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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

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ABSTRACT

Previous studies on non-diadromous euryhaline teleosts introduced a hypothesis that the lowest level of gill Na^+/K^+-ATPase (NKA) activity occurs in the environments with salinity close to the primary natural habitats of the studied species. To provide more evidence of the hypothesis, two medaka species, *Oryzias latipes* and *O. dancena*, whose primary natural habitats are fresh water (FW) and brackish water (BW) environments, respectively, were compared from levels of mRNA to cells in this study. The plasma osmolalities of *O. latipes* and *O. dancena* were lowest in the FW individuals. The muscle water contents of *O. latipes* decreased with elevated external salinities, but were constant among FW-, BW-, and seawater (SW)-acclimated *O. dancena*. Expression of NKA, the primary driving force of ion transporters in gill ionocytes, revealed different patterns in the two *Oryzias* species. The highest NKA α-subunit mRNA abundances were found in the gills of the SW *O. latipes* and the FW *O. dancena*, respectively. The pattern of NKA activity and α-subunit protein abundance in the gills of *O. latipes* revealed that the FW group was the lowest, while the pattern in *O. dancena* revealed that the BW group was the lowest. Immunohistochemical staining showed similar profiles of NKA immunoreactive (NKIR) cell activities (NKIR cell number×cell size) in the gills of these two species among FW, BW, and SW groups. Taken together, *O. latipes* exhibited better hyposmoregulatory ability, while *O. dancena* exhibited better hyperosmoregulatory ability. Our results corresponding to the hypothesis indicated that the lowest branchial NKA activities of these two medaka species were found in the environments with salinities similar to their natural habitats.

1. Introduction

Euryhaline teleosts are able to tolerate a broad range of environmental salinities by using efficient osmoregulatory mechanisms to maintain homeostasis. Osmoregulation and ionoregulation in fish is mainly accomplished by the gills, kidneys, and intestines. Among them, the gills, which are exposed to external environments, are the major site for ion transport. In fresh water (FW), teleostean gills actively transport Na^+ and Cl^- from the external media into the circulatory system to compensate for the passive loss of ions in the urine or due to diffusion through the body surface. In contrast, teleosts residing in seawater (SW) drink external water to counterbalance the osmotic loss of water and secrete excess Na^+ and Cl^- through the gill. In the branchial epithelium, mitochondrion-rich cells (MR cells, i.e., chloride cells) are the ionocytes responsible for ion transport. MR cells play the role of absorbing ions in FW and secreting ions in SW (Wood and Marshall, 1994; Evans et al., 1999; Marshall, 2002; Hirose et al., 2003; Hwang and Lee, 2007). Immunohistochemical studies have demonstrated that, in the gills, MR cells contained most of their Na^+/K^+-ATPase (NKA) on the basolateral membrane (Dang et al., 2000; Lee et al., 2000; Sakamoto et al., 2001b; Brauer et al., 2005).

NKA is a ubiquitous membrane-bound enzyme that couples the exchange of two extracellular K^+ for three intracellular Na^+ to the hydrolysis of one molecule of ATP. It is a P-type ATPase consisting of an $(\alpha\beta)_2$ protein complex, which contains four catalytic α (α1-4) and three glycosylated β (β1-3) isoforms. The molecular weight of the catalytic α-subunit and the smaller glycosylated β-subunit are about 100 and 55 kDa, respectively (Scheiner-Bobis, 2002). NKA plays an important role in sustaining intracellular homeostasis by providing a driving force for many ion transporting systems in the gills (Marshall And Bryson, 1998; Hirose et al., 2003; Lin et al., 2004a; Hwang and Lee, 2007).

Previous studies reported that euryhaline teleosts, including diadromous and non-diadromous fish, exhibited adaptive changes in gill NKA response following an osmotic challenge (Marshall, 2002; Evans et al., 2005). Increases in the NKA activity upon salinity

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challenge were attributed to the precedence or accompaniment of increased NKA α-subunit mRNA abundance (Scott et al., 2004a; Seidelin et al., 2000; Singer et al., 2002), protein amounts (Tipsmark et al., 2002; Lee et al., 2000; Lin et al., 2003), or both (D'Cotta et al., 2000; Lin et al., 2004a, 2006). The gill NKA response to a salinity challenge, which is crucial for the salinity adaptation of euryhaline teleosts, consists of the activation of mRNA or proteins of NKA, as well as the size and number of NKA-immunoreactive (NKA-IR) cells (Marshall, 2002; Hirose et al., 2003; Lin et al., 2003). The non-diadromous euryhaline teleosts have different patterns of gill NKA responses correlated with their primary natural habitats (Hwang and Lee, 2007). The Mozambique tilapia (*Oreochromis mossambicus*), which naturally inhabits FW, expressed the lowest gill NKA activity in FW, and NKA activity increased in hyperosmotic media (Hwang et al., 1989; Lee et al., 2003; Lin et al., 2003; Lin and Lee, 2005). On the other hand, the milkfish (*Chanos chanos*), naturally residing in SW, expressed the lowest gill NKA activity in SW, and increasing activity was found in hyposmotic environments (Lin et al., 2003, 2006). In addition, the green spotted pufferfish (*Tetraodon nigroviridis*), which naturally inhabits estuaries (brackish water; BW), revealed a U-shaped pattern of gill NKA activity. The lowest gill NKA activity was found in BW individuals, and the activity increased in milieus with salinities other than the natural habitats (i.e., FW or SW) (Lin et al., 2004b). Hence, we hypothesized that, in non-diadromous euryhaline teleosts acclimated to a range of environmental salinities, the lowest gill NKA activity occurs in the environments with salinities close to the primary natural habitats of the studied species.

Different species of medaka from diverse natural habitats with various salinities were found to have different salinity tolerances (Inoue and Takei, 2002, 2003). *Oryzias latipes* (Japanese medaka) is a euryhaline teleost which resides in FW environments, such as ponds, marshes, and paddy fields (Haruta et al., 1991; Sakamoto et al., 2001a; Inoue and Takei, 2002, 2003). It is a model fish with a genomic database (National Institute of Genetics medaka genome database, http://dolphin.lab.nig.ac.jp/medaka/) that provides sufficient bioinformation (Wittbrodt et al., 2002). Meanwhile, *O. dancena* (marine medaka) is also a euryhaline teleost that mainly inhabits river mouths and estuaries (BW; Roberts, 1998). In hyperosmotic environments, *O. dancena* showed better tolerance than *O. latipes*, including survival rates of adult fish and hatched rates of oosperm (Inoue and Takei, 2002, 2003). In previous studies, comparisons of gill NKA activity among different salmonid species or killifish with different primary natural habitats revealed that the patterns of the gill NKA responses were influenced by their salinity tolerance (Scott et al., 2004b; Bystriansky et al., 2006; Hiroi and McCormick, 2007; Nilsen et al., 2007). With different salinity tolerances, these two medaka species with different primary natural habitats are perfect for testing the hypothesis mentioned above; this will be accomplished by comparing plasma analyses, muscle water content, gill NKA responses (including the levels of mRNA, protein, and enzyme activity), and the activities of the NKA-IR cells between *O. latipes* and *O. dancena* acclimated to salinities ranging from a hyperosmotic environment (SW) to a hypoosmotic environment (FW).

2. Materials and methods

2.1. Fish and experimental environments

Adult reddish orange Japanese medaka (*Oryzias latipes*, hi-medaka) with a 2.6±0.3 cm standard length were inbreed raised in the laboratory. Adult marine medaka (*O. dancena*) obtained from a local aquarium were 2.5±0.3 cm in standard length. Seawater (35‰; SW) and brackish water (15‰; BW) were prepared from local tap water with proper amounts of the synthetic sea salt "Instant Ocean" (Aquarium Systems, Mentor, OH, USA). *O. latipes* and *O. dancena* were maintained in fresh water (FW) and BW, respectively. For experiments, these two medaka species were acclimated to either FW, BW, or SW for at least three weeks under 14L:10D at 28±1 °C. The medaka fish were then analyzed as described below. The water was continuously circulated through fabric-floss filters and partially refreshed every week. Fish were fed a daily diet of commercial pellets.

2.2. Plasma analysis

Blood was collected from the chamber of the swim bladder by destroying the dorsal artery and vein using heparinized 1 mL syringes and 27 gauge needles. Each plasma sample was pooled from the blood of 10 individuals. After centrifugation at 1000 *g* and 4 °C for 15 min, the plasma was stored at − 80 °C. Plasma osmolality was determined by the Wescor 5520 vapro osmometer (Logan, UT, USA).

2.3. Muscle water contents (MWC)

The muscles from the tails of the FW-, BW-, and SW-medaka fish were excised. Water and blood on the muscle were removed with tissue paper. The muscle water content was determined as the percentage of weight loss after drying at 100 °C for 48 h.

2.4. Total RNA extraction and reverse transcription

Each total RNA sample from the gills was extracted from four medaka by using the RNA-Bee™ (Tel-Test, Friendwood, TX, USA) following the manufacturer's instructions. The RNA pellet was dissolved in 50 μL DEPC-H_2O and treated with the RNA clean-up protocol from the RNAspin Mini RNA isolation kit (GE Health Care, Piscataway, NJ, USA), following the manufacturer's instructions, to eliminate genomic DNA contamination. RNA integrity was verified by 0.8% agarose gel electrophoresis. Extracted RNA samples were stored at − 80 °C after isolation. First-strand cDNA was synthesized by reverse transcribing 5 μg of the total RNA using a 1 μL Oligo(dT) (0.5 μg/μL) primer and a 1 μL PowerScript™ Reverse Transcriptase (Clontech, Palo Alto, CA, USA) following the manufacturer's instructions.

2.5. Primers used for real-time PCR

The cDNA sequence of *O. latipes* β-actin (GenBank accession no. S74868) and the Na^+/K^+-ATPase (NKA) α-subunit were searched from the cDNA library of the Esemble medaka genomic database browser (http://www.ensembl.org/Oryzias_latipes/index.html). In addition, the cDNA sequence of *O. dancena* NKA α-subunit (EU490421) and β-actin (EU490422) were established. The NKA and β-actin gene sequences of these two medaka species were aligned and compared with each other. Then, the highly homologous region of the gene sequence between the two species was selected as a reference to design the sequence of the primer for real-time PCR with the Primer3 software (Rozen and Skaletsky, 2000). The NKA α-subunit primer sequences were as follows (5′ to 3′): forward — GGAAGACAGC-TACGGACAGC and reverse — GAGTTCCTCCTGGTCTTGCA. The β-actin primer sequences were as follows (5′ to 3′): forward — CTGGACTTC-GAGCAGGAGAT and reverse — AGGAAGGAAGGCTGGAAGAG.

2.6. Real-time PCR analysis

The analysis was carried out according to Lin et al. (2006) with little modification. The NKA α-subunit mRNA was quantified with an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). PCR reactions contained 8 μL of cDNA (2000×), 2 μL of either NKA α-subunit primer mixture or β-actin primer mixture (both F and R, 5 μM), and 10 μL of 2× SYBR Green PCR Master Mix (Toyobo, Tokyo, Japan). The real-time PCR reactions were performed as follows: 1 cycle at 50 °C for 2 min and 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. All samples were run in

triplicate. Reactions to quantify the β-actin copy number were performed exactly as described above except for the use of different primers. The NKA mRNA values were normalized using the expression of the β-actin mRNA from the same DNA samples. The occurrence of secondary products and primer-dimers was inspected using melting curve analysis and electrophoresis to confirm that the amplification was specific. One gill cDNA sample from the BW-acclimated *O. latipes* and *O. dancena* was used as the internal control among different groups. For each unknown sample, the comparative Ct method with the formula 2^ − [(CtNKA,*n* − Ctβ-actin,*n*) − (CtNKA,*c* − Ctβ-actin,*c*)] was used to obtain the corresponding NKA α-subunit and β-actin values, where Ct corresponded to the threshold cycle number.

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

A mouse monoclonal antibody (α5) against the α-subunit of the avian NKA (Takeyasu et al., 1988) applied in the present study was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). The secondary antibody was alkaline phosphate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

2.8. Immunoblotting of NKA

Four pairs of gills from the medaka were excised and blotted dry. The gill scrapings were suspended in a mixture of homogenization medium (100 mM imidazole–HCl, 5 mM Na_2EDTA, 200 mM sucrose, 0.1% sodium deoxycholate, pH 7.6) and proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 mL aprotinin) (vol/vol: 50/1). Homogenization was performed in micro-centrifuge tubes with a Polytron PT1200E (Lucerne, Switzerland) at the maximal speed for 25 rotations on ice. The homogenates were then centrifuged at 13,000 *g* and 4 °C for 20 min. Protein concentrations of the supernatant were determined by reagents from the Protein Assay Kit (Bio-Rad, Hercules, CA, USA), and bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard. The pre-stained protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD, USA). Aliquots containing 30 μg of the gill homogenates were heated at 37 °C for 30 min and fractionated by electrophoresis on SDS-containing 7.5% polyacrylamide gels. Separated proteins were transferred from the unstained gels to PVDF (Millipore, Bedford, MA, USA) using a tank transfer system (Bio-Rad, Mini Protean 3). Blots were preincubated for 2 h in PBST (phosphate buffer saline with Tween 20) 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; they were then incubated at 4 °C with primary antibody (α5) diluted in PBST (1:4,000) overnight. The blot was washed with PBST, followed by a 1 h incubation with AP-conjugated secondary antibody, and then developed after incubation with a NBT/BCIP kit (Zymed, South San Francisco, CA, USA). The images of the scanned immunoblots were imported in the TIFF format into a commercial software package (Kodak Digital Science 1D, 1995) and converted to numerical values to compare the relative intensities of the immunoreactive bands. Gill homogenates from one tilapia (*Oreochromis mossambicus*) were used as internal controls among different immunoblots (data not shown). The intensity of the immunoreactive band of the internal control was converted and adjusted to a numerical value of 100 in each immunoblot. The values for the average relative intensities of each environmental group were from the immunoblots of eight individuals.

2.9. Na^+/K^+-ATPase (NKA) activity

The gill NKA activities of *O. latipes* and *O. dancena* were measured according to the NADH-linked methods (McCormick, 1993) with some modifications. ADP derived from the hydrolysis of ATP by ATPase was enzymatically coupled to the oxidation of reduced NADH using lactate dehydrogenase (LDH) and pyruvate kinase (PK). The four pairs of gills from each sample were dissected quickly and stored in a microcentrifuge tube at − 80 °C. The tissue was rapidly thawed and homogenized in 500 μL SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.1% sodium deoxycholate, pH 7.5) containing protease inhibitor with a Polytron PT1200E at maximal speed for 10 s on ice. The homogenates were then centrifuged at 5000 *g* and 4 °C for 2 min. The supernatants were assayed for NKA activity and protein concentration. The assay solution (50 mM imidazole, 0.5 mM ATP, 2 mM phosphoenolpyruvate (PEP), 0.32 mM NADH, 3.3 U LDH mL^{-1}, and 3.6 U PK mL^{-1}, pH 7.5) was mixed with salt solution (189 mM NaCl, 10.5 mM $MgCl_2$, 42 mM KCl, 50 mM imidazole, pH 7.5) in a 3:1 ratio. Before the assay, a standard curve was determined from 0 to 30 nmol ADP per well at 340 nm at 28 °C after adding 200 μL assay mixture for at least 5 min in a 96-well plate. The slope of the standard curve should be − 0.012 to − 0.015 absorbance units $nmol\ ADP^{-1}$. A 10 μL sample from one fish was loaded in a well, and 200 μL assay mixture was added with or without 1 mM ouabain; each sample was assayed in triplicate. The plate was detected every 15 s for up to 10 min in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA,

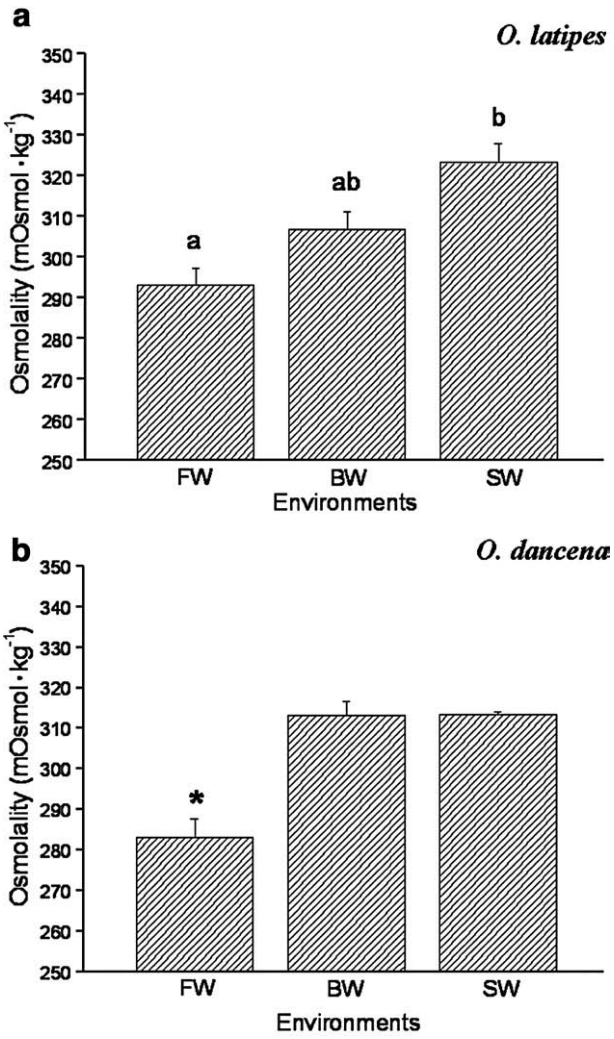

Fig. 1. Plasma osmolality ($mOsmol\text{-}kg^{-1}$) of *O. latipes* and *O. dancena* acclimated to FW, BW, or SW (n=4 for all groups). (a) The higher salinity of environments, the higher plasma osmolality of *O. latipes*; (b) the osmolality of FW *O. dancena* was significantly lower than the other groups. Different letters and the asterisk indicate significant differences (P<0.05) using Tukey's multiple-comparison test following one-way ANOVA. Values are means ± S.E.M. FW, fresh water; BW, brackish water; SW, seawater.

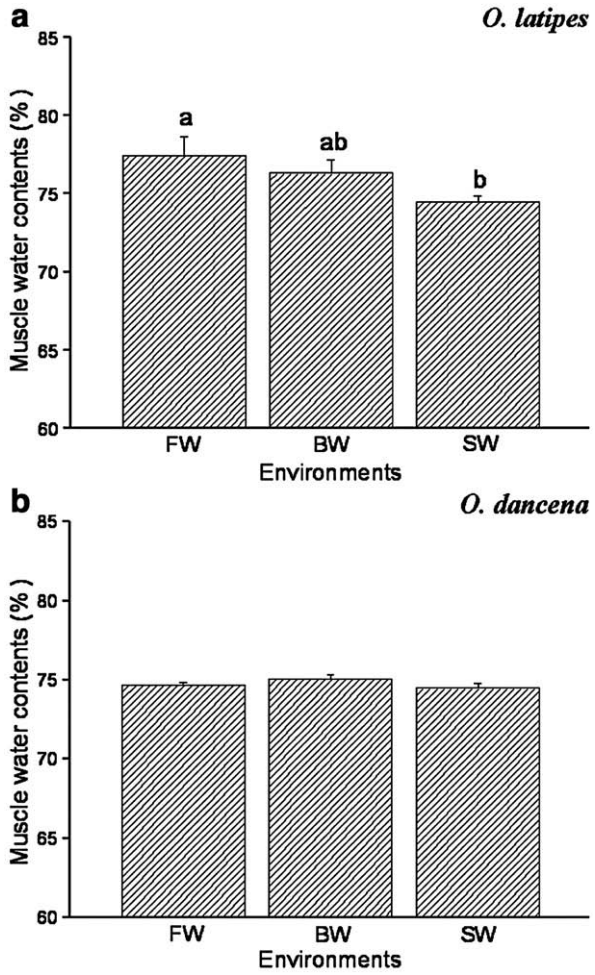

Fig. 2. Muscle water contents of *O. latipes* and *O. dancena* acclimated to FW, BW, or SW (n=15 for all groups). (a) The higher salinity of environments, the lower muscle water contents of *O. latipes*; (b) no significant difference was found among different salinity groups of *O. dancena*. Different letters indicate significant differences (P<0.05) using Tukey's multiple-comparison test following one-way ANOVA. Values are means±S.E.M. FW, fresh water; BW, brackish water; SW, seawater.

USA) at 340 nm and 28 °C. The linear rate from 2 to 10 min for each pair of triplicate wells was determined. Protein concentrations of the samples were determined by the Protein Assay Dye (Bio-Rad) using bovine serum albumin (Sigma) as a standard. The NKA activity was calculated as the difference in slope of ATP hydrolysis (NADH reduction) in the presence and absence of ouabain, and is expressed as μmol ADP per mg protein per hour.

2.10. Immunohistochemical detection of NKA immunoreactive (NKA-IR) cells

The gills were excised and fixed in Bouin solution at room temperature for 48 h. The samples were dehydrated through a graded ethanol series, infiltrated with xylene, and embedded in paraffin. Cross sections of the gills were cut at 5 μm thickness and mounted on poly-L-lysine-coated glass slides. The deparaffinized sections were immunohistochemically stained with a monoclonal antibody (α5) to the NKA α-subunit followed by a commercial kit (PicTure™, Zymed, South San Francisco, CA, USA) for visualization of the immunoreaction. The immunostained sections were then counterstained with Hematoxylin (Merck, Hohenbrunn, Germany) and observed with a microscope (Olympus BX50, Tokyo, Japan). Negative control experiments, in which normal mouse serum was used to replace the primary antibody, were conducted (data not shown) to confirm the above positive results.

The number and size of the NKA-IR cells in the first and secondary pairs of the medaka gills were quantified to determine the NKA-IR cell activity, defined as the cell size×cell number. The average value of the cell size and number were determined from 20 filaments. Only those cross sections of filaments about 175±25 μm in length were randomly selected to minimize the effects of cutting angles on the sections, and micrographs of the sections were taken with a digital camera (Nikon COOLPIX 5000, Tokyo, Japan) for subsequent measurement. The cell size of each filament was obtained from the average size of all NKA-IR cells in that filament quantified by the Image-Pro Plus software (Image-Pro Plus version 4.5.0.29, Media Cybernetics). Meanwhile, the number of NKA-IR cells per filament was counted. In each individual, the values for the size, number and NKA-IR cell activity (cell size×cell number) was divided by standard body length (cm) to exclude the influence of body size. These values for different groups of fish were then calculated and compared.

2.11. Statistical analysis

Values were compared using a one-way analysis of variance (ANOVA) (Tukey's pair-wise method), and P<0.05 was set as the

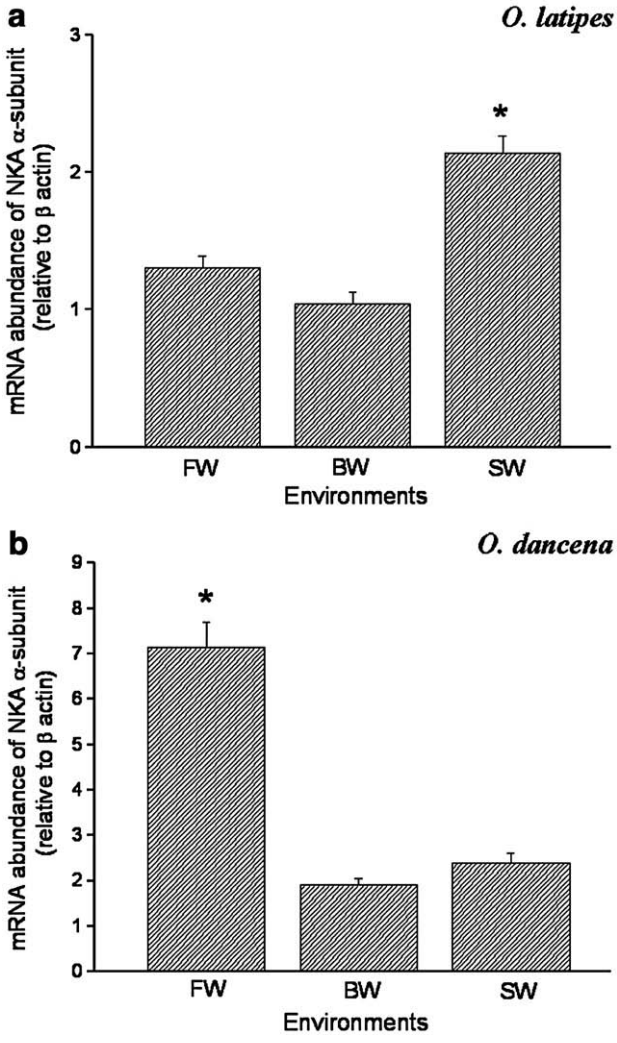

Fig. 3. Gill Na^+/K^+-ATPase (NKA) α-subunit mRNA expression in gills of *O. latipes* and *O. dancena* acclimated to FW, BW, or SW (n=7 for all groups). (a) Relative mRNA abundance of SW acclimated *O. latipes* was significantly higher than the other groups; (b) relative mRNA abundance of FW acclimated *O. dancena* was significantly higher than the other groups. The asterisk indicates a significant difference (P<0.05) using Tukey's multiple-comparison test following one-way ANOVA. Values are means±S.E.M. FW, fresh water; BW, brackish water; SW, seawater.

significant level. Values were expressed as the mean±S.E.M. (the standard error of the mean) unless stated otherwise.

3. Results

3.1. Plasma analysis and muscle water contents (MWC)

Both *Oryzias latipes* and *O. dancena* osmoregulated efficiently for homeostasis upon salinity challenge. Changes in their plasma osmolality or MWC, however, were different. The plasma osmolality of *O. latipes* increased with environmental salinity (Fig. 1a), while the osmolality of fresh water (FW)-acclimated *O. dancena* was significantly lower than the other two groups (Fig. 1b). On the other hand, the MWC of *O. latipes* decreased with environmental salinity (Fig. 2a), but no significant difference in MWC among FW-, brackish water (BW)- or seawater (SW)-acclimated *O. dancena* was found (Fig. 2b).

3.2. Gill Na^+/ K^+-ATPase (NKA) expression

The gill NKA α-subunit mRNA abundance of the SW–*O. latipes* was significantly higher (approximately 1.6-fold) than the other two groups (Fig. 3a). In *O. dancena*, however, the highest relative mRNA abundance was found in the FW-acclimated individuals and was approximately 3.8-fold higher than the abundance of the other groups (Fig. 3b). Immunoblots of the gills from these two medaka species indicated a single immunoreactive band at 110 kDa in all three groups (Fig. 4a and b). Quantification of the immunoreactive bands of different groups revealed that the lowest relative abundance of the NKA α-subunit in *O. latipes* was found in the FW group, and BW and SW individuals were about 2.3- and 2.5-fold higher (Fig. 4c). In *O. dancena*, the BW group showed the lowest level, and the FW and SW groups were about 1.6- and 1.7-fold higher compared to the BW group (Fig. 4d). Comparisons between samples of these two medaka species

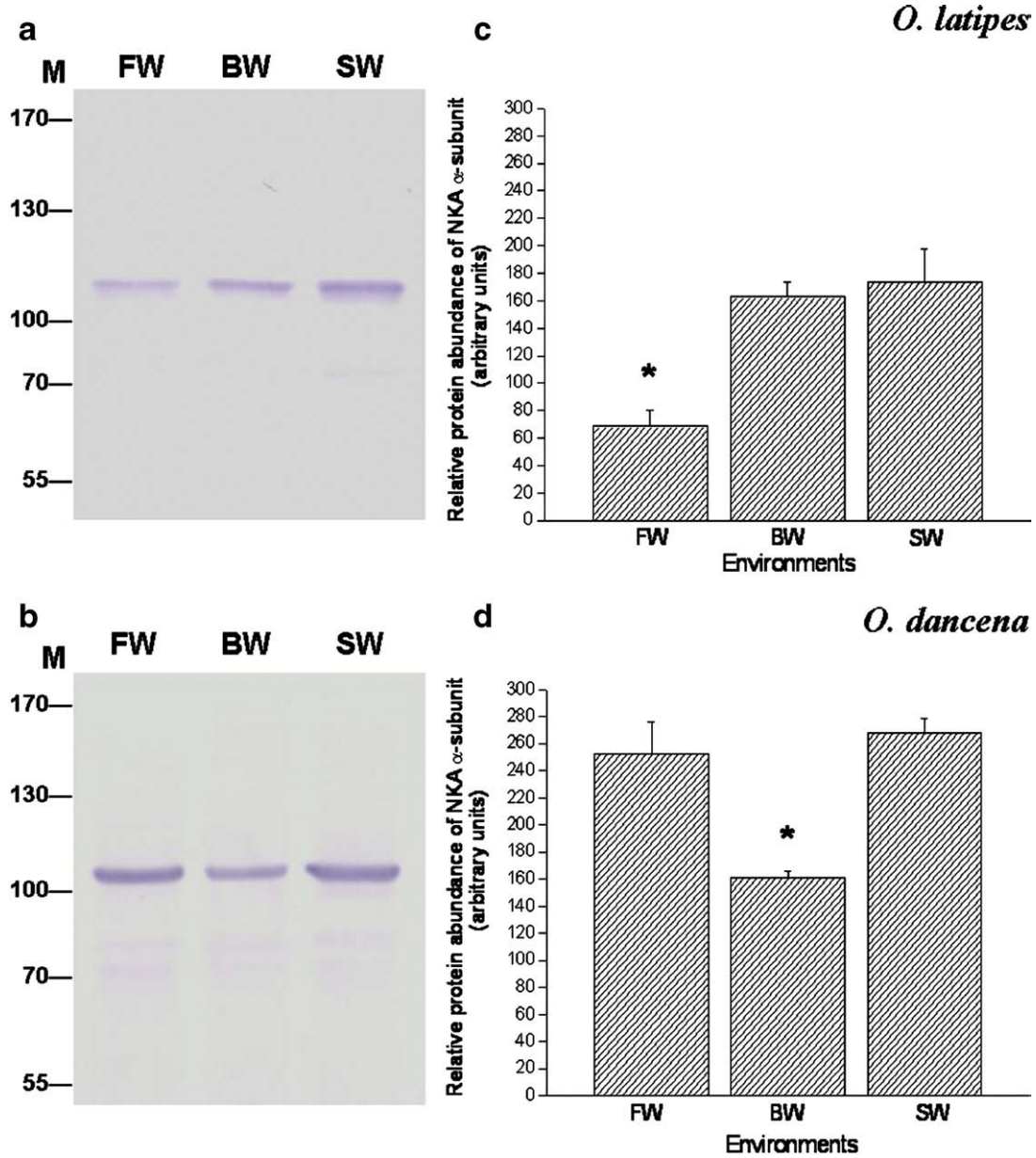

Fig. 4. Expression of Na^+/K^+-ATPase (NKA) α-subunit protein in gills of *O. latipes* and *O. dancena* acclimated to FW, BW, or SW. Immunoblots of *O. latipes* (a) and *O. dancena* (b) gills probed with the α5 antibody indicated single immunoreactive bands at 110kDa in all environment groups. Intensities of immunoreactive bands of NKA α-subunit in gills of different salinity groups ($n=8$ for all groups) revealed that FW-acclimated *O. latipes* was significantly lower than the other groups (c) while BW-acclimated *O. dancena* was significantly lower than the other groups (d). The asterisk indicates a significant difference ($P<0.05$) using Tukey's multiple-comparison test following one-way ANOVA. Values are means±S.E.M. FW, fresh water; BW, brackish water; SW, seawater.

by the same control revealed that, in 30 μg gill total lysates, the NKA α-subunit protein abundance of *O. dancena* was more than that of *O. latipes* except for in the BW-acclimated groups. In addition, the specific activities of the BW- and SW-acclimated *O. latipes* were significantly higher (approximately 1.4- and 3.9-fold, respectively) than that of the FW group (Fig. 5a). Meanwhile, the specific activities of the FW- and SW-acclimated *O. dancena* were about 4.5- and 3.7-fold higher than that of the BW group, respectively (Fig. 5b).

3.3. NKA immunoreactive (NKA-IR) cell activity

The gill NKA immunoreactive (NKA-IR) cells of two medaka species acclimated to FW, BW, or SW were distributed mainly in the afferent epithelium of the gill filaments (Fig. 6). The sizes of the NKA-IR cells from the FW-acclimated *O. latipes* and BW-acclimated *O. dancena* were significantly smaller than those of the other groups, respectively (Table 1). There was significant difference in the number of NKA-IR cells in the BW-acclimated *O. latipes* (Table 1), while the number of NKA-IR cells for the FW-acclimated *O. dancena* was significantly more than that of the other groups (Table 1). The NKA-IR cell activities of the BW- and SW-acclimated *O. latipes* were approximately 1.2- and 1.4-fold higher than that of the FW group, respectively (Fig. 7a). On the other hand, the NKA-IR cell activities of the FW- and SW-acclimated *O. dancena* were approximately 2.4- and 1.5-fold higher than that of the BW group, respectively (Fig. 7b).

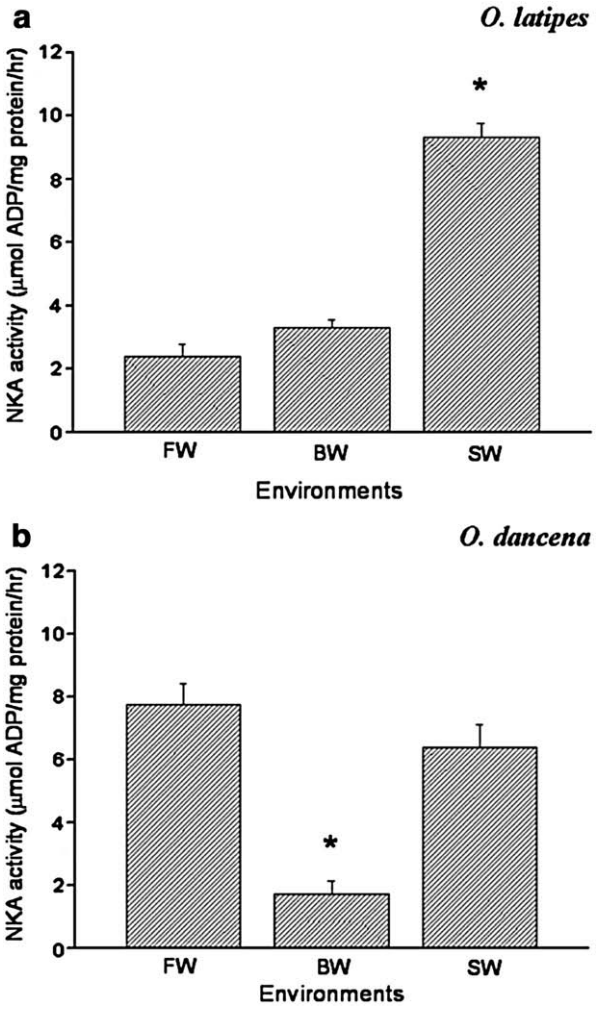

Fig. 5. Gill Na^+/K^+-ATPase (NKA) activity of *O. latipes* and *O. dancena* acclimated to FW, BW, or SW ($n=6$ for all groups). (a) The activity of SW *O. latipes* was significantly higher than the other groups; (b) the activity of BW *O. dancena* was significantly lower than the other groups. The asterisk indicates a significant difference ($P<0.05$) using Tukey's multiple-comparison test following one-way ANOVA. Values are means±S.E.M. FW, fresh water; BW, brackish water; SW, seawater.

4. Discussion

There are many euryhaline species in the genus *Oryzias* with different hyperosmotic tolerances for environments of different salinities (Inoue and Takei, 2002, 2003). Among them, the Japanese medaka (*O. latipes*) has been used as a model animal for laboratory use with abundant molecular-biological and genetic information (Wittbrodt et al., 2002). Therefore, the *Oryzias* species are good models for osmoregulatory studies (Sakamoto et al., 2001a; Inoue and Takei, 2002, 2003). The present study compares the expression of the gill Na^+/K^+-ATPase (NKA) from levels of mRNA to cells between the *O. latipes* and *O. dancena* acclimated to freshwater (FW), brackish water (BW), and seawater (SW).

In this study, we established a method for collecting blood from the swim bladder to accurately detect the plasma osmolality of the FW, BW, or SW-acclimated medaka species, *O. latipes* and *O. dancena*, which are small in size. The plasma osmolalities of both species were from 285 to 320 mOsm kg^{-1} (Fig. 1), the range normally found in euryhaline teleosts, indicating that they are successful osmoregulators during salinity challenge. Plasma osmolalities of the euryhaline teleosts, however, are influenced by their salinity tolerance and primary habitats (Kato et al., 2005; Bystriansky et al., 2006). The patterns of plasma osmolality among FW-, BW-, and SW-acclimated *O. latipes* (Fig. 1a) are similar to those of FW-residing euryhaline fish, e.g., tilapia (*Oreochromis mossambicus*; Lee et al., 2000; Uchida et al., 2000). Their plasma osmolalities increase at the tolerated range in environments with higher salinity. On the other hand, the patterns of plasma osmolality found among FW-, BW-, and SW-acclimated *O. dancena* (Fig. 1b) are similar to those of BW- or SW-residing euryhaline teleosts, such as sea bass (*Dicentrarchus labrax*; Jensen et al., 1998), black seabream (*Mylio macrocephalus*; Kelly et al., 1999), killifish (*Fundulus heteroclitus*; Katoh et al., 2002), milkfish (*Chanos chanos*; Lin et al., 2004a), spotted green pufferfish (*Tetraodon nigroviridis*; Lin et al., 2004b), and takifugu (*Takifugu niphobles*; Kato et al., 2005). The plasma osmolalities of those species are efficiently maintained in the hyperosmotic environments, but decrease in hyposmotic environments. Therefore, these SW euryhaline teleosts must have specific osmoregulatory mechanisms to tolerate the stress of decreasing plasma osmolality in hyposmotic environments. Additionally, the muscle water contents (MWC) were assayed in this study to indicate the osmoregulatory capacity of the medaka. The MWC of different euryhaline teleosts usually changed at the tolerated range with environmental salinity. The black seabream (Kelly et al., 1999) and gilthead sea bream (*Sparus aurata*, Kelly and Woo, 1999) increased their MWC after a transfer from SW to FW. The emperor angelfish (Pomacanthus imperator) exhibited constant levels of MWC upon a salinity challenge (Woo and Chung, 1995). Sakamoto et al. (2001a) reported the MWC of *O. latipes* decreased temporarily after a transfer from FW to SW, and was restored at seven days post-transfer. Our results, however, revealed that after acclimation to BW and SW for three weeks, the MWC values of *O. latipes* decreased 1.4% and 3.1%, respectively, compared to that of the FW-acclimated individuals (Fig. 2a). Meanwhile, the MWC of *O. dancena* was more stable than those of *O. latipes* in FW, BW, or SW (Fig. 2b). Comparisons of plasma osmolality and MWC between *O. latipes* and *O. dancena* acclimated to FW, BW, or SW indicated that the osmoregulatory mechanisms of the *O. dancena* are more efficient than those of the *O. latipes* in hyperosmotic environments.

Most euryhaline teleosts exhibit adaptive changes in gill NKA activity in response to salinity changes (Marshall, 2002; Evans et al., 2005; Hwang and Lee, 2007). The changing pattern of the branchial NKA activity of the euryhaline teleosts was hypothesized to be related

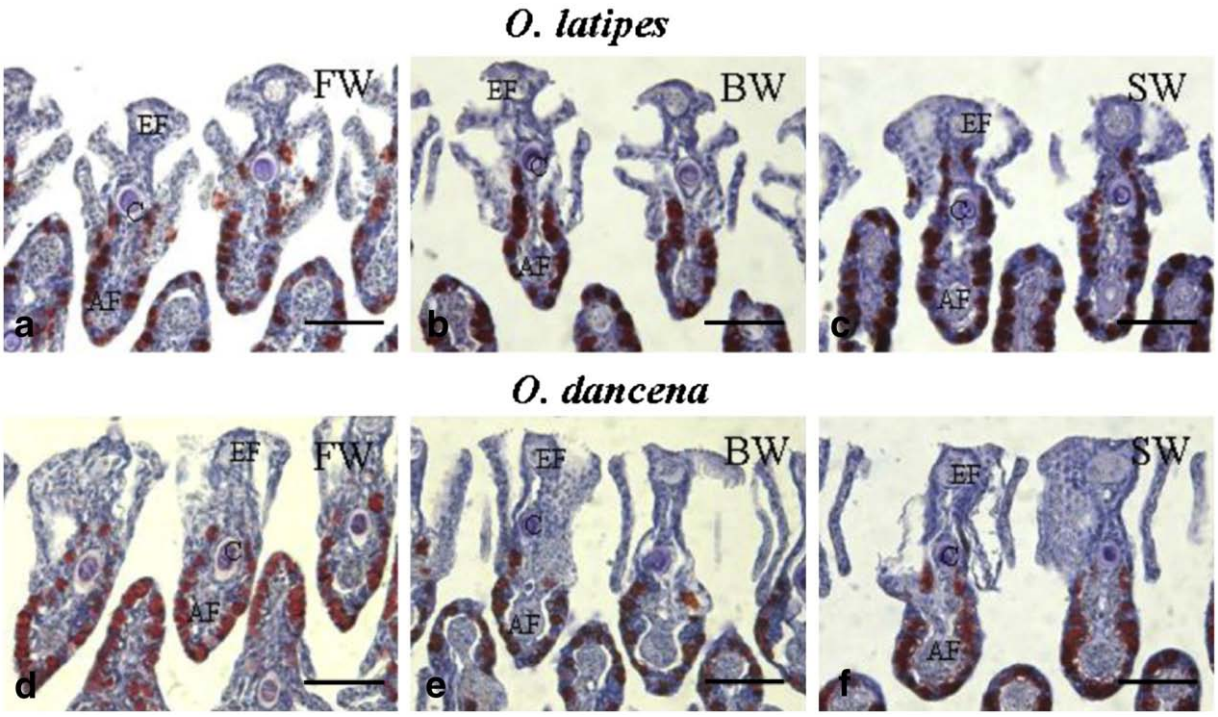

Fig. 6. Imunohistochemical staining of Na^+/K^+-ATPase (NKA) α-subunit on cross sections of gills of *O. latipes* and *O. dancena* acclimated to FW (a; d), BW (b; e), or SW(c; f). The Na^+/K^+-ATPase immunoractive (NKA-IR) cells (red) were mainly distributed in the afferent epithelium of the filaments (AF). C, cartilage; EF, efferent epithelium of the filament; L, lamellae. Bar = 50 μm. FW, fresh water; BW, brackish water; SW, seawater.

to their primary natural habitats (Hwang and Lee, 2007). In the present study, the method of the NADH-linked NKA activity assay was applied in the medaka gills. This method was also reported for analyzing the gill NKA activity of the other euryhaline teleosts (McCormick, 1993; Uchida and Kaneko, 1996; Uchida et al., 2000; Jensen et al., 1998; Imsland et al., 2003; Scott et al., 2004b; Tipsmark et al., 2004; Bystriansky et al., 2006; Arjona et al., 2007). Compared to the inorganic phosphate assay for NKA activity (Peterson, 1978), the temperature for the NADH-linked NKA activity assay was closer to the natural breeding temperature of the medaka (28 °C) and revealed similar results (data not shown). The patterns of NKA activity in the gills of FW-, BW-, and SW-acclimated *O. latipes* and *O. dancena* were different (Fig. 5). In *O. latipes*, whose natural habitat is FW, NKA activity in the gills of the SW-acclimated individuals increased (3.9-fold; Fig. 5a) to provide more driving force for preventing the internal excess ions from escaping to the environment upon hyperosmotic challenge, similar to the patterns of euryhaline tilapia (Lee et al., 2000; Uchida et al., 2000) and diadromous teleosts, e.g., eel (Marsigliante et al., 2000) and salmon (Bystriansky et al., 2006; Nilsen et al., 2007). On the other hand, the *O. dancena*, residing naturally in BW, revealed a U-shaped pattern of the gill NKA activity among FW, BW, and SW groups (Fig. 5b). The pattern is similar to other BW-residing species, e.g., sea bass (Jensen et al., 1998) and spotted green pufferfish (Lin et al., 2004b). The gill NKA activity of *O. dancena* increased to provide the driving force for absorbing ions in FW and excluding ions in SW.

Since the activity of branchial NKA changed with environmental salinities, it is suggested that the difference may be due to the switches of NKA α-subunit isoforms. Three NKA α1-subunit-like sequences (ENSORLG**00000002122**, ENSORLG**000000018577**, ENSORL**G00000002047**), one α2-subunit-like sequence (ENSORLG**00000002639**), and two α3-subunit-like sequences (ENSORLG**00000007036**, ENSORLG**00000013191**) in the Esembl medaka genomic database browser were searched in the present study. These six searched NKA α-subunit sequences of *O. latipes* and the cloned NKA α1-subunit sequence of *O. dancena* (GenBank accession no. **EU490421**) were aligned to compare the highly conserved regions of the nucleotide sequence and to design specific primers for detecting the NKA α-subunit mRNA abundances of these two medaka species. Previous studies revealed that FW euryhaline tilapia (Feng et al., 2002) and diadromous teleosts, e.g., salmon (Bystriansky et al., 2006; Nilsen et al., 2007) and eel (Tse et al., 2006), exhibited increased levels of the gill NKA α-subunit mRNA in hyperosmotic environments. On the other hand, the BW- or SW-residing euryhaline teleosts, e.g., sea bass (Jensen et al., 1998), killifish (Scott et al., 2004a; b), milkfish (Lin et al., 2006), and black porgy (Choi and An, 2008), exhibited an increased

Table 1
The size and number of Na^+/K^+-ATPase immunoreacive (NKA-IR) cells in the afferent epithelium of gill filaments of *O. latipes* and *O. dancena* acclimated to FW, BW, or SW

Environments		FW	Salinity BW	SW
Species				
O. latipes				
	Size (μm^2) / body length (cm)	25.34±0.72*	38.07±1.33	33.07±1.24
	Number / body length (cm)	7.50±0.20	6.25±0.43*	7.74±0.41
O. dancena				
	Size (μm^2)/body length (cm)	29.82±1.82	23.44±0.85*	33.76±1.85
	Number/body length (cm)	8.52±0.98*	4.66±0.35	4.74±0.33

Values are means±S.E.M.(n=5). The asterisks indicated significant differences (P<0.05) using Tukey's multiple-comparison test following one-way ANOVA. FW, fresh water; BW, brackish water; SW, seawater.

abundance of the gill NKA α-subunit mRNA in hyposmotic environments. Our results indicated that the SW-acclimated *O. latipes* expressed the highest level of NKA α-subunit mRNA, similar to those reported in FW euryhaline and diadromous fish (Fig. 3a). *O. dancena*, however, revealed the highest level of NKA α-subunit mRNA in FW, similar to BW- or SW-residing euryhaline fish (Fig. 3b). Recently, the reciprocally expressed mRNA abundances of the NKA α-subunit isoforms were found in salmon and trout: α1a increased in FW, while α1b increased in SW (Richards et al., 2003; Bystriansky et al., 2006; Nilsen et al., 2007). In the gills of *O. latipes* and *O. dancena*, the amplified PCR products of these primers were similar to the sequence of the NKA α1b-subunit of the salmon (unpublished data). It is possible that the different levels of NKA activity and mRNA expression of the NKA (α1b)-subunit in SW *O. dancena* compared to the BW group were due to the reciprocal expression, as found in the salmon.

Previous studies revealed that the higher NKA activity accompanied an increase of the NKA α-subunit protein abundance in the gills of the euryhaline teleosts, e.g., tilapia (Lee et al., 2000; 2003), salmon (D'Cotta et al., 2000), milkfish (Lin et al., 2004a; 2006), and spotted green pufferfish (Lin et al., 2004b). In the gills of these two medaka species, the patterns of the NKA protein abundance among the FW, BW, or SW groups were similar to the patterns of the NKA activity. Discrepancies, however, were found in BW-acclimated *O. latipes*: the measured gill NKA activities in the BW and FW individuals revealed no significant difference (Fig. 5a), while a significant difference in the NKA protein abundance was observed between the two groups (Fig. 4a). Inoue and Takei (2003) reported in their salinity-transfer study that *O. latipes* was not able to survive after a direct transfer from FW to SW, while the capability for hyperosmotic regulation was induced after transfer from FW to BW for 24 h, similar to the Mozambique tilapia (Hwang et al., 1998). Since the FW Japanese medaka survived after direct transfer to BW but died after SW transfer (Inoue and Takei, 2003), our data suggested that the BW-acclimated *O. latipes* required higher NKA protein expression for establishing the hyperosmotic capacity, but an insufficient level of the NKA activity remained for the driving force of ion regulation. Meanwhile, the NKA protein abundance and NKA activity in the gills of the FW- and SW-acclimated *O. dancena* were higher than in the BW fish (Figs. 4b and 5b). This pattern of the NKA expression conformed to the U-shape pattern found in the sea bass (Jensen et al., 1998) and spotted green pufferfish (Lin et al., 2004b). Our data indicated that the BW-residing *O. dancena* reinforced gill NKA activity by increasing NKA protein abundance with changes in environmental salinity.

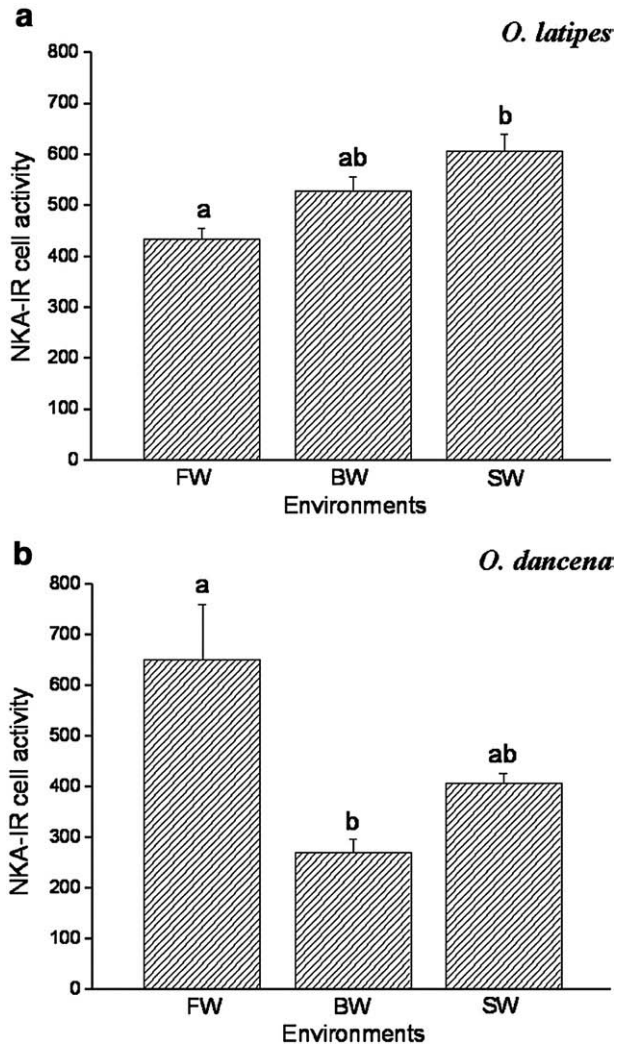

Fig. 7. Gill Na^+/K^+-ATPase immunoreacive (NKA-IR) cell activity (cell size × cell number/body length) of *O. latipes* and *O. dancena* acclimated to FW, BW, or SW (n=5 for all groups). (a) The cell activity of *O. latipes* showed significant differences among all groups (SW > BW > FW); (b) the cell activity of *O. dancena* showed significant differences among all groups (FW > SW > BW). Different letters indicated significant differences (P<0.05) using Tukey's multiple-comparison test following a one-way ANOVA. Values are means ± S.E.M. FW, fresh water; BW, brackish water; SW, seawater.

Mitochondrion-rich (MR) cells (chloride cells) are ionocytes in the gills of the teleosts (Marshall, 2002; Hirose et al., 2003; Hwang and Lee, 2007). Immunocytochemical studies on the gill sections, as well as biochemical studies on isolated MR cells, have demonstrated that the ionocytes express the most NKA in the teleostean gills (Dang et al., 2000; Lee et al., 2000; Sakamoto et al., 2001b; Brauer et al., 2005). Hence, the NKA immuonreactive (NKA-IR) cells in this study represent the MR cells using the same antibody as in the immunoblots. The cross-sections of the gill filaments showed that the NKA-IR cells of these two medaka species acclimated to FW, BW, or SW were distributed mainly in the afferent epithelium and scarcely observed on the lamellar epithelium in the FW group (Fig. 6). In euryhaline teleosts, the number and size of the NKA-IR/MR cells usually change with external salinities (Hirose et al., 2003). Previous studies reported that the size of the NKA-IR/MR cells increased in the SW-acclimated Japanese medaka (Sakamoto et al., 2001a), guppy (*Poecilia reticulata*; Shikano and Fujio, 1998a,b), tilapia (Shiraishi et al., 1997; Hiroi et al., 1999; Lee et al., 2003), salmon (Uchida and Kaneko., 1996; Hiroi and McCormick, 2007), and eel (Wong and Chan, 1999), whereas the NKA-IR/MR cells of the BW-inhabiting killifish are larger in FW than in SW (Katoh and Kaneko, 2003). Our results revealed that the size of the NKA-IR cells in the BW- or SW-acclimated *O. latipes* was larger than that in the FW group, while the number of cells was the lowest in the BW group (Table 1). Meanwhile, the smallest NKA-IR cells were observed in BW-acclimated *O. dancena*, and the number of NKA-IR cells increased in the FW group (Table 1). Similarly, scanning electron microscopy or immunocytochemical observation indicated that the number of NKA-IR/MR cells in the gills of the other marine euryhaline teleosts, including black sea bream (Kelly et al., 1999), sea bass (Hirai et al., 1999; Versamos et al., 2002), and milkfish (Lin et al., 2003; 2006), was elevated when acclimated to a hyposomotic milieu or to FW. In this study, the size and number of the NKA-IR cells were combined to show NKA-IR cell activity. The highest NKA-IR cell activity was found in SW-acclimated *O. latipes* (Fig. 7a), while FW- and SW-acclimated *O. dancena* had higher NKA-IR cell activity than the BW group (Fig. 7b). In the gills of these two medaka species, the patterns of the NKA-IR cell activity among different salinity groups were similar to those of the protein abundance (Fig. 4) and enzyme activity (Fig. 5) of NKA. The results suggest that the higher NKA-IR cell activity provides an expansion of the tubular system where the gill NKA is located. Therefore, increased NKA-IR cell activity was found in this study to parallel with the protein abundances and enzyme

activities of *O. latipes* and *O. dancena* in response to the osmotic stress from environments with non-habitat salinities.

In summary, differential patterns of NKA expression from mRNA to cell activity were found in the gills of the *O. latipes* and *O. dancena* acclimated to FW, BW, and SW. The expression of NKA in the gills of *O. latipes* was similar to other FW-residing euryhaline teleosts, which exhibited elevated levels for ion exclusion with higher environmental salinities. In the gills of *O. dancena*, the NKA pattern was similar to other BW-residing euryhaline teleosts, which expressed the lowest levels in BW and required more NKA expression for ion absorption and secretion in FW and SW. These results confirm the hypothesis that the lowest NKA activity in the gills of non-diadromous euryhaline teleosts is found in individuals acclimated to environments with salinities similar to their habitats. Our data also suggest that FW- residing *O. latipes* exhibit better hyposmoregulatory ability, while *O. dancena* reside in river mouths or estuaries where salinity changes are more violent possessing efficient hyperosmoregulatory ability for adaptation. Future work will focus on the expression patterns of other ion transporters in the gills to compare the ionoregulatory mechanisms of these two medaka species with different salinity tolerances and primary habitats.

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References

Arjona, F.J., Vargas-Chacoff, L., Ruiz-Jarabo, I., Martín del Río, M.P., Mancera, J.M., 2007. Osmoregulatory response of *Senegalese sole* (*Solea senegalensis*) to changes in environmental salinity. Comp. Biochem. Physiol. A 148, 413–421.

Brauer, P.R., Sanmann, J.N., Petzel, D.H., 2005. Effects of warm acclimation on Na^+,K^+-ATPase alpha-subunit expression in chloride cells of antarctic fish. Anat. Rec. 285A, 600–609.

Bystriansky, J.S., Richards, J.G., Schulte, P.M., Ballantyne, J.S., 2006. Reciprocal expression of gill Na^+/K^+-ATPase alpha-subunit isoforms alpha1a and alpha1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. J. Exp. Biol. 209, 1848–1858.

Choi, C.Y., An, K.W., 2008. Cloning and expression of Na^+/K^+-ATPase and osmotic stress transcription factor 1 mRNA in black porgy, *Acanthopagrus schlegeli* during osmotic stress. Comp. Biochem. Physiol. B 149, 91–100.

Dang, Z.C., Balm, P.H.M., Flik, G., Bonga, S.E.W., Lock, R.A.C., 2000. Cortisol increases Na^+/K^+-ATPase density in plasma membranes of gill chloride cells in the freshwater tilapia *Oreochromis mossambicus*. J. Exp. Biol. 203, 2349–2355.

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.

Evans, D.H., Piermarini, P.M., Potts, W.T.W., 1999. Ionic transport in the fish gill epithelium. J. Exp. Zool. 283, 641–652.

Evans, D.H., Piermarini, P.M., Choe, K.P., 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, S.H., Leu, J.H., Yang, C.H., Fang, M.J., Huang, C.J., Hwang, P.P., 2002. Gene expression of Na^+-K^+-ATPase α1 and α3 subunits in gills of the teleost *Oreochromis mossambicus*, adapted to different environmental salinities. Mar. Biotechnol. 4, 379–391.

Haruta, K., Yamashita, T., Kawashima, S., 1991. Changes in arginine vasotocin content in the pituitary of the medaka (*Oryzias latipes*) during osmotic sress. Gen. Comp. Endocrinol. 83, 327–336.

Hirai, N., Tagawa, M., Kaneko, T., Seikai, T., Tanaka, M., 1999. Distributional changes in branchial chloride cells during freshwater adaptation in Japanese sea bass *Lateolabrax japonicus*. Zool. Sci. 16, 43–49.

Hiroi, J., McCormick, S.D., 2007. Variation in salinity tolerance, gill Na^+/K^+-ATPase, Na^+/K^+/2Cl-cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. J. Exp. Biol. 210, 1015–1024.

Hirose, S., Kaneko, T., Naito, N., Takei, Y., 2003. Molecular biology of major components of chloride cells. Comp. Biochem. Physiol. B 136, 593–620.

Hwang, P.P., Lee, T.H., 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem. Physiol. A 148, 479–497.

Hwang, P.P., Sun, C.M., Wu, S.M., 1989. Changes of plasma osmolarity, chloride concentration and gill Na^+, K^+-ATPase activity in tilapia *Oreochromis mossambicus* during seawater acclimation. Mar. Biol. 100, 295–299.

Hwang, P.P., Fang, M.J., Tsai, J.C., Huang, C.J., Chen, S.T., 1998. Expression of mRNA and protein of Na^+-K^+-ATPase alpha subunit in gills of tilapia (*Oreochromis mossambicus*). Fish Physiol. Biochem. 18, 363–373.

Imsland, A.K., Gunnarsson, S., Foss, A., Stefansson, S.O., 2003. Gill Na^+, K^+-ATPase activity, plasma chloride and osmolality in juvenile turbot (*Scophthalmus maximus*) reared at different temperatures and salinities. Aquaculture 218, 671–683.

Inoue, K., Takei, Y., 2002. Diverse adaptability in Oryzias species to high environmental salinity. Zool. Sci. 19, 727–734.

Inoue, K., Takei, Y., 2003. Asian medaka fishes offer new models for studying mechanisms of seawater adaptation. Comp. Biochem. Physiol. B 136, 635–645.

Jensen, M.K., Madsen, S.S., Kristiansen, K., 1998. Osmoregulation and salinity effects on the expression and activity of Na^+, K^+-ATPase in the gills of European sea bass, *Dicentrarchus labrax* (L.). J. Exp. Zool. 282, 290–300.

Kato, A., Doi, H., Nakada, T., Sakai, H., Hirose, S., 2005. *Takifugu obscurus* is a euryhaline fugu species very close to *Takifugu rubripes* and suitable for studying osmoregulation. BMC Physiol. 5, 18.

Katoh, F., Kaneko, T., 2003. Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established time-differential double fluorescent staining technique. J. Exp. Biol. 206, 4113–4123.

Katoh, F., Shimizu, A., Uchida, K., Kaneko, T., 2002. Shift of chloride cell distribution during early life stages in seawateradapted killifish, *Fundulus heteroclitus*. Zool. Sci. 17, 11–18.

Kelly, S.P., Woo, N.Y.S., 1999. Cellular and biochemical characterization of hyposmotic adaptation in a marine teleost, *Sparus sarba*. Zool. Sci. 16, 505–514.

Kelly, S.P., Chow, I.N.K., Woo, N.Y.S., 1999. Haloplasticity of black seabream (*Mylio macrocephalus*): hypersaline to freshwater acclimation. J. Exp. Zool. 283, 226–241.

Kodak Digital Science 1D, 1995. Kodak 1D image analysis software.Windows Version 2.0.3. Eastman Kodak Company, Rochester, NY.

Lee, T.H., Hwang, P.P., Shieh, Y.E., Lin, C.H., 2000. The relationship between 'deep-hole' mitochondria-rich cells and salinity adaptation in the euryhaline teleost, *Oreochromis mossambicus*. Fish Physiol. Biochem. 23, 133–140.

Lee, T.H., Feng, S.H., Lin, C.H., Hwang, Y.H., Huang, C.L., Hwang, P.P., 2003. Ambient salinity modulates the expression of sodium pumps in branchial mitochondria-rich cells of Mozambique tilapia, *Oreochromis mossambicus*. Zool. Sci. 20, 29–36.

Lin, C.H., Lee, T.H., 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, Y.M., Chen, C.N., Lee, T.H., 2003. The expression of gill Na^+, K^+-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and fresh water. Comp. Biochem. Physiol. A 135, 489–497.

Lin, C.H., Huang, C.L., Yang, C.H., Lee, T.H., Hwang, P.P., 2004a. Time-course changes in the expression of Na^+, K^+-ATPase and the morphometry of mitochondrion-rich cells in gills of euryhaline tilapia (*Oreochromis mossambicus*) during freshwater acclimation. J. Exp. Zool. 301A, 85–96.

Lin, C.H., Tsai, R.S., Lee, T.H., 2004b. Expression and distribution of Na^+, K^+-ATPase in gills and kidneys of the green spotted pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. Comp. Biochem. Physiol. A 138, 287–295.

Lin, Y.M., Chen, C.N., Yoshinaga, T., Tsai, S.C., Shen, I.D., Lee, T.H., 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.

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

Marshall, W.S., Bryson, S.E., 1998. Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. Comp. Biochem. Physiol. A 119, 97–106.

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, S.D., 1993. Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Can. J. Fish. Aquat. Sci. 50, 656–658.

Nilsen, T.O., Ebbesson, L.O.E., Madsen, S.S., McCormick, S.D., Andresson, E., Björnsson, B.T., Prunet, P., Stefansson, S.O., 2007. Differential expression of gill Na^+, K^+-ATPase alpha- and beta-subunits, Na^+,K^+,2Cl-cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. J. Exp. Biol. 210, 2885–2896.

Peterson, G.L., 1978. A simplified method for analysis of inorganic phosphate in the presence of interfering substances. Anal. Biochem. 84, 164–172.

Richards, J.G., Semple, J.W., Bystriansky, J.S., Schulte, P.M., 2003. Na^+/K^+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. J. Exp. Biol. 206, 4475–4486.

Roberts, T.R., 1998. Systematic observations on tropical Asian medakas or ricefishes of the genus Oryzias with descriptions of four new species. Ichthyol. Res. 45, 213–224.

Rozen, S., Skaletsky, H.J., 2000. Primer3 on theWWWfor general users and for biologist programmers. In: Krawetz, S., Misener, S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 365–386.

Sakamoto, T., Kozaka, T., Takahashi, A., Kawauchi, H., Ando, M., 2001a. Medaka (*Oryzias latipes*) as a model for hypoosmoregulation of euryhaline fishes. Aquaculture 193, 347–354.

Sakamoto, T., Uchida, K., Yokota, S., 2001b. Regulation of the ion-transporting mitochondrial-rich cell during adaptation of teleost to different salinities. Zool. Sci. 18, 1163–1174.

Scheiner-Bobis, G., 2002. The sodium pump — its molecular properties and mechanics of ion transport. Eur. J. Biochem. 269, 2424–2433.

Scott, G.R., Richards, J.G., Forbush, B., Isenring, P., Schulte, P.M., 2004a. Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. Am. J. Physiol. 287, C300–309.

Scott, G.R., Rogers, J.T., Richards, J.G., Wood, M.C., Schulte, P.M., 2004b. Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation. J. Exp. Biol. 207, 3399–3410.

Seidelin, M., Madsen, S.S., Blenstrup, H., Tipsmark, C.K., 2000. Time-course changes in the expression of Na^+, K^+-ATPase in gills and pyloric caeca of brown trout (*Salmo trutta*) during acclimation to seawater. Physiol. Biochem. Zool. 73, 446–453.

Singer, T.D., Clements, K.M., Semple, J.W., Schulte, P.M., Bystriansky, J.S., Finstad, B., Fleming, I.A., McKinley, R.S., 2002. Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. Can. J. Fish. Aquat. Sci. 59, 125–135.

Shikano, T., Fujio, Y., 1998a. Immunolocalization of Na^+, K^+-ATPase and morphological changes in two types of chloride cells in the gill epithelium during seawater and freshwater adaptation in a euryhaline teleost, *Poecilia reticulata*. J. Exp. Zool. 281, 80–89.

Shikano, T., Fujio, Y., 1998b. Immunolocalization of Na^+/K^+-ATPase in branchial epithelium of chum salmon fry during seawater and freshwater acclimation. J. Exp. Biol. 201, 3031–3040.

Shiraishi, K., Kaneko, T., Hasegawa, S., Hirano, T., 1997. Development of multicellular complexes of chloride cells in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) embryos and larvae in seawater. Cell Tissue Res. 288, 583–590.

Takeyasu, K., Tamkum, M.M., Renaud, K.J., Fambrough, D.M., 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.

Tipsmark, C.K., Madsen, S.S., Seidelin, M., Christensen, A.S., Cutler, C.P., Cramb, G., 2002. Dynamics of Na^+, K^+,2Cl-cotransporter and Na^+, K^+-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). J. Exp. Zool. 293, 106–118.

Tipsmark, C.K., Madsen, S.S., Borski, R.J., 2004. Effect of salinity on expression of branchial ion transporters in striped bass (*Morone saxatilis*). J. Exp. Zool. 301A, 979–991.

Tse, W.K.F., Au, D.W.T., Wong, C.K.C., 2006. Characterization of ion channel and transporter mRNA expressions in isolated gill chloride and pavement cells of seawater acclimating eels. Biochem. Biophys. Res. Comm. 346, 1181–1190.

Uchida, K., Kaneko, T., 1996. Enhanced chloride cell turnover in the gills of chum salmon fry in seawater. Zool. Sci. 13, 655–660.

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.

Versamos, S., Diaz, J.P., Charmantier, G., Flik, G., Blasco, C., Connes, R., 2002. Branchial chloride cells in sea bass (*Dicentrarchus labrax*) adapted to fresh water, seawater, and doubly concentrated seawater. J. Exp. Zool. 293, 12–26.

Wittbrodt, J., Shima, A., Schartl, M., 2002. Medaka — a model organism from the far East. Nat. Rev. Genet. 3, 53–64.

Wong, C.K.C., Chan, D.K.O., 1999. Chloride cell subtypes in the gill epithelium of Japanese eel *Anguilla japonica*. Am. J. Physiol. 277, R517–R522.

Woo, N.Y.S., Chung, K.C., 1995. Tolerance of *Pomacanthus imperator* to hypoosmotic salinities: changes in body composition and hepatic enzyme activities. J. Fish Biol. 47, 70–81.

Wood, C.M., Marshall, W.S., 1994. Ion balance, acid-base regulation, and chloride cell function in the common killifish, *Fundulus heteroclitus* — a euryhaline estuarine teleosts. Estuaries 17, 34–52.