J Comp Physiol B (2016) 186:485–501
DOI 10.1007/s00360-016-0972-6

ORIGINAL PAPER

Does Japanese medaka (*Oryzias latipes*) exhibit a gill Na^+/K^+-ATPase isoform switch during salinity change?

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Received: 18 November 2015 / Revised: 2 February 2016 / Accepted: 12 February 2016 / Published online: 26 February 2016

Abstract Some euryhaline teleosts exhibit a switch in gill Na^+/K^+-ATPase (Nka) α isoform when moving between fresh water (FW) and seawater (SW). The present study tested the hypothesis that a similar mechanism is present in Japanese medaka and whether salinity affects ouabain, Mg^{2+}, Na^+ and K^+ affinity of the gill enzyme. Phylogenetic analysis classified six separate medaka Nka α isoforms (α1a, α1b, α1c, α2, α3a and α3b). Medaka acclimated long-term (>30 days) to either FW or SW had similar gill expression of *α1c, α2, α3a* and *α3b,* while both *α1a* and *α1b* were elevated in SW. Since a potential isoform shift may rely on early changes in transcript abundance, we conducted two short-term (1–3 days) salinity transfer experiments. FW to SW acclimation induced an elevation of *α1b* and *α1a* after 1 and 3 days. SW to FW acclimation reduced *α1b* after 3 days with no other α isoforms affected. To verify that the responses were typical, additional transport proteins were examined. Gill *ncc* and *nhe3* expression were elevated in FW, while *cftr* and *nkcc1a* were up-regulated in SW. This is in accordance with putative roles in ion-uptake and secretion. SW-acclimated medaka had higher gill Nka V_{max} and lower apparent K_m for Na^+ compared to FW fish, while apparent affinities for K^+, Mg^{2+} and ouabain were unchanged. The present study showed that the Japanese medaka does not exhibit a salinity-induced α isoform switch and therefore suggests that Na^+ affinity changes involve altered posttranslational modification or intermolecular interactions.

Keywords α-Subunit · Euryhaline teleost · Na^+/K^+-ATPase enzymatic activity · Osmoregulation · Substrate affinity

Communicated by I.D. Hume.

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Introduction

Teleost fishes maintain internal osmotic and ionic balance independent of external salinity and ion and water homeostasis are consequently under constant threat. In fresh water (FW), fish experience diffusive ion loss and osmotic water gain while seawater (SW) causes ion gain and dehydration (Marshall and Grosell 2006). Ion homeostasis is dependent on key osmoregulatory organs (gill, kidney and intestine) and in euryhaline fishes the phenotypic plasticity of the gill is especially remarkable. Here a complete reversal of active monovalent ion transport occurs, rendering the tissue from adsorptive in FW to secretory in SW (Evans et al. 2005).

Secretion of NaCl by SW ionocytes involves a basolaterally located Na^+/K^+-ATPase (Nka) and Na^+, K^+, $2Cl^-$ cotransporter (Nkcc) working in conjunction with an apical cystic fibrosis transmembrane conductance regulator chloride channel (Cftr: Hiroi and McCormick 2012; Marshall and Singer 2002; Silva et al. 1977) and a cation-selective paracellular exit path for sodium (Degnan and Zadunaisky 1980; Hwang and Hirano 1985). This model appears general and is largely confirmed in a series of teleosts species such as tilapia (*Oreochromis mossambicus*: Hiroi et al. 2005, 2008; Tipsmark et al. 2011), killifish (*Fundulus heteroclitus*: Marshall and Singer 2002), Japanese medaka (*Oryzias latipes*: Hsu et al. 2014) and a number of salmonids (Bystriansky

et al. 2006; McCormick et al. 2009; Nilsen et al. 2007; Tipsmark and Madsen 2009). Ion absorption by branchial FW ionocytes is less well understood and has been a topic of controversy for quite some time, possibly due to species differences and diverse methods being applied to classify these cells (Dymowska et al. 2012; Evans et al. 2005; Hirose et al. 2003; Hwang et al. 2011; Marshall and Singer 2002; Perry et al. 2003). In the gill of FW medaka, three distinct ionocytes were recently classified, all of which express a basolateral Nka (Hsu et al. 2014) but characterized by presence of other specific ion transport proteins. One cell type has high expression of apical Na^+,Cl^- cotransporter (Ncc) while another shows high apical Na^+/H^+ exchanger (Nhe3), both with a putative role in monovalent ion absorption. A third FW ionocyte with a putative role in calcium homeostasis has strong apical expression of the epithelial Ca^{2+} channel protein. Apical expression of Nhe3 has also been found in FW gill of rainbow trout (*Oncorhynchus mykiss*: Hiroi and McCormick 2012; Ivanis et al. 2008) and tilapia (*O. mossambicus*: Hiroi et al. 2008; Inokuchi et al. 2008). An apical vacuolar-type H^+-ATPase (Vata) was observed in the FW gill of rainbow trout (*O. mykiss*: Wilson et al. 2000) and was also expressed in FW Atlantic salmon gill (*Salmo salar*: Bystriansky and Schulte 2011). Gill ionocytes with putative function in ion uptake and apical Ncc immunostaining have been reported in tilapia (*O. mossambicus*: Hiroi et al. 2008) and zebrafish (*Danio rerio*: Wang et al. 2009).

The Nka appears to be the primary driving force for ion uptake in FW and ion secretion in SW gills (Foskett and Scheffey 1982). The Nka exchanges three intracellular Na^+ for two extracellular K^+ ions at the cost one ATP, maintaining ion gradients favorable for vectorial transepithelial ion transport (Glynn 1985, 1993). In most euryhaline teleosts, gill Nka activity and abundance increase after SW acclimation (Johnston and Saunders 1981; Kelly and Woo 1999; Madsen and Naamansen 1989; McCormick et al. 1989). A switch in gill expression between specific α-subunit genes has been documented in several teleosts after transfer from hyposmotic FW to hyperosmotic SW or vice versa, and seems in these species to be instrumental for acclimation to the new environment (*O. myskiss*: Bystriansky et al. 2006; Richards et al. 2003; *S. salar*: Bystriansky et al. 2006; Madsen et al. 2009; McCormick et al. 2009; *Salvelinus alpinus*: Bystriansky et al., 2006; *O. mossambicus*: Feng et al. 2002; Lee et al. 1998; Tipsmark et al. 2011; *Anabus testudineus*: Ip et al. 2012; *Galaxias maculatus*: Urbina et al. 2013). Recent molecular analyses of these salinity responsive α1 isoforms suggest that they developed through parallel evolution in different fish species (Dalziel et al. 2014). The nomenclature in these fishes is based on similar regulation by salinity with Nka *α1b* elevated in SW and *α1a* in FW. Furthermore a conserved amino acid substitution (Lys-Arg) in Nka α1a transmembrane domain 5 (TM5) of salmonids and tilapia could render it energetically suitable for Na^+ transport against steep electrochemical gradients as in FW (Dalziel et al. 2014; Jorgensen 2008). This suggests that in these species, α1a provides the driving force for ion uptake in FW, while α1b appears critical to ion secretion in SW. In climbing perch the Lys-Arg substitution is present in both α1a and α1b but absent in α1c. However, in this species, expression of *α1a* is highest in FW while both *α1b* and *α1c* increase in SW suggesting an important role of the latter isoforms in the hyperosmotic environment (Ip et al. 2012).

Japanese medaka have been used for a while as a model for understanding ionoregulatory function in euryhaline fishes (Bossus et al. 2015; Hsu et al. 2014; Inoue and Takei 2002, 2003; Madsen et al. 2014; Sakamoto et al. 2001). This FW species is native to marshes, ponds and irrigation canals of rice fields in Japan, Korea and China (Takehana et al. 2003) and is capable of adapting to a wide range of salinities (Haruta et al. 1991; Inoue and Takei 2002; Miyamoto et al. 1986; Shen et al. 2011). An essential step in developing this model is characterization of the molecular driving force for gill ion transport in FW and SW ionocytes. The hypothesis tested in the current study is that medaka exhibit α1 isoform shift in the gill when switching between hypo- and hyperosmoregulation. Given the scarcity of species on which studies have been published so far, this is by no means a trivial hypothesis as testing this occurrence is important for our understanding of euryhalinity. Therefore, the primary aim of the present study was to identify α isoforms expressed in Japanese medaka and examine if salinity-induced α isoform switch occurs in medaka gill during salinity acclimation. The expression of additional transport proteins (*ncc*, *nhe3*, *cftr*, *nkcc1a*, *vata*, *fxyd9*, *fxyd11*) was examined in parallel to confirm that medaka gill respond in accordance with studies in other teleosts (Dymowska et al. 2012; Hiroi and McCormick 2012). A final goal addressed the potential of functional differences in gill Nka activity in FW and SW fish by investigating the apparent enzyme affinity for Na^+, K^+, Mg^{2+} and ouabain.

Materials and methods

Fish and maintenance

Adult Japanese medaka (*O. latipes*, Temmink and Schlegel; size range 25–35 mm, weight range 250–350 mg) were obtained from Aquatic Research Organisms (Hampton, NH, USA) and acclimated to recirculating de-chlorinated tap water, mechanically and biologically filtered (0.34 mM Na^+, 0.64 mM Ca^{2+}, 0.09 mM Mg^{2+}, 0.03 mM K^+). Fish were maintained at 20 °C with a 14 h light/10 h dark photoperiod. They were fed daily with Tetramin tropical flakes (Tetra, United Pet Group, Blacksburg, VA, USA) or frozen brine shrimp (San Fanscisco Bay Brand, Inc., Newark, CA,

Table 1 Primer sequences for quantitative PCR of medaka transcript targets

Target name	Forward primer	Reverse primer	GenBank Accession #
α1a	ATGACAAAGAACCGCATCCT	GTACATTCTGAGGGCGGTGT	XM_004084864
α1b	GAACCGTCACCATCCTCTGT	TGTCGCTCTCAGCTTCTTCA	XM_004066527
α1c	GATCGAGCACCATCCTCTGT	CCAAGGATGAGGGAGAGGAT	XM_004066525
α2	TTCAGTGGGCGGATCTTATC	CAGAGCCGTCTCAACAAACA	XM_004078573
α3a	CGTCATCATGGCTGAAAATG	ATTGCTGGCCATAGCTGTCT	XM_004074068
α3b	TTGCCCCCTTAATGTCACTC	GGGGCAGTTGTGATGAAAAT	XM_004078003
fxyd-9	GGTGTTTGCAGAAGAGACTTG	TGATGCCAATGAGACAAAGG	JX565422
fxyd-11	TGGAAACTGAAGCAAATCCA	TATCAACAGGCCGACAATGA	JX624726
cftr	GGGAAGAGGTGATGGAGACA	CACAATGGCGGAGAAGAAGT	XM_004086223
nhe-3	CATTTTTCGTGGTTGCCTTT	TTTTTGGTGCATCTGGTCAA	XM_004074310
ncc	GGGCCTATGTGCTCTGCTAC	TTGAGATCAAGGCTGCAATG	XM_004084987
nkcc-1a	CCCATCATCTCTGCTGGAAT	TTACACAGGGCCTGGAAAAC	XM_004084607
vat-a	GTGGCAACGAGATGTCTGAA	CAGGGCCGTTCTCTTCATAA	XM_004076355
s18	ACCCAGCTGCTGTCTCAGTT	TTGGACACCTCCTTCTGCTT	XM_004085258
rpl-7	CAGATGGAGAGGCGTGAGAT	CCTGATGACAAAGGCCAGTT	NM_001104870
actb	GAGAGGGAAATTGTCCGTGA	CTTCTCCAGGGAGGAAGAGG	NM_001104808
ef1a	ACGTGTCCGTCAAGGAAATC	TGATGACCTGAGCGTTGAAG	NM_001104662

USA). Food was withheld during the short-term salinity transfer experiments from one day before and throughout the experiment. All handling and experimental procedures were approved by the Animal Care and Use Committee of the University of Arkansas (IACUC 11005).

Experimental design and sampling

While Japanese medaka are capable of acclimating to full strength SW, they require a gradual transition to maintain good survival rates (Inoue and Takei 2002). Therefore, a salinity of 28–30 ppt was chosen as in a previous study by Sakamoto et al. (2001). In this way, the fish were hyperosmotically challenged with osmolalities 2.5- to 3-fold ordinary plasma levels while not compromising the 3 day experiments. For tissue distribution, 4 FW and 4 SW (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) medaka were long-term acclimated to their respective salinities for a month prior to sampling. Food was withheld one day before sampling. Fish were anesthetized in 100 mg L^{-1} tricaine methanesulfonate (Western Chemical Inc., Ferndale, WA) and killed by cervical dislocation. The following tissues were dissected: gill, kidney, intestine, muscle and liver. Tissues were immediately placed on dry ice and stored at −80 °C until further use. Gills from these fish were also used for comparison of specific targets between long-term acclimated FW and SW medaka. In the short-time course experiments (72 h), medaka were acclimated to respective salinities for at least a month prior to experimentation. They were then transferred from FW to SW or from SW to FW. Sampling occurred 24 and 72 h after transfer ($n = 6$) and each experiment included a sham-transfer group to serve as control. Gill filaments were dissected and promptly frozen on dry ice.

RNA extraction, cDNA synthesis and real-time qPCR

Tissues were homogenized in TRI Reagent® (Sigma Aldrich, St. Louis, MO, USA) using a Power Max 200 rotating knife homogenizer (Advanced Homogenizing System; Manufactured by PRO Scientific for Henry Troemner LLC, Thorofare, NJ, USA). Total RNA was extracted following the manufacturer's protocol. RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water. NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to estimate quantity and purity (A_{260}/A_{280}) of each sample. cDNA was synthesized from 1 µg total RNA in a final volume of 20 µL using Applied Biosystems high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocol. mRNA sequences for Japanese medaka target transcripts were identified in the Ensembl genome browser (Flicek et al. 2014) and used to design specific primers (Table 1) to detect the following transcripts: *nka α1a*, *α1b*, *α1c*, *α2*, *α3a*, *α3b*, *nkcc1a*, *ncc*, *nhe3*, *vata*, *cftr*, *fxyd9 and fxyd11*. Elongation factor 1-alpha (*ef1a*), β-actin (*actb*) and ribosomal protein PO (*rplp0*) were used as normalization genes (Vandesompele et al. 2002). Primers were generated using Primer3 software (Koressaar and Remm 2007; Untergasser et al. 2012). Quantitative real-time qPCR was performed in a final volume of 15 µL using BioRad CFX96 platform (BioRad, Hercules, CA, USA) and SYBR® Green JumpStar™ (Sigma Aldrich). The following thermocycling protocol was used:

3 min initial denaturation/activation phase (94 °C), 40 cycles of a 15 s denaturation step and an annealing/elongation steps for 60 s (60 °C), followed by a melting curve analysis at an interval of 5 s per degree from 55 to 94 °C. Amplification efficiency of each primer set was analyzed using the standard curve method and sequential dilutions from 2 to 16 times. Relative copy numbers of individual target genes was calculated using the primer set amplification efficiency. Relative copy numbers were calculated as $E_a^{\Delta Ct}$, where C_t is the threshold cycle number and E_a is the amplification efficiency (Pfaffl 2001). GeNorm software (Biogazelle, Zwijnaarde, Belgium) was used to calculate a geometric mean of the three normalization genes. Normalized units were calculated by dividing the relative copy number of each target gene by the geometric mean of normalization genes.

Nka enzyme assay

Gill Nka activity was measured in gill homogenates using a NADH-coupled assay as described by McCormick (1993) with modifications for use with microplate spectrophotometer (SpectraMax® Plus384, Molecular Devices, Sunnyvale, CA, USA; (Tipsmark and Madsen 2001). Whole gill apparatus was dissected and quickly frozen in SEI buffer (300 mM sucrose, 20 mM Na_2EDTA, 50 mM imidazole, pH 7.5). Prior to start the assay, gill apparatus was thawed on ice and homogenized in 0.5 mL ice-cold SEIDM buffer (SEI buffer with 0.1 % Na-deoxycholate and 10 mM mercaptoethanol) with the Power Max homogenizer. Samples were then centrifuged at 8000 rpm for 60 s. Supernatant was transferred to a new tube and diluted with 1.0 mL ice-cold SEIDM.

Maximal Nka activity (V_{max}) was measured by coupling ATP hydrolysis to the conversion of NADH to NAD^+ by pyruvate kinase (PK) and lactate dehydrogenase (LDH) and analyzed by kinetic readings at 340 nm for 10 min at 25 °C. Each homogenate was assayed under the following conditions with or without the presence of ouabain (0.5 mM) in triplicate: (in mM) 49.1 NaCl, 10.4 KCl, 1.8 $MgCl_2$, 0.5 Na_3-phosphoenolpyruvate, 0.5 Na_2-ATP, 0.16 Na_2NADH, 0.4 KCN, 2.6 units mL^{-1} PK, 2.0 units mL^{-1} LDH, in 50 mM imidazole buffer, pH 7.5. Protein content of tissue homogenates was measured by the Bradford method modified for microplate reader (Bradford 1976). Enzyme activity was normalized to protein content and expressed as μmoles ADP mg $protein^{-1}$ h^{-1} using the following equation: NKA Activity $= \frac{\frac{ABS_{340}}{min} \times \frac{60min}{h}}{\left(\frac{\Delta OD_{340}}{nmol\ ADP}\right) \times \left(0.01mL \times \frac{mg\ protein}{mL}\right)}$. Where ΔABS_{340} is the difference in slopes between assays of the same sample with and without ouabain and ΔOD_{340} $nmol^{-1}$ ADP is the calibration factor.

A series of assays were performed to examine the apparent affinity of Nka to the substrates Na^+ and K^+, the cofactor Mg^{2+} and inhibitor ouabain. In the salt substrate assays, the concentration of the cation examined was varied while the other two were held constant. Apparent K_m for Na^+ was measured (as described above) with varying concentrations of Na^+ (4.2, 9.2, 19.2, 34.2, 79.2, 104.2, 154.2 mM) while holding [K^+] (15 mM) and [Mg^{2+}] (2 mM) constant. Apparent K_m for K^+ was measured as described above holding [Na^+] (50 mM) and [Mg^{2+}] (2 mM) with varying concentrations of K^+ (0.4, 1.4, 3.4, 6.4, 10.4, 25.4, 50.4 mM). Additionally, apparent K_m for Mg^{2+} was measured as described above holding [Na^+] (50 mM) and [K^+] (15 mM) constant with varying concentrations of Mg^{2+} (0, 1.0, 1.5, 4.0, 8.0, 15.0 mM). Apparent Nka affinity to ouabain was evaluated by measuring Nka activity under conditions described above with varying concentrations of ouabain (0, 1.0×10^{-6}, 3.0×10^{-6}, 1.0×10^{-5}, 3.0×10^{-5}, 1.0×10^{-4}, 3.0×10^{-4}, 1.0×10^{-3}, 3.0×10^{-3}, 0.01, 0.03, 0.1, 0.3, 1.0 mM).

Phylogenetic analysis

Amino acid sequences of Nka from other species were obtained from GenBank using the following accession numbers: the climbing perch (*A. testudineus*) *α1a* (JN180940), *α1b* (JN180941), *α1c* (JN180942); rainbow trout (*O. mykiss*) *α1a* (NP001117933.1), *α1b* (NP001117932.1), *α1c* (NP001117931.1), *α2* (NP001117930.1), *α3* (NP001118102.1); inanga (*G. maculatus*) *α1a* (AFM73918.1), *α1b* (AFM73919.1), *α1c* (AFM73917.1), *α2* (AFM73922.1), *α3a* (AFM73920.1), *α3b* (AFM73921.1); tilapia (*O. niloticus*) *α1a* (XP_005452412.1), *α1b* (XP_003446597.1), *α1-3* (XP_005452414.1), *α1-4* (XP_003446598.1), *α1-5* (XP_003446653.1) *α2,* (XP_003447505.1), *α3-1* (XP_005459144.1), *α3-2*(XP_003450710.1); and the sea urchin (*Strongylocentrotus purpuratus*) *α* (NP_001116982.1). Based on the distant relationship of the sea urchin Nka, this protein was used as an outgroup in the analysis. Predicted sequences were aligned using ClustalW. The maximum likelihood consensus tree was generated using SEQBOOT, PROML and CONSENSE, all programs in the PHYLIP package (Felsenstein 1989). Alignment was also used to compare Nka α1 isoform transmembrane domain amino acid sequences. As only a partial sequence for inanga α1b was available from GenBank, this sequence was excluded from the transmembrane domain comparison.

Alignment of select functional areas of medaka α-subunits

Amino acid sequences of Nka were obtained from GenBank using the following accession numbers: *O. latipes* α1a (XM004084864), α1b (XM004066527), α1c (XM004066525); *O. niloticus* α1a (XP005452412.1), α1b (XP003446597.1), α1-3 (XP005452414.1), α1-4

(XP003446598.1), α1-5 (XP003446653.1); *O. mykiss* α1a (AY319391), α1b (AY319390) and α1c (AY319389); *G. maculatus* α1a (AFM73918.1) and α1c (AFM73917.1); *A. testudineus* α1a (JN180940), α1b (JN180941) and α1c (JN180942). Sequences were aligned using ClustalW.

Statistical analysis

Tissue expression data were analyzed by one-way ANOVA followed by Tukey's Honestly Significant Difference post hoc test. Time course experiments were analyzed by two-way ANOVA. When significant interaction between factors occurred this was followed by Bonferroni-adjusted Fisher's least significant difference test. When required, transformation of data was done to meet the ANOVA assumption of homogeneity of variances, as tested by Bartlett's test. Expression and Kinetics data of long-term acclimated FW and SW fish were compared using a Student's *t* test. A significance level of P value <0.05 was used throughout. All tests were performed using GraphPad Prism 5.0 software (San Diego, CA, USA).

Results

Phylogenetic analysis

We identified three Nka α1 isoforms (a, b and c), one α2 isoform and two α3 isoforms (a and b) in the genome of the Japanese medaka. The phylogenetic relationship of medaka Nka α isoforms was examined by constructing a tree including sequences from trout, inanga, Nile tilapia and climbing perch (Fig. 1). The α1c isoform was named

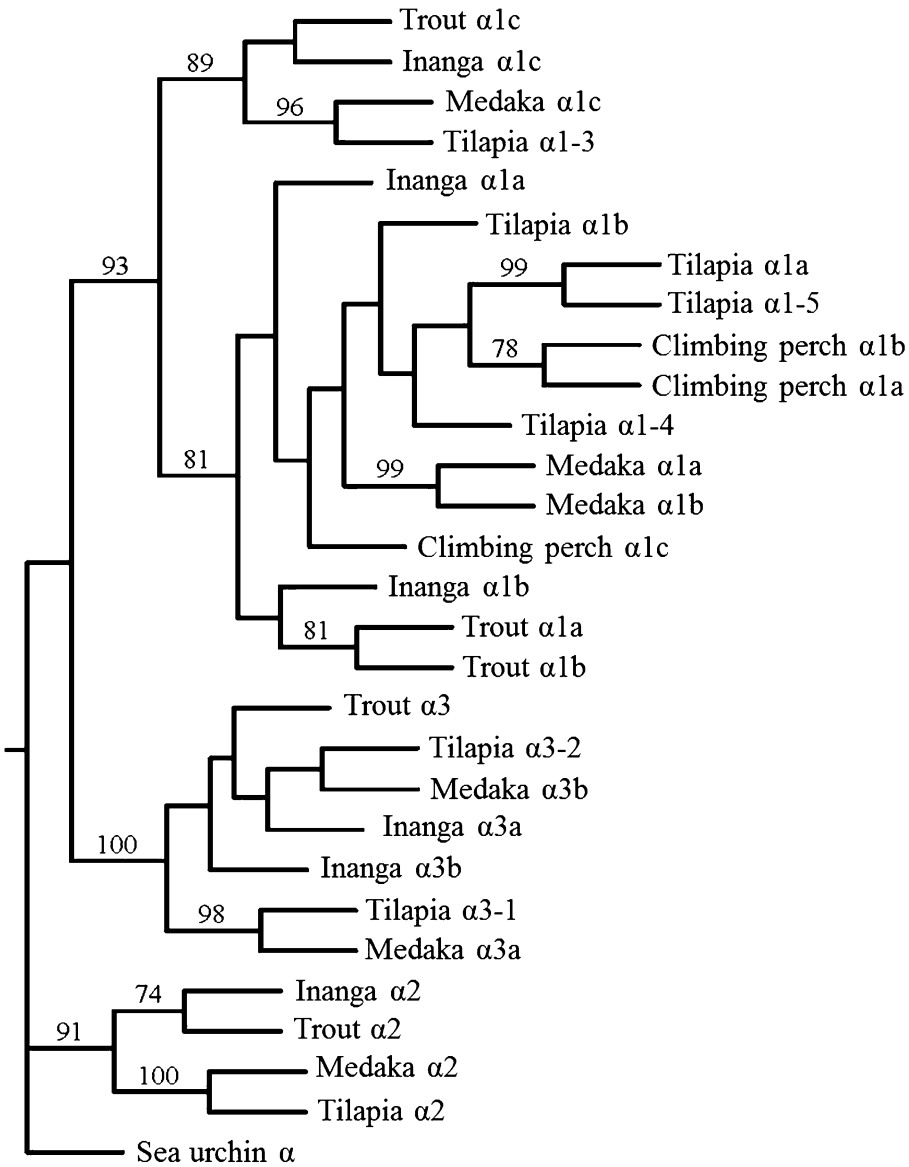

Fig. 1 The consensus tree was assimilated based on homology of medaka (*Oryzias latipes*) with other teleosts using maximum likelihood; trout (*Oncorhynchus mykiss*), inanga (*Galaxius maculatus*) and climbing perch (*Anabus testudineus*). Numbers represent bootstrap values in percent of 1000 replicates. Sea urchin (*Strongylocentrotus purpuratus*) Nka α-subunit was used as outgroup

based on its homology with tilapia α1-3, trout α1c and inanga α1c. The medaka α1a and b were clearly grouped together, separately from the other α1 isoforms and arbitrarily named a and b. Designation of medaka α2 was supported by the formation of a strong clade with inanga, trout and tilapia Nka α2. Medaka α3 isoforms were named based on their homology with the other teleost α3 isoforms.

Alignment of selected functional areas of medaka α-subunits

Amino acid sequences of Nka from medaka, rainbow trout, tilapia, inanga and climbing perch were aligned for comparison of the fifth (TM5), eight (TM8) and ninth (TM9) transmembrane domains (Fig. 2). Specifically, it shows that a lysine (Lys) substitution for asparagine 783 (Asn783) in TM5, a valine (Val) substitution for aspartate 933 (Asp933) in TM8 and a serine (Ser) substitution for glutamate 961 (Glu961) in TM9 are present in trout α1a compared with trout α1b and α1c. The Asn783-Lys substitution present in rainbow trout Nka α1a was also observed in tilapia α1a and α1.5 (latter not shown), inanga α1a (not shown) and climbing perch α1a and α1b. None of the medaka Nka α1 isoforms had this substitution. In TM8, the Asp933-Val substitution was only observed in rainbow trout α1a, while climbing perch α1a contained a threonine instead of Asp933. Finally, in TM9, only rainbow trout α1a exhibited a Glu961-Ser substitution while climbing perch α1b has a Val substitution at this position.

TM5	Ola.atp-α1a	IAYSLTS**N**IPELSPFLLFILASIP
	Ola.atp-α1b	IAYTLTS**N**IPEISPFLLFILASIP
	Ola.atp-α1c	IAYTLTS**N**IPEITPFLLFIIANIP
	Oni.atp-α1a	IAYTLTS**K**IPEMSPFLLFVIANIP
	Oni.atp-α1b	IAYTLTS**N**IPEISPFLLFIIANIP
	Omy.atp-α1a	ITYTLSS**K**IPEMTPFLFLLLANIP
	Omy.atp-α1b	IAYTLTS**N**IPEISPFLLFIIANIP
	Omy.atp-α1c	IAYTLTS**N**IPEITPFLFFIIANIP
	Ate.atp-α1a	IVYTLSS**K**IPEMSPFFFFAIANIP
	Ate.atp-α1b	IAYTLTS**K**IPEMSPFLFFVVASMP
	Ate.atp-α1c	IAYTLTS**N**IPEISPFLLFIIANIP
TM8	Ola.atp-α1a	CHTAFFISIVVVQWA**D**LIICK
	Ola.atp-α1b	CHTAFFTSIVIVQWA**D**LIICK
	Ola.atp-α1c	CHTAFFASIVIVQWA**D**LIICK
	Oni.atp-α1a	CHTAFFSSIVIVQVA**D**LLICK
	Oni.atp-α1b	CHTAFFASIVIVQWA**D**LIICK
	Omy.atp-α1a	CHTAYFAAVVIAQWA**V**LIVCK
	Omy.atp-α1b	CHTAFFASIVVVQWA**D**LIICK
	Omy.atp-α1c	CHTAFFASIVVVQWA**D**LIICK
	Ate.atp-α1a	CHTAYFVNIVVIRWF**T**LIIAK
	Ate.atp-α1b	CHTAFFISIVIVQWT**D**LLICK
	Ate.atp-α1c	CHTAFFVSIVIVQWA**D**LIICK
TM9	Ola.atp-α1a	LIFGLF**E**ETALAAFLSYCP
	Ola.atp-α1b	LIFGLI**E**ETALAAFLSYCP
	Ola.atp-α1c	LIFGLF**E**ETALAAFLSYCP
	Oni.atp-α1a	LIFGMF**E**ELALAVFLSYCP
	Oni.atp-α1b	LIFGLF**E**ETALAAFLSYCP
	Omy.atp-α1a	LIFGLC**S**ESALALFLSYCP
	Omy.atp-α1b	LIFGLF**E**ETALAVFLSYCP
	Omy.atp-α1c	LIFGLF**E**ETALAAFLSYCP
	Ate.atp-α1a	LIFGLF**E**ETALATFLSYCP
	Ate.atp-α1b	LIFGLF**V**ETALAAFLSYCP
	Ate.atp-α1c	LIFGLF**E**ETALAAFLSYCP

Fig. 2 Sequence alignment of Nka TM5, TM8 and TM9 segments. Nka aligned protein sequences include: Japanese medaka α1a, α1b and α1c (*Ola.*); tilapia α1a and α1b (*Oni.*); rainbow trout α1a, α1b and α1c (*Omy.*); and climbing perch α1a, α1b and α1c (*Ate.*). *Bold letters* indicate the Asn783 → Lys in TM5, Asp933 → Val in TM8 and Glu961 → Ser or Val in TM9. Alignment numbers are set according to the rainbow trout sequence (see Jorgensen 2008)

Tissue distribution

The *α1a* and *α1b* isoforms had a mRNA expression more than 10-fold higher in kidney, intestine and gill than in muscle and liver (Fig. 3a, b), while *α1c* levels were significantly higher in kidney than in the other tissues examined (Fig. 3c). Nka *α2* was prominently expressed in muscle with a transcript level from 20- to 100-fold higher than in any other examined tissues (Fig. 3d). Both *α3a* and *α3b* had highest expression in gill and kidney (Fig. 3e, f). Transcript levels of *fxyd9* were 2-fold higher in gill than in the other tissues (Fig. 4a). *fxyd11* showed more than 1000-fold higher expression in gill than in kidney and intestine and was not detected in muscle or liver (Fig. 4b). The *cftr* chloride channel was expressed in all five examined tissues with the highest levels in intestine > kidney > gill (Fig. 4c). *nhe3* had high transcript expression in gill and kidney with very low levels in the remaining tissues (Fig. 4e). Gill expression of *ncc* and *nkcc1a* were, respectively, 1000- and 10-fold higher than in the other examined tissues (Fig. 4d, f). Finally, the expression of *vata* was highest in gill and intestine (50–80 % higher; Fig. 4g). Figure 5 shows the relative abundance of the six Nka α isoforms in the gill of 12 FW and 12 SW medaka with *α1b* levels being 6-fold higher than *α1a* and 2–5 orders of magnitude higher than any other isoforms.

mRNA expression in FW and SW

In fish acclimated long-term to either FW or SW, expression of *α1a*, *α1b*, *fxyd11*, *cftr* and *nkcc1a* were elevated in SW gill (Fig. 6). FW-acclimated fish had a significantly higher gill transcript expression of *nhe3* and *ncc* when compared to the SW group (Fig. 6). No significant difference was exhibited in the transcript levels of *α1c*, *α2*, *α3a*, *α3b*, *fxyd9* or *vata* (Fig. 6).

Transcript levels of those genes were also analyzed in the gill during the initial acclimation stages after FW to SW transfer (Figs. 7, 8) or SW to FW transfer (Figs. 9, 10). Transfer to SW induced a 1.5-fold increase in *α1a* gill expression after

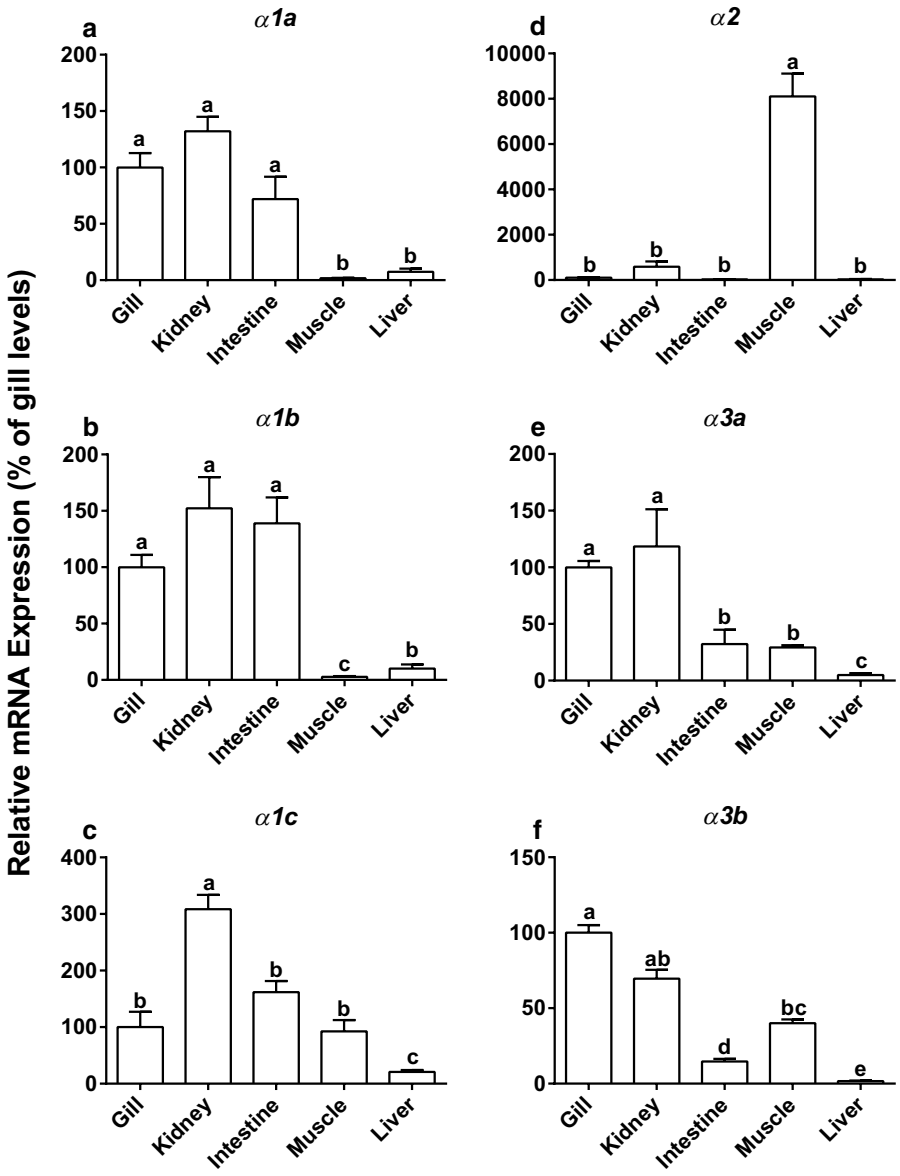

Fig. 3 Transcript levels of Nka *α1a* (**a**), *α1b* (**b**), *α1c* (**c**), *α2* (**d**), *α3a* (**e**) and *α3b* (**f**) in various tissues from medaka. Expression levels represent the mean value ± SEM of both FW- and SW-acclimated fish ($n = 8$) in 100 % of the gill levels. Significant difference between means is indicated by *different letters above bars* $P < 0.05$

72 h (Fig. 7a) while *α1b* increased 2–3 orders of magnitude from FW controls (Fig. 7b). There was no effect of salinity on gill *α1c* in the short-term SW transfer experiment (Fig. 7c). Transfer to SW had no significant effect on *α2, α3a* or *α3b* expression (Fig. 7d–f). However, SW induced a significant increase in expression of *fxyd11, cftr* and *nkcc1a* (Fig. 8b, c, f). *ncc* and *nhe3* both exhibited a significant decrease in SW (Fig. 8d, e). Short-term transfer to FW did not induce any significant change in *α1a*, *α1c*, *α2*, *α3a* or *α3b* (Fig. 9a, c–f). Additionally, there was no effect of FW on *fxyd9*, *fxyd11* or *vata* (Fig. 10a, b, g). After 72 h in FW, a decrease in *α1b* was observed (Fig. 9b) along with *cftr* and *nkcc1a* (Fig. 10c, f). Both *ncc* and *nhe3* increased in the gill during the short-term FW transfer experiment (Fig. 10d, e).

Gill Nka kinetic analysis

Maximal gill Nka activity (V_{max}) and apparent affinities for Na^+, K^+, Mg^{2+} and ouabain were analyzed in gill homogenates from long-term FW- and SW-acclimated fish (Table 2; Fig. 11). The apparent K_m for Na^+ was significantly higher in preparations from FW than SW gill. The other apparent affinities were not significantly different between the two salinities; however, V_{max} was highest in SW gill. Due to substrate inhibition at 150 mM Na^+ this data point was excluded in K_m calculation (Table 2; Fig. 11a). Similarly, cofactor inhibition by Mg^{2+} at 8.0 and 15 mM (latter not shown) were observed and these data points excluded from K_m calculations (Table 2; Fig. 11c).

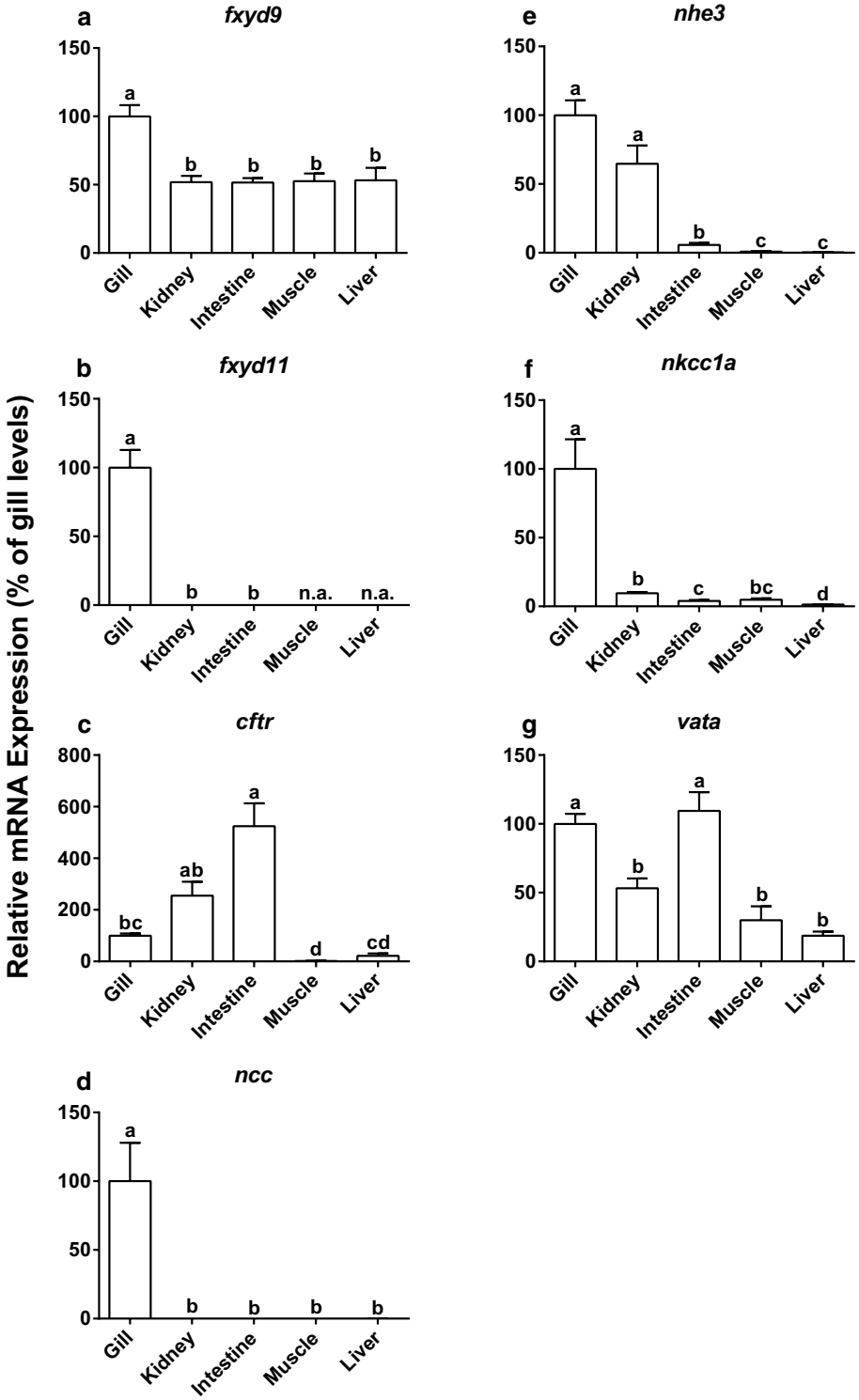

Fig. 4 Transcript levels of *fxyd9* (**a**), *fxyd11* (**b**), *cftr* (**c**), *ncc* (**d**), *nhe3* (**e**), *nkcc1a* (**f**) and *vata* (**g**) in various tissues from medaka. Expression levels represent the mean value ± SEM of both FW- and SW-acclimated fish ($n = 8$) in 100 % of the gill levels. Significant difference between means is indicated by *different letters above bars* $P < 0.05$

Discussion

Over the last decade it has become clear that in some euryhaline teleosts, such as salmonids and tilapia, there is a salinity-specific effect on the expression of gill Nka α-subunit isoforms. In these species, the α1a isoform seems to be the prominent isoform in FW ionocytes, whereas the α1b isoform becomes predominant during SW acclimation (Dalziel et al. 2014). The present study demonstrated that among the six medaka Nka α-subunit isoforms (α1a, α1b, α1c, α2, α3a and α3b) only *α1b* and, to a lesser extent, *α1a* were regulated by salinity; however,

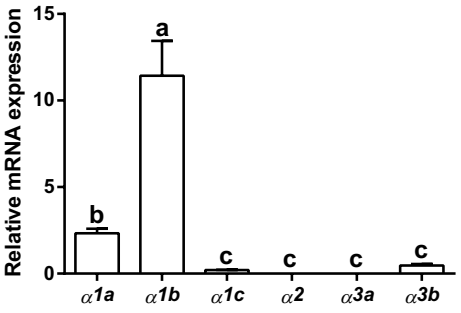

Fig. 5 Relative mRNA expression of Nka *α1a, α1b, α1c, α2, α3a* and *α3b* in medaka gill. mRNA expression values are normalized to normalization genes and represent the mean of 12 FW and 12 SW acclimated fish. Expression levels represent the mean value ± SEM. Significant difference between means is indicated by *different letters above bars*: $P < 0.05$

they were both stimulated by SW. When compared to relative transcript abundance *α1b* appears to be the most significant isoform in the gill, with *α1a* levels being somewhat lower and the other *α* isoforms detected at much lower levels. Sequence alignment of Nka α1 isoforms from medaka and other teleosts revealed that none of the medaka α isoforms has the amino acid substitution present in salmonids and tilapia α1a that is suggested to be critical to FW adaptation (Jorgensen 2008; Tipsmark et al. 2011). While emphasizing the role of these α1 isoforms in the medaka gill, it also shows that Nka isoform shift is not part of the medaka acclimation strategy. As expected, medaka gill exhibited increased expression of marker genes for FW ionocytes (nhe3 and ncc: Hiroi et al. 2008; Hsu et al. 2014; Inokuchi et al. 2008) in FW and for SW ionocytes (cftr and nkcc1a: Hiroi and McCormick 2012; Marshall and Singer 2002) in SW. Enzyme analysis revealed that gills from SW-acclimated fish have higher maximal Nka activity and higher apparent affinity for Na^+ compared to FW-acclimated fish, while K^+, Mg^{2+} and ouabain affinity were unaffected by salinity.

In silico analysis of medaka Nka isoforms

The phylogenetic analysis grouped medaka α1c, α2, α3a and α3b with their corresponding α isoforms of the other species included. According to the present phylogenetic analysis and recently published molecular analysis performed by Dalziel et al. (2014), α1a and α1b isoforms may have developed separately by parallel evolution. In a functional study, Jorgensen (2008) examined amino acid substitutions in trout α1a and α1b by site-directed mutagenesis in critical ion-binding sites of the Nka α-subunit (TM5, TM8 and TM9). Using porcine α1-subunit as a template, the substitution of lysine (Lys) in trout α1a for asparagine (Asn) at site 783, a critical cation binding site in TM5, resulted in decreased binding affinity for Na^+ and K^+. In TM8, the Asp933-Val substitution, as observed in rainbow trout α1a, decreased the binding affinity for K^+ (Jorgensen 2008). The combination of these two substitutions is suggested to diminish the affinity for K^+, more than that for Na^+, thus allowing the Nka to preferentially pump Na^+. Additionally, this results in the insertion of the Lys ε-amino group of TM5 in the cation binding site, possibly reducing the Na^+/ATP ratio from $3Na^+$/ATP to $2Na^+$/ATP which could render sodium uptake from a dilute media more feasible for the FW-type Nka (α1a) in salmonids. Molecular reconstruction of Nka TM9 revealed that the Glu961 side chain points away from cation binding sites toward the regulatory Fxyd subunit. Therefore, the Glu961-Ser substitution in rainbow trout α1a may interfere with and/or change interactions with the regulatory subunit. The amino acid substitution in TM5 of Nka α1a is found in salmonids, climbing perch and tilapia, supporting the notion of an adaptive advantage of this substitution during FW acclimation. This thus lends

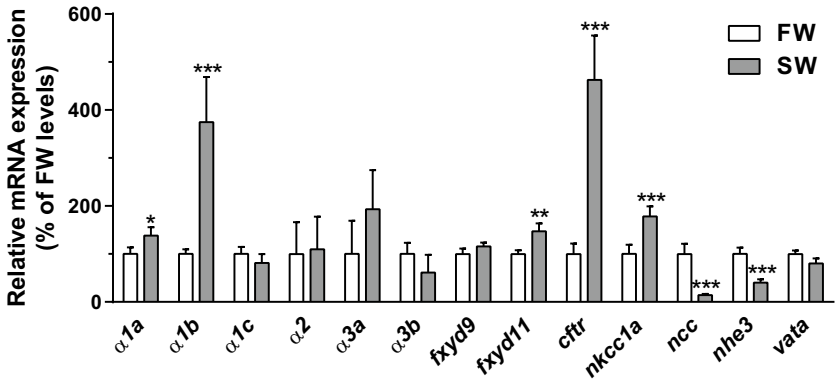

Fig. 6 Transcript levels of Nka *α1a*, *α1b*, *α1c*, *α2*, *α3a*, *α3b*, *fxyd9*, *fxyd11*, *cftr*, *nkcc1a*, *ncc*, *nhe3*, *vata* in gill from medaka acclimated to fresh water (FW) or seawater (SW). Fish were acclimated to the respective salinities for at least one month prior to sampling ($n = 12$). Expression levels represent the mean value ± SEM relative to 100 % of FW levels. *Asterisks* indicate a significant difference from FW expression: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

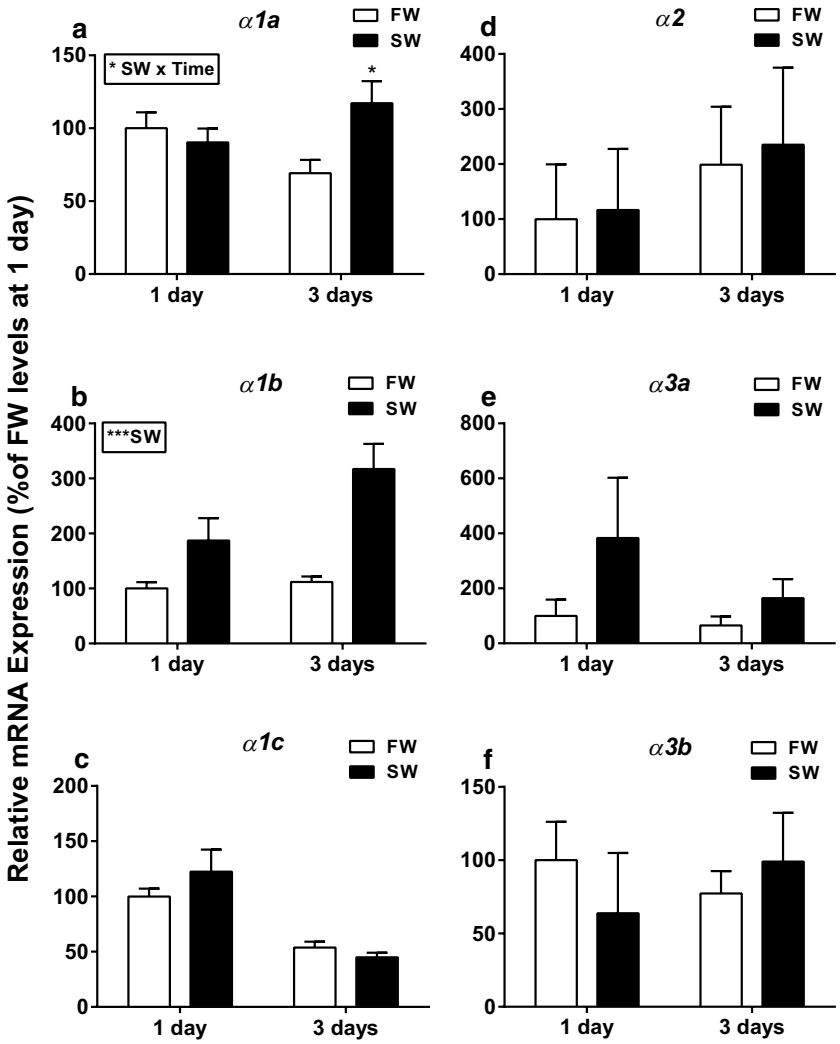

Fig. 7 Effect of FW-to-SW transfer on gill transcript levels of Nka *α1a* (**a**), *α1b* (**b**), *α1c* (**c**), *α2* (**d**), *α3a* (**e**) and *α3b* (**f**). Fish were transferred from FW to SW or FW to FW as a control and sampled at 24 and 72 h ($N = 6$). Expression levels represent the mean value ± SEM relative to 100 % of the 24 h-FW group. "SW" and "SW × Time" refers to overall effects and statistical interaction between factors as indicated by *asterisks*: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. When the interaction between factors are significant *asterisk* are placed above SW group at specific time-point ($*P < 0.05$)

insight as to why the α1 isoform switch is observed in some teleosts upon salinity challenge. In our study, the alignment of Nka α1 isoforms included the TM5, TM8 and TM9 domains of medaka, rainbow trout, tilapia, climbing perch and inanga (Ip et al. 2012; Urbina et al. 2013). The medaka Nka α1 isoforms did not exhibit any of the TM5, TM8 or TM9 substitutions observed in FW-type α1a in rainbow trout and other salmonids. If these amino acid substitutions in α1a are instrumental to ion absorption in trout ionocytes, the lack thereof in any medaka α isoforms may be important to our understanding of this euryhaline model. Thus, these solely in silico considerations do not support our initial hypothesis about salinity-induced Nka isoform shift in medaka.

Nka and Fxyd expression in the gills

The medaka Nka α isoforms identified were expressed in all tissues examined, however, with variable transcript levels. *α1a* and *α1b* were mostly expressed in osmoregulatory organs which is similar to *α1a* and *α1b* expression in rainbow trout (Richards et al. 2003), tilapia (Tipsmark et al. 2011) and climbing perch (Ip et al. 2012). Additionally, *α1c* exhibited ubiquitous expression and was unchanged by salinity in the gill which is also similar to findings in salmonids (Richards et al. 2003). Based on its wide tissue distribution, we suggest that *α1c* may play a universal role as a general housekeeping gene. In addition, this isoform is possibly involved in driving transepithelial ion transport in the gut and kidney (Tipsmark et al. 2010b). The transcript of *α2* was mostly found in muscle, which parallels *α2* expression in rats (Mobasheri et al. 2000). *α3a* and *α3b* were expressed at approximately the same level in the tested tissues. This is in accordance with findings in rainbow trout (Richards et al. 2003) where ubiquitous expression of *α3* was reported.

In the few euryhaline fishes examined, gill α1 isoform expression is strongly influenced by salinity. The predominant FW and SW isoforms have been named α1a and α1b, respectively (Bystriansky et al. 2006; Ip et al. 2012;

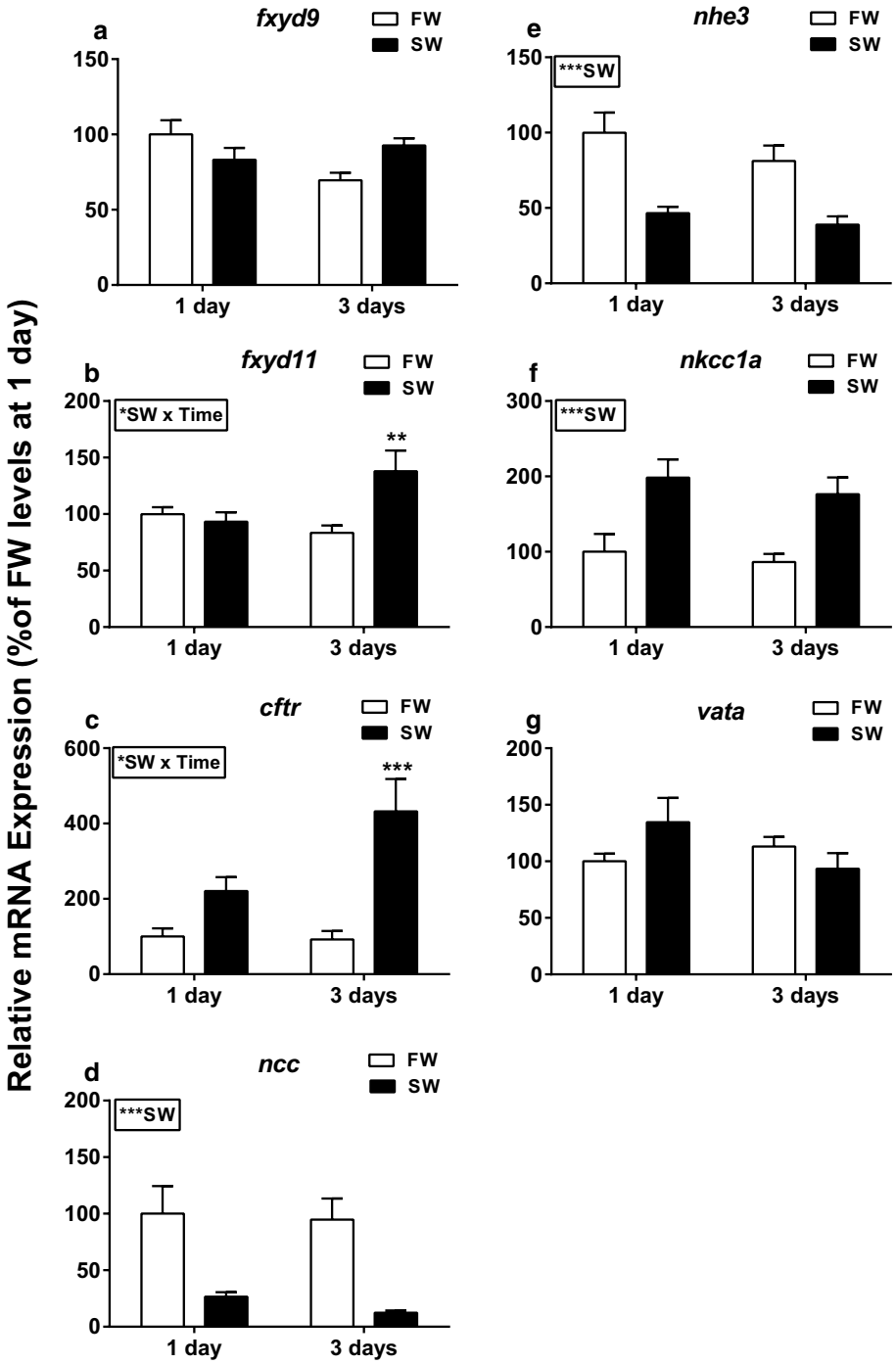

Fig. 8 Effect of FW-to-SW transfer on gill transcript levels of fxyd9 (**a**), fxyd11 (**b**), cftr (**c**), ncc (**d**), nhe3 (**e**) nkcc1a (**f**) and vata (**g**). Fish were transferred from FW to SW or FW to FW as a control and sampled at 24 and 72 h ($N = 6$). Expression levels represent the mean value ± SEM relative to 100 % of the 24 h-FW group. "SW" and "SW × Time" refers to overall effects and statistical interaction between factors as indicated by *asterisks*: $*P < 0.05$, $***P < 0.001$. When the interaction between factors are significant *asterisks* are placed above SW group at specific time-point ($**P < 0.01$, $***P < 0.001$)

Madsen et al. 2009; Richards et al. 2003; Tipsmark et al. 2011; Urbina et al. 2013). Our initial long-term acclimation experiment suggested that an upregulation of *α1a* and *α1b* is associated with SW acclimation in medaka with no apparent isoform shift. To further validate this observation, two short-term transfer experiments were conducted (1–3 days; FW-to-SW and SW-to-FW). It was evident from these data that no switch in α1, α2 or α3 isoforms occurs as part of the osmoregulatory strategy in this species. Furthermore, α1a and α1b are the only salinity-sensitive isoforms suggesting that they are central to ionocyte function in the gill. In the present study, medaka *α1b,* and to a smaller extent *α1a,* showed elevated transcript expression in the SW gill. This is similar to expression of *α1b* exhibited in trout gill (Richards et al. 2003) with an increase in SW and decrease in FW. The present study showed that the medaka gill does not exhibit a salinity-dependent Nka α1 isoform switch. While this deviates from observations in

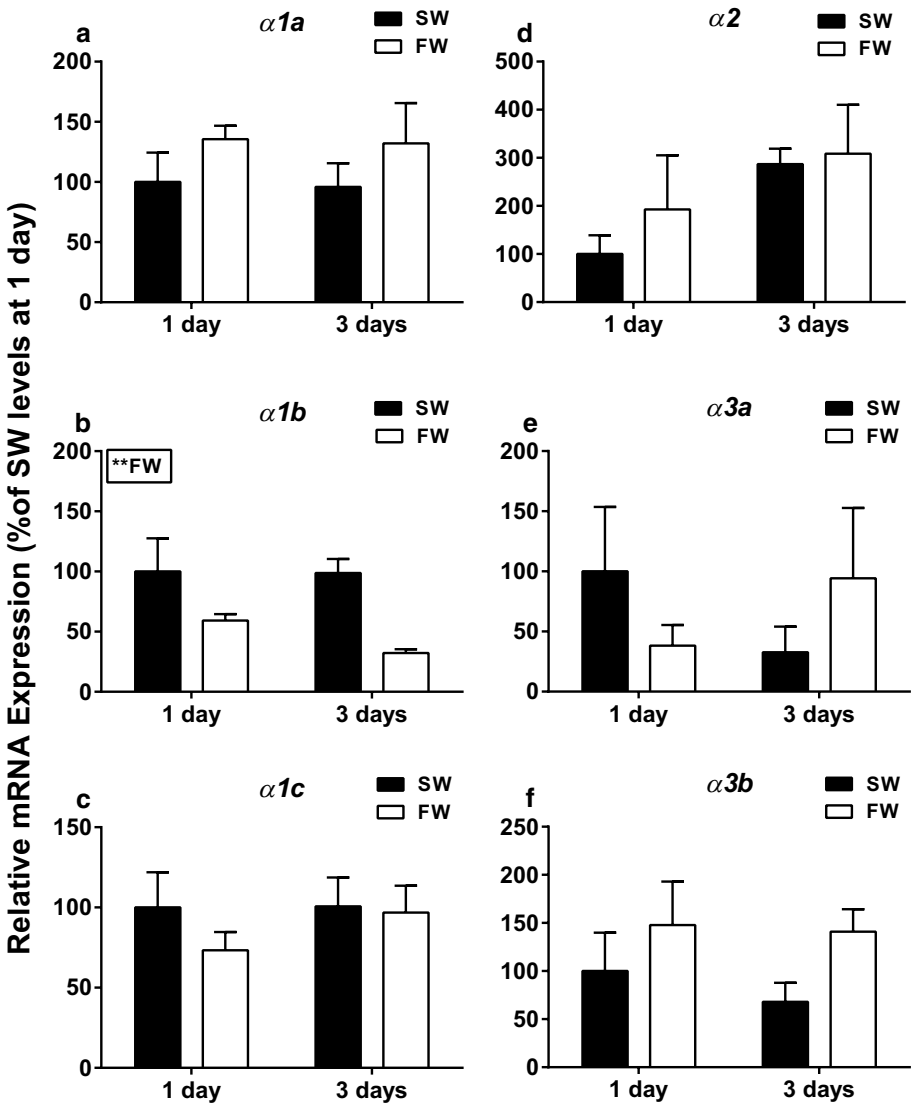

Fig. 9 Effect of SW-to-FW transfer on gill transcript levels of Nka *α1a* (**a**), *α1b* (**b**), *α1c* (**c**), *α2* (**d**), *α3a* (**e**) and *α3b* (**f**). Fish were transferred from SW to FW or SW to SW as a control and sampled at 24 and 72 h ($N = 6$). Expression levels represent the mean value ± SEM relative to 100 % of the 24 h-SW group. "FW" refer to overall effects as indicated by *asterisks*: $^{**}P < 0.01$

some other euryhaline fishes examined to date, it is similar to observations during salinity acclimation in the three-spine stickleback (*Gastrerostereus aculeatus*) where there is no sign of isoform shift, however, there is an isoform-specific stimulation after SW entry (Judd 2012; Madsen unpublished observations). In this species, mRNA levels of *atp1a1* isoform are highest in SW and lowest in FW as is the case for medaka *α1b* in the current study.

The Nka regulatory subunit is a single transmembrane protein that is often referred to as FXYD for its conserved extracellular motif: phenylalanine-X-tyrosine-aspartate (Sweadner and Rael 2000). The family of FXYD proteins has been shown to interact with and modulate kinetic properties of Nka (Garty and Karlish 2006). In Atlantic salmon eight FXYD isoforms were identified (Tipsmark 2008) and of these, *fxyd11* was almost exclusively expressed in the gills. Elevated expression of *fxyd11* was also demonstrated in SW gill of two medaka species (*O. dancena* and *O. latipes*: Yang et al. 2013), in SW-acclimated Atlantic salmon (Tipsmark et al. 2010a) as well as in zebrafish exposed to ion-poor FW (Saito et al. 2010); all cases were correlated with elevated *nka* expression. Gill Fxyd11 has been shown to interact specifically with the Nka α-subunit in Atlantic salmon (Tipsmark et al. 2010a) and brackish medaka (Yang et al. 2013). In the present study, gill Nka *α1a* and *α1b* mRNA levels along with *fxyd11* were elevated during and after SW acclimation suggesting co-expression and co-localization as demonstrated in other species. It is possible that divergent interaction of Nka with Fxyd11 in FW and SW gill is responsible for the difference in apparent kinetic properties we observed at the two salinities.

Other ion transporters in the gill

The mechanism of ion absorption in the FW gill is still under debate; therefore, the current study measured several

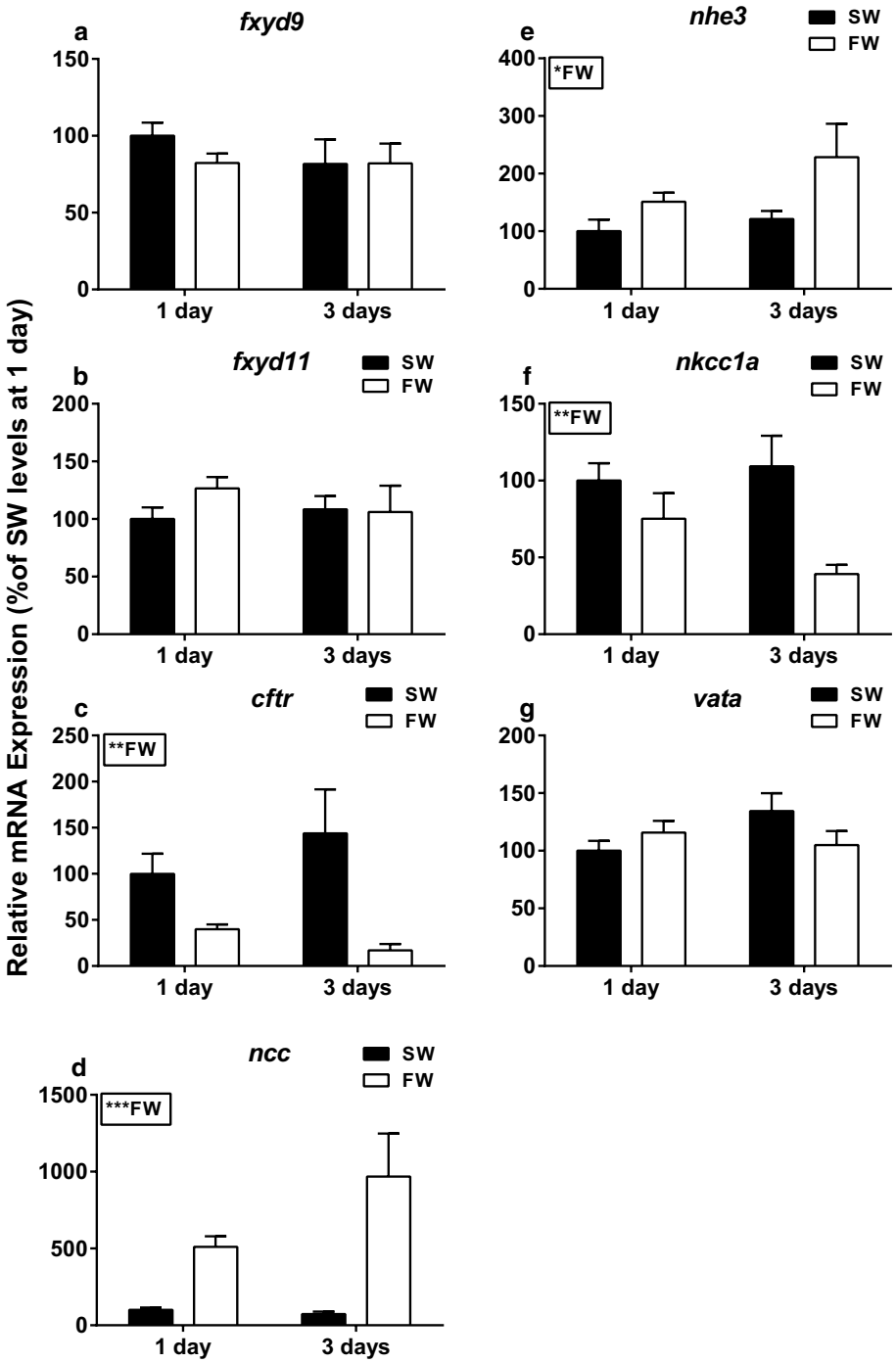

Fig. 10 Effect of SW-to-FW transfer on gill transcript levels of *fxyd9* (**a**), *fxyd11* (**b**), *cftr* (**c**), *ncc* (**d**), *nhe3* (**e**) *nkcc1a* (**f**) and *vata* (**g**). Fish were transferred from SW to FW or SW to SW as a control and sampled at 24 and 72 h ($N = 6$). Expression levels represent the mean value ± SEM relative to 100 % of the 24 h-FW group. "FW" refers to overall effects as indicated by *asterisks*: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

FW-type ion transporters. Gill expression of *nhe3* and *ncc* decreased rapidly upon exposure to SW and were significantly lower in SW long-term acclimated medaka which is similar to the salinity effect on *nhe3* and *ncc* in tilapia (Hiroi et al. 2008; Inokuchi et al. 2008). Expression of *vata* seems to be unresponsive to salinity as there was no difference between FW and SW gills at the transcript level. In contrast, *vata* expression increased in gill of Atlantic salmon when transferred to FW (Bystriansky and Schulte 2011). Our findings showing FW-induced *ncc* and *nhe3* for ion-absorption in gill is consistent with a recent study by Hsu and co-workers (2014), demonstrating apical localization of Ncc and Nhe3 in two separate populations of FW ionocytes in medaka. Furthermore, exposure of adult medaka to low Na^+ FW (0.03–0.05 mM) exhibited gill expression levels of *ncc-like2* comparable to those exposed to high Na^+ FW (9.2–10.5 mM; Hsu et al. 2014). It was previously hypothesized that transport of Na^+ against a steep gradient from

Table 2 Nka apparent affinities (Na^+, K^+, Mg^{2+} and ouabain) and maximal activity in gill samples of fresh water (FW) and seawater (SW) acclimated medaka

	FW	SW
K_m (mM)		
Na^+	8.97 ± 0.92	6.34 ± 0.67*
K^+	1.03 ± 0.25	0.86 ± 0.16
Mg^{2+}	1.03 ± 0.13	0.98 ± 0.10
V_{max} (μmol ADP mg^{-1} protein hr^{-1})	3.13 ± 0.42	4.54 ± 0.50*
IC_{50} (μM ouabain)	1.82 ± 0.74	1.09 ± 0.28

Values are expressed as mean ± SEM; $n = 12$. Significantly difference is indicated by asterisks (* $P < 0.05$)

FW (0.01–0.1 mM Na^+) may require the Asn783 to Lys substitution in Nka seen in a number of teleosts (Jorgensen 2008). Therefore, the inability of medaka to express a Nka α-subunit with a Lys substitution suggests that Nhe3 and Ncc may be more efficient in FW than previously expected.

In teleosts, the model for ion secretion in the branchial SW-ionocyte involves basolateral Nka and Nkcc1a, apical Cftr and a leaky paracellular pathway (Hiroi and McCormick 2012). Accordingly, we showed that transfer of medaka to SW increased gill *cftr* and *nkcc1a* while expression was down-regulated within 72 h in FW. Additionally, in long-term SW-acclimated medaka, gill Nka *α1a, α1b*, *cftr* and *nkcc1a* were significantly higher than in FW-acclimated medaka. This transcriptional data supports the idea that the SW medaka gill achieves ion secretion, at least in part, by the combined efforts of Nka α1b, Nkcc1a, Cftr and possibly Nka α1a.

Kinetic analysis

The present study revealed that SW-acclimated medaka display higher V_{max} than those acclimated to FW which is in accordance with previous findings in Japanese medaka (Kang et al. 2008). This is similar to findings in rainbow trout (Pagliarani et al. 1991), brown trout (Tipsmark and Madsen 2001), tilapia (Lin and Lee 2005), sea bass (Jensen et al. 1998), climbing perch (Ip et al. 2012) and the giant mudskipper (Chew et al. 2014). Substrate affinity assays for Na^+, K^+ and Mg^{2+} revealed the apparent affinity for Na^+ to be salinity-dependent as SW gills exhibited a higher binding affinity for Na^+. There was no difference in apparent affinities for K^+, Mg^{2+} or ouabain. According to Jorgensen (2008), the combined effect of the Asn783-Lys and Asp933-Val substitutions in TM5 and TM8, respectively, decrease the binding affinity for K^+ thus allowing trout Nka α1a to preferentially bind Na^+. The Japanese medaka lacks these substitutions that may increase Nka affinity for Na^+ in other euryhaline teleosts while in FW. However, in the present study we observed a lower affinity for Na^+ in FW than in SW medaka which could possibly be due to interactions with Fxyd11. In contrast, we have previously found a higher affinity for Na^+ in gill of FW-acclimated brown trout and Atlantic salmon when compared to SW-acclimated fish (Madsen and Tipsmark, unpublished) similar to findings in rainbow trout (Pagliarani et al. 1991), which could be a result of TM5 substitutions. Furthermore, in European sea bass no salinity difference in apparent ion affinities was observed (Jensen et al. 1998).

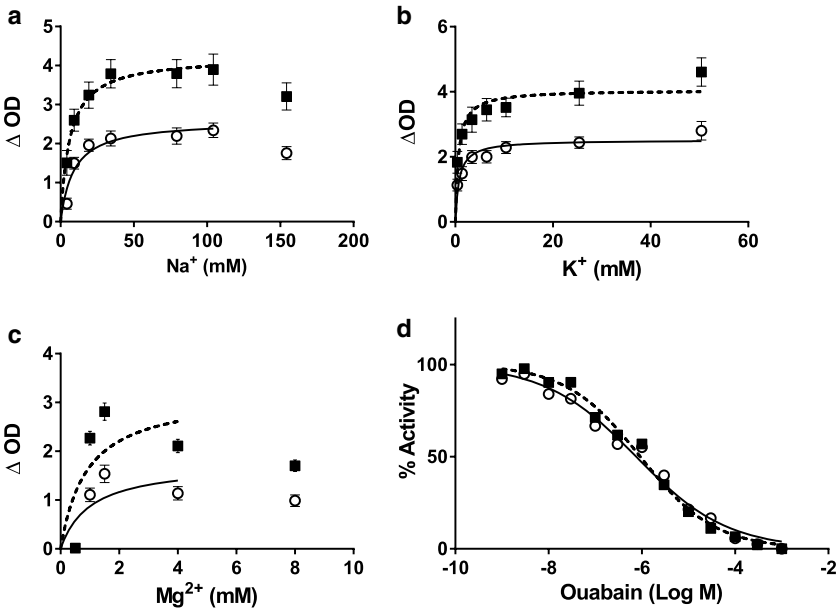

Fig. 11 Kinetic analysis of apparent Nka affinity (Na^+, K^+, Mg^{2+} and ouabain) in gill samples from fresh water (FW) and seawater (SW) acclimated medaka. Effects of varying concentrations of K^+ (**a**), Na^+ (**b**), Mg^{2+} (**c**) and ouabain (**d**) on Nka activity in gill homogenates from FW- (*open circle*) and SW-acclimated (*filled square*) Japanese medaka. Values are means of 12 fish ± SEM K_m and IC_{50} values are listed in Table 2. Substrate and cofactor inhibition resulted in the exclusion of some data points in the curve fitting for Na^+ and Mg^{2+}, respectively

Conclusions

The regulation of Nka α-subunits during salinity acclimation observed in Japanese medaka in the present study does not include a Nka isoform switch. While the current data are on the level of mRNA expression, the results from the long-term experiment are carefully verified by two separate short-term experiments that support the general conclusion. Furthermore, the mRNA levels of the dominant gill isoform, *α1b,* are stimulated during SW acclimation, suggesting a causal relationship to the elevated gill Nka activity observed. The regulatory pattern of Nka differs from the isoform shift observed in most euryhaline species examined so far (salmonid species like *O. myskiss*: Bystriansky et al. 2006; *A. testudineus*: Ip et al. 2012; Richards et al. 2003; *O. mossambicus*: Tipsmark et al. 2011; *G. maculatus*: Urbina et al. 2013). However, such different osmoregulatory patterns may not be altogether surprising especially given that euryhaline adaptation, including diadromous life cycles, is a reoccurring phenomenon in teleost evolution (Hiroi and McCormick 2012; Dalziel et al. 2014; Kultz 2015). The regulatory pattern in Japanese medaka is similar to that of the diadromous stickleback (Judd 2012) and emphasizes the importance of understanding Nka regulation at other levels, including interaction with other membrane components. The higher affinity for Na^+ observed in SW medaka may not be directly associated to changes in the primary structure of the catalytic α-subunit. The observed effects may instead relate to other mechanisms such as posttranslational modifications or intermolecular interactions with other membrane proteins or lipids (Cornelius and Mahmmoud 2007). In this context, it will be important to understand the functional significance of Fxyd11-Nka interactions previously demonstrated in branchial ionocytes of various species (zebrafish: Saito et al. 2010; Atlantic salmon: Tipsmark et al. 2010a; brackish medaka: Yang et al. 2013) in chronic and rapid regulation of Nka kinetics.

Acknowledgments This work was supported by a grant from the National Science Foundation [IBN 12-51616] and the Arkansas Biosciences Institute to C. K. T. The Fulbright Commission supported S. S. M. with a visiting professor scholarship. The Cell and Molecular Biology program at the University of Arkansas supported R. J. B. with a research stipend. The authors would like to thank Ms. R. T. Trubitt for technical help.

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