expression of Na, K-ATPase α-subunit was significantly higher in the FW-adapted group than the other groups. Different letters indicate a significant difference ($p < 0.05$) using Tukey's multiple-comparison test following a one-way ANOVA. Values are means ± S.E.M.

than in the other groups, with about 3.2-fold higher expression than BW-acclimated fish and 5.8-fold higher than SW-acclimated fish (Fig. 4b). The specific activity of kidney NKA of fish acclimated to FW was significantly higher than that of fish acclimated to BW and SW (2.2- and 2.7-fold, Fig. 5). There was no significant difference between kidney NKA activity of BW and SW-acclimated fish (Fig. 5).

3.4. Immunohistochemistry of kidney NKA

NKA was immunohistochemically stained by using α5 antibody in kidneys of different salinity-acclimated (FW, BW and SW) fish. In all salinity groups, the immunoreaction was found to be localized mainly in the collecting tubules, distal convoluted tubules and proximal convoluted tubules (Fig. 6a–c). In the collecting tubules, weak immunoreactivity was exhibited throughout the epithelial cells with exception of the basal membrane, which demonstrated a slightly more prominent response (Fig. 6a). On the other hand, the NKA antibody strongly stained entire cells in distal convoluted tubules (Fig. 6b), yet was only present on the basolateral surface of proximal convoluted tubules (Fig. 6c). NKA distribution in kidney of pufferfish adapted to various milieus was identical (Fig. 6d–f). The glomeruli were not stained with the antibody.

3.5. Plasma analysis

There was no significant difference among plasma sodium concentrations of spotted green pufferfish acclimated to FW, BW or SW (Fig. 7). However, plasma osmolality and chloride concentration were significantly lower in the FW-acclimated group than in the other groups (BW and SW, Fig. 7).

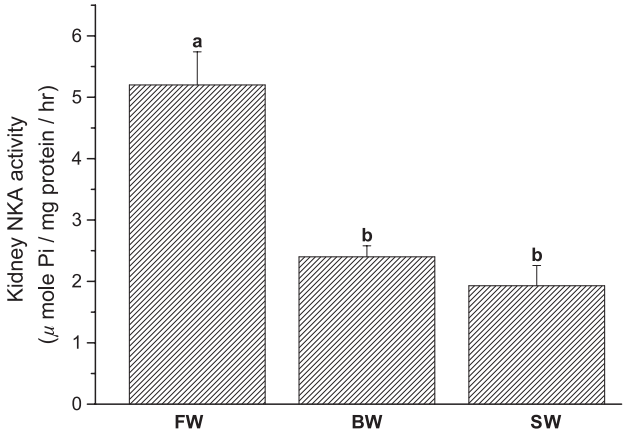

Fig. 5. Na,K-ATPase activity of kidney in spotted green pufferfish (*T. nigroviridis*) adapted to different salinities; $n = 6$ for all groups. FW-adapted group was significantly higher than other groups. Different letters indicate a significant difference ($p < 0.05$) using Tukey's multiple-comparison test following a one-way ANOVA. Values are means ± S.E.M.

4. Discussion

The significance of the role played by branchial Na,K-ATPase (NKA) in ion transport has been confirmed through years and species (Marshall, 2002) since the first studies of Epstein et al. (1967) on killifish, *Fundulus* spp., and Kamiya and Utida (1968) on eels, *Anguilla japonica*. Similar to euryhaline tilapia, *Oreochromis mossambicus*, (Lee et al., 2000; 2003), salmon, *Salmo salar* (D'Cotta et al., 2000), and milkfish, *Chanos chanos* (Lin et al., 2003), salinity-dependent alterations of NKA activity and the amounts of NKA protein were found in spotted green pufferfish, *T. nigroviridis*. In vitro activity assays demonstrated that gill NKA of euryhaline teleosts, e.g., eel, trout, tilapia, and sea bass, is sensitive to ionic strength (Johnson et al., 1977; Ho and Chan, 1980; Trigari et al., 1985; Hwang et al., 1988; Pagliarani et al., 1988). Moreover, Pfeiler and Kirschner (1972) and Pagliarani et al. (1991) gave evidences to describe that the optimal conditions for maximal activation of NKA activity in FW- and BW-adapted trout gills are different. The affinities of gill NKA in euryhaline teleosts to various concentrations of ions in environments of varied salinities may thus be different and lead to changes of NKA activity. Protein abundance as well as the activity of branchial NKA is at a maximum in the SW-acclimated pufferfish (Figs. 1 and 2). According to current model of salt excretion in gills of SW teleosts, the driving force for Cl^- secretion is the Na^+ electrochemical gradient established by NKA, and Na^+ secretion occurs down its electrochemical gradient via a cation-selective paracellular pathway (Marshall, 2002). The highest level of NKA activity and protein abundance makes the pufferfish secret excess salts efficiently and thus acclimate smoothly into SW. Meanwhile, measured amounts of gill NKA protein in FW-acclimated pufferfish, showed significant increase in comparison to BW-acclimated fish (Fig. 1), although an insignificant difference in NKA activity was found between the two groups (Fig. 2). Pufferfish exposed to BW experienced little osmotic stress because the osmolality of plasma was isotonic with the external environment, and showed the lowest level of NKA expression in gills. On the other hand, FW-acclimated pufferfish have to absorb ions actively from the hypotonic external milieus to compensate for those passively lost ions. In gills of FW pufferfish, the higher but insignificant NKA activity, compared to the significantly increasing levels of NKA protein (3.8-fold greater), may be restrained by characteristics of NKA itself, e.g., different affinities for ions in various milieus, as described above.

Expression of gill NKA is ascribed to Na,K-ATPase immunoreactive (NKIR) cells distributed in epithelia. NKIR cells in FW-, BW-, or SW-acclimated spotted green pufferfish (Fig. 3), as well as in tilapia, occurred only in gill filaments (Lee et al., 1996, 2003; Uchida et al., 2000). NKIR cells are normally abundant in filament epithelia of both FW and SW teleosts (Wilson and Laurent, 2002) and effective at secreting ions in hypertonic SW as well as

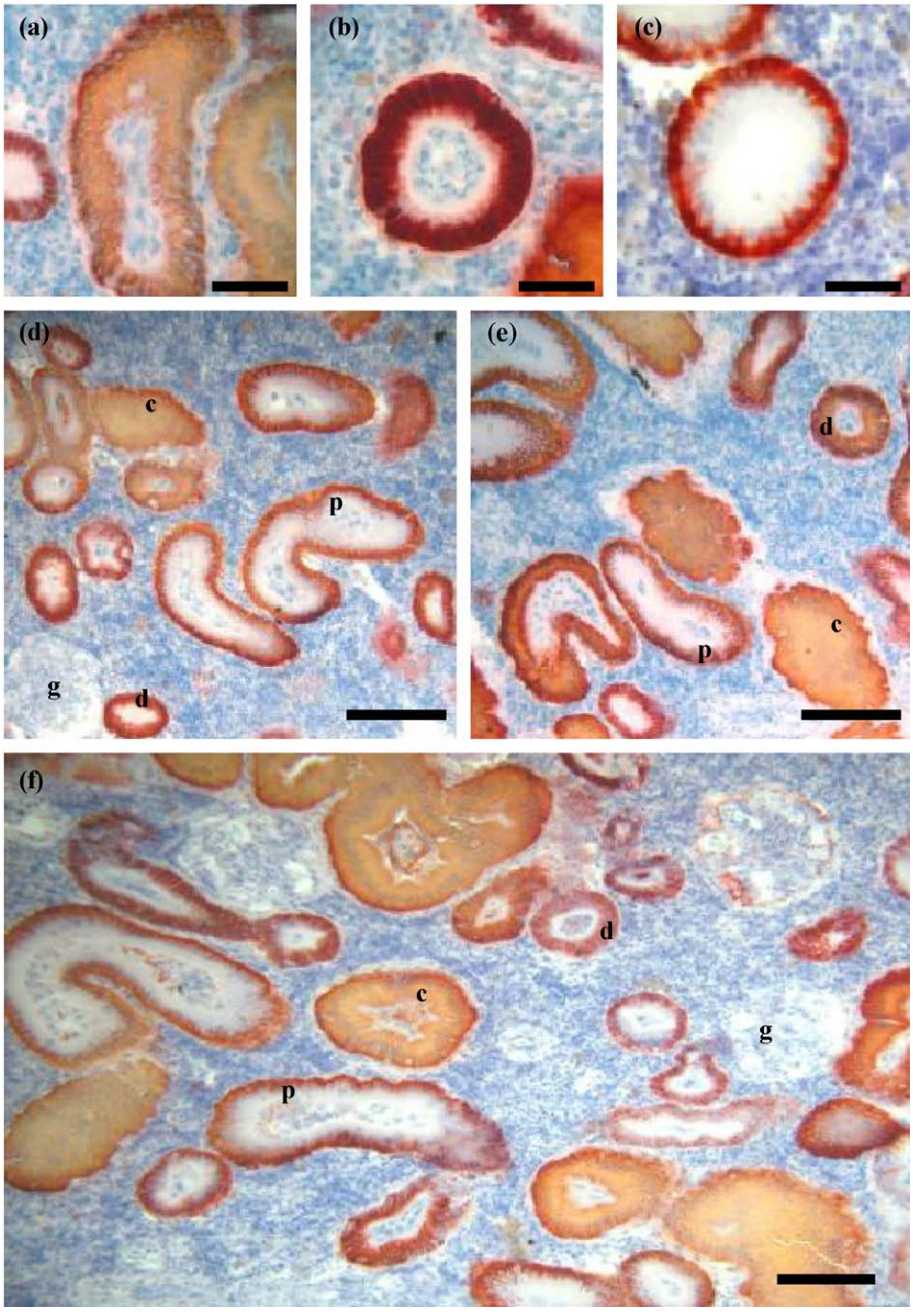

Fig. 6. Immunohistochemical localization of Na,K-ATPase recognized by α5 antibody in dorsal-ventral sections of spotted green pufferfish (*T. nigroviridis*) kidney adapted to different salinities. Panels (a), (b) and (c) showed collecting tubule, distal convoluted tubule and proximal convoluted tubule at high magnification (the objective lens was 40 ×). Panels (d), (e) and (f) showed SW-adapted fish, FW-adapted fish and BW-adapted fish at low magnification (the objective lens was 20 ×). c, collecting tubule; d, distal convoluted tubule; g, glomerus; p, proximal convoluted tubule. Bar is 20 μm in panels (a), (b) and (c), and 50 μm in panels (d), (e) and (f).

uptaking ions in hypotonic FW (reviewed by Marshall, 2002). In some FW-adapted euryhaline fish, however, NKIR cells were redistributed in epithelia of both filaments and lamellae (Sakamoto et al., 2001). The occurrence of lamellar NKIR cells is thought to meet the physiological requirement of ion-uptake in some freshwater-adapted euryhaline teleosts (Avella et al., 1987; Uchida et al., 1996; Sasai et al., 1998; Hirai et al., 1999; Versamos et al., 2002) but not in others (Laurent and Perry, 1990; Lin and Sung, 2003). Thus, utilizing the multi-ionoregulatory functions of existing NKIR cells in filaments may reflect the rapid physiological demands of the spotted green pufferfish which resides in environments with changing salinities.

NKA protein abundance as well as specific activity in kidneys of the FW pufferfish increased significantly (Figs. 4 and 5), while the patterns of NKA expression in gills proved to have an opposite response. In FW, the primary function of kidneys is to excrete excess water, while reabsorbing most

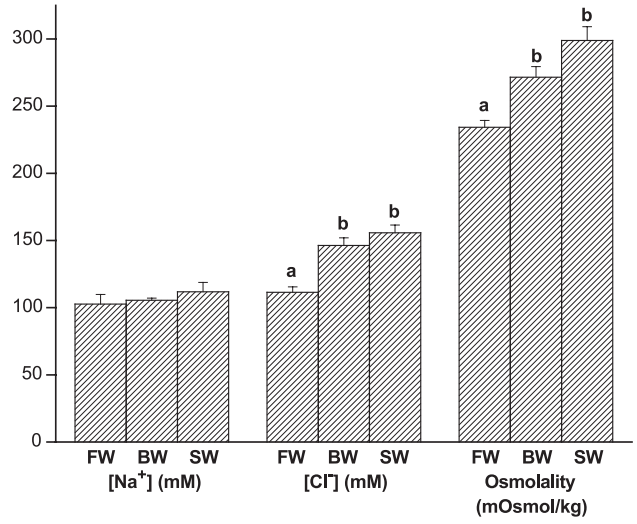

Fig. 7. Plasma sodium, chloride and osmolality concentrations of spotted green pufferfish (*T. nigroviridis*) adapted to water of different salinities; $n=6$ for all groups. No significant difference was found in plasma sodium concentration. Plasma chloride and osmolality concentrations were significantly lower in the FW-adapted group than the other groups. Different letters indicate a significant difference ($p<0.05$) using Tukey's multiple-comparison test following a one-way ANOVA. Values are means ± S.E.M.

of the filtered solutes. To minimize salt loss, FW teleosts reabsorb approximately 95% of the NaCl that enters the glomerular filtrate, and therefore, they produce dilute urine (Miyazaki et al., 2002). Due to the low levels of ions in FW, higher NKA activity in kidneys of the euryhaline teleosts thus provides one mechanism by which such an enhancement of reabsorbing tubular NaCl destined for large volumes of urinary output might be accomplished (Perry et al., 2003). Renal NKA activity was also found to be higher in FW individuals than in SW specimens in several other euryhaline species (Epstein et al., 1969; Lasserre, 1971; Gallis and Bourdichon, 1976; Venturini et al., 1992; Madsen et al., 1994). Meanwhile, recent studies on rainbow trout acclimated to ion-deficient water revealed higher renal NKA activity compared to FW-acclimated individuals (Sloman et al., 2001). To the best of our knowledge, however, the positive correlation between increasing NKA activity and protein abundance in kidneys of euryhaline teleosts has been demonstrated here for the first time.

The kidneys of spotted green pufferfish acclimated to FW, BW or SW, all exhibit the structure of typical freshwater fish (Endo and Kimura, 1984) with complete glomerulus, proximal convoluted tubules, distal convoluted tubules and collecting tubules in the nephron (Fig. 7). Using the same antibody, NKA protein was found in the tubular sections and is responsible for reabsorption of salts in kidneys of FW-, BW-, or SW-acclimated individuals (Fig. 7). In mammalian kidneys, NKA protein was localized in the basolateral surface of the nephron segments (Seigel et al., 1984). In spotted green pufferfish, however, NKA was localized in the cytoplasm of epithelial cells of distal and collecting tubules, in addition to the basolateral immunostaining of proximal tubular cells. Similar results were also reported in other euryhaline teleosts (Ura et al., 1996). Different immunolocalization of NKA, an integral membrane protein, among proximal, distal, and collecting tubules, may be due to different extents of the tubular systems extended from basolateral membrane to cytoplasms. Ultrastructural observations of the nephron segments of the pufferfish are thus warranted in future studies.

Plasma osmolalities, and Na^+ and Cl^- levels (Fig. 7) observed in FW-, BW-, and SW-acclimated spotted green pufferfish are well within the ranges reported for FW as well as SW teleosts (Withers, 1992). Unlike the euryhaline FW tilapia (*O. mossambicus*; Lee et al., 2000) or the euryhaline marine milkfish (*C. chanos*; Lin et al., 2003), salinity-induced changes are found in the circulating electrolytes of the spotted green pufferfish, a river and estuarine resident. This observation, taken together with the changes of NKA expression in gills and kidneys, demonstrated that spotted green pufferfish is capable of osmoregulating in different salinity milieus.

In conclusion, these characteristics as well as its general availability and economical maintenance in the laboratory make this pufferfish an excellent model for future research of osmoregulation. In response to salinity challenge, the opposing patterns of NKA expression in gill and kidney of spotted green pufferfish could be attributed to different roles of these two osmoregulatory organs. Further exploration of rapid acclimation to changing salinities will be continued to depict the ionoregulatory mechanisms of this euryhaline freshwater/estuarine teleost.

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