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Enhanced expression of *ncc1* and *clc2c* in the kidney and urinary bladder accompanies freshwater acclimation in Mozambique tilapia

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ABSTRACT

Euryhaline fishes maintain hydromineral balance in a broad range of environmental salinities via the activities of multiple osmoregulatory organs, namely the gill, gastrointestinal tract, skin, kidney, and urinary bladder. Teleosts residing in freshwater (FW) environments are faced with the diffusive loss of ions and the osmotic gain of water, and, therefore, the kidney and urinary bladder reabsorb Na^+ and Cl^- to support the production of dilute urine. Nonetheless, the regulated pathways for Na^+ and Cl^- transport by euryhaline fishes, especially in the urinary bladder, have not been fully resolved. Here, we first investigated the ultrastructure of epithelial cells within the urinary bladder of FW-acclimated Mozambique tilapia (*Oreochromis mossambicus*) by electron microscopy. We then investigated whether tilapia employ Na^+/Cl^- cotransporter 1 (Ncc1) and Clc family Cl^- channel 2c (Clc2c) for the reabsorption of Na^+ and Cl^- by the kidney and urinary bladder. We hypothesized that levels of their associated gene transcripts vary inversely with environmental salinity. In whole kidney and urinary bladder homogenates, *ncc1* and *clc2c* mRNA levels were markedly higher in steady-state FW- versus SW (seawater)-acclimated tilapia. Following transfer from SW to FW, *ncc1* and *clc2c* in both the kidney and urinary bladder were elevated within 48 h. A concomitant increase in branchial *ncc2*, and decreases in *$Na^+/K^+/2Cl^-$ cotransporter 1a* (*nkcc1a*) and *cystic fibrosis transmembrane regulator 1* (*cftr1*) levels indicated a transition from Na^+ and Cl^- secretion to absorption by the gills in parallel with the identified renal and urinary bladder responses to FW transfer. Our findings suggest that Ncc1 and Clc2c contribute to the functional plasticity of the kidney and urinary bladder in tilapia.

1. Introduction

The majority of teleost fishes maintain plasma osmolality (270–400 mOsm/kg), Na^+ (130–180 mM), and Cl^- (100–150 mM) levels within narrow ranges despite inhabiting aquatic environments that are markedly dissimilar from their internal set points (Evans and Claiborne, 2008; Marshall and Grosell, 2006). Osmotic and ionic homeostasis is achieved via the functional coordination of multiple organs, namely the gill, gastrointestinal tract, skin, kidney, and urinary bladder. The osmotic gain of water from the external environment by fish inhabiting fresh water (FW) is counterbalanced by the production of large volumes of dilute urine. High glomerular filtration rates combined with solute reabsorption across renal and urinary bladder epithelia underlie the production of urine that approximates 50 mOsm/kg (Beyenbach, 1995; Hickman and Trump, 1969; Nishimura et al., 1983; Schmidt-Nielsen and Renfro, 1975). To mitigate their passive losses from the body, Na^+, Cl^-, and Ca^{2+} are simultaneously absorbed from the external environment and diet via branchial, integumentary, and gastrointestinal processes (Flik et al., 1996; Guh et al., 2015; Kaneko et al., 2008). Teleosts residing in marine environments, on the other hand, must continuously defend against dehydration and salt loading. They replace water lost via osmosis to the external environment by drinking ambient seawater (SW). Consumed SW is initially desalinated in the esophagus so that a fluid closer to the osmolality of the blood is passed to the stomach and intestine (Grosell, 2014; Hirano and Mayer-Gostan, 1976). The intestine can then absorb water through solute-linked transport processes (Grosell, 2014). Branchial ionocytes constitute the primary sites of active Na^+ and Cl^- secretion into the surrounding environment (Kaneko et al., 2008). Teleosts cannot produce urine that is hyperosmotic to body fluids as a means to conserve water (Nishimura and Fan, 2003). Rather, SW-

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acclimated fish produce isosmotic urine by combining Na^+, Cl^-, and water reabsorption in the kidney and urinary bladder (Dantzler, 2003; Hickman and Trump, 1969).

As a dilation of the fused archinephric ducts, the teleost urinary bladder is considered a functional continuation of the mesonephric kidney because it exhibits osmoregulatory activities similar to those which operate in the collecting tubule and duct (Curtis and Wood, 1991; Demarest and Machen, 1984; Hickman and Trump, 1969; Hirano et al., 1971; Johnson et al., 1972; Kato et al., 2011; Marshall, 1995; Renfro, 1975; Utida et al., 1972). Enabling, at least in part, their capacity to tolerate dramatic shifts in external salinity, euryhaline fishes modulate solute reabsorption and osmotic permeability within the urinary bladder to modify urine in a fashion that promotes hydromineral balance. For instance, the urinary bladders of mudsucker (*Gillichthys mirabilis*) and flounder (*Platichthys sp.*) reabsorb water at higher rates when isolated from fish acclimated to SW versus FW (Foster, 1975; Hirano et al., 1971; Hirano et al., 1973; Johnson et al., 1972; Utida et al., 1972). The conservation of water via reabsorption in the urinary bladder decreases the amount of SW that must be imbibed (and the obligatory salt load) to sustain organismal water balance (Howe and Gutknecht, 1978).

For decades, a series of teleost models have helped to elucidate the molecular and cellular underpinnings of active Na^+ and Cl^- reabsorption in the distal nephron and urinary bladder of fishes (Takvam et al., 2021). In particular, the discovery of electroneutral active Na^+ and Cl^- transport in the urinary bladder of winter flounder (*Pseudopleuronectes americanus*) by Renfro (1975) led to the cloning of a Na^+/Cl^- cotransporter (Ncc) (Gamba et al., 1993). This 'conventional' Ncc, denoted Ncc1 (Slc12a3) in teleosts (Hiroi et al., 2008; Takei et al., 2014), mediates Na^+ and Cl^- reabsorption in the distal nephron and urinary bladder (Kato et al., 2011). Accordingly, when euryhaline species such as European eel (*Anguilla anguilla*), Japanese eel (*A. japonica*), and mefugu (*Takifugu obscurus*) acclimate to FW, renal *ncc1* (denoted *nccα* in eel) expression is increased in parallel with insertion of the translated protein into the apical membrane of collecting duct cells (Cutler and Cramb, 2008; Kato et al., 2011; Teranishi et al., 2013). While Na^+/K^+-ATPase completes the transcellular movement of Na^+ through Ncc1-expressing cells (Kato et al., 2011), the basolateral pathway for Cl^- to exit these cells remains unknown. One member of the Clc Cl^- channel family, Clc-K, is highly expressed in the kidney of FW-acclimated Mozambique tilapia (*Oreochromis mossambicus*) but is undetectable in SW-acclimated fish (Miyazaki et al., 2002). Furthermore, *ncc1* and *clc-k* gene expression overlap in the distal segment of pronephric tubules in zebrafish (*Danio rerio*) (Wingert et al., 2007); and thus, Clc-K was proposed to support the basolateral exit of Cl^- from Ncc1-expressing cells in teleosts (Kato et al., 2011). Whether the expression of two additional Clc-family members, Clc2c and Clc3, in the kidney and/or urinary bladder is dependent upon environmental salinity stands unresolved. Clc2c and Clc3 are of particular interest given their presence within branchial/epidermal ionocytes that employ the 'fish-specific' Ncc2 (Slc12a10) (Hiroi et al., 2008; Pérez-Ruis et al., 2015; Takei et al., 2014; Tang and Lee, 2011; Wang et al., 2015). Finally, while Na^+/H^+ exchanger 3 (Nhe3) was linked with Na^+ and water reabsorption in teleost proximal renal tubules, it has not been assessed in tilapia kidney or urinary bladder (Ivanis et al., 2008; Madsen et al., 2020; Teranishi et al., 2013).

The functional plasticity of their osmoregulatory systems enables Mozambique tilapia to reside in environments ranging from FW to double-strength SW (Fiess et al., 2007). In turn, tilapia continue to provide an appropriate model from which to identify the cellular and molecular underpinnings of euryhalinity (Kaneko et al., 2008). In the current study, we first determined whether epithelial cells of the tilapia urinary bladder exhibit ultrastructure associated with ion transport (Nagahama et al., 1975; Nebel et al., 2005). We then characterized *ncc1*, *nhe3*, *clc2c*, and *clc3* mRNA levels in the kidney and urinary bladder of tilapia undergoing FW acclimation to assess the involvement of their encoded proteins in salinity acclimation.

2. Materials and methods

2.1. Experimental animals and rearing conditions

Urinary bladders for electron microscopic analyses were collected from male tilapia (60–130 g) housed at the Skidmore College Animal Care Facility. Fish were maintained in FW (conditioned deionized water; <1‰, 5.31 mM Na^+, 5.25 mM Cl^-, 0.10 mM Ca^{2+}) with particle and charcoal filtration at 23–25 °C under 12:L:12D. Fish were fed twice daily with Omega One cichlid pellets (Omega Sea, Painesville, OH). For all other experiments, tilapia (50 g-1.1 kg) of both sexes were obtained from a population maintained at the Hawai'i Institute of Marine Biology, University of Hawai'i. Fish were reared in outdoor tanks with a continuous flow of FW (municipal water; <1‰, 1.05 mM Na^+, 0.55 mM Ca^{2+}) or SW (Kaneohe Bay, Hawai'i, USA; 34‰, 582 mM Na^+, 545 mM Cl^-, 10.7 mM Ca^{2+}) at 24–26 °C under natural photoperiod. SW-acclimated tilapia employed in this study were spawned, and continuously reared in SW, having never been previously exposed to FW. Fish were fed twice daily with Trout Chow (Skretting, Tooele, UT). The Institutional Animal Care and Use Committees of Skidmore College and the University of Hawai'i approved all housing and experimental procedures.

2.2. Transmission electron microscopy

Urinary bladders were excised from fish lethally anesthetized with 2-phenoxyethanol (2-PE; 0.3 ml/l, Sigma-Aldrich, St. Louis, MO) and initially washed in ice-cold phosphate-buffered saline. The tissue was then immediately placed in a fixative solution containing 1% glutaraldehyde, 5 mM $CaCl_2$, and 0.1 M Sorensen's phosphate buffer (SPB; pH 7.2). The tissue was fixed for 1 h at 4 °C, washed three times with ice cold SPB, and then post-fixed with 1% OsO_4 in SPB overnight at 4 °C in the dark. The tissue was washed three times with SPB, dehydrated in acetone over 4 h, and then infiltrated/embedded in Spurr's low viscosity resin (polymerization, 8 h at 70 °C). 80 nm sections were cut with a Leica EM UC6 ultramicrotome, collected on Formvar coated copper grids, conventionally stained with uranyl acetate/lead citrate, and viewed with a Zeiss Libra 120 transmission electron microscope at 120 kV. All microscopy reagents were obtained from EMS Microscopy (Ft. Washington, PA).

2.3. Tissue and steady-state gene expression

A series of tissues were collected from tilapia maintained in FW for >1 year (males; $n = 6$). Fish were lethally anesthetized with 2-PE as described above and the following tissues were collected: whole brain, gill, esophagus, stomach, anterior intestine, body kidney, urinary bladder, and muscle. Tissues were stored in TRI Reagent (MRC, Cincinnati, OH) at −80 °C until RNA isolation. To compare plasma osmolality and *ncc1*, *nhe3*, *clc2c*, and *clc3* mRNA levels between SW- and FW-acclimated animals (mixed sex; $n = 16$), blood plasma, urinary bladder, and kidney were collected from animals acclimated to the two environmental salinities for >1 month. Blood was collected from the caudal vasculature by a needle and syringe treated with heparin ammonium salt (200 U/ml, Sigma-Aldrich). Plasma was separated by centrifugation for measurement of plasma osmolality using a vapor pressure osmometer (Wescor 5100C, Logan, UT, USA).

2.4. Effect of salinity transfer (SW to FW) on plasma osmolality and gene expression in gill, urinary bladder, and kidney

Sixty-four tilapia maintained in SW for >2 years (males) were distributed across eight 700-l tanks supplied with SW (8 fish/tank). Fish were fed daily to satiation and acclimated to the experimental tanks for >2 weeks prior to salinity transfer. On day 0, all fish were sampled from two tanks. Then, water supplies to three tanks were changed to FW (SW-

FW treatment) while three tanks were maintained as time-matched controls (SW-SW treatment). FW conditions were reached after 60 min. At the time of sampling (6, 24, and 48 h after transfer), all fish housed in one SW-SW and one SW-FW tank were quickly netted and lethally anesthetized with 2-PE. Blood plasma, gill filaments, urinary bladder, and body kidney were collected and stored as described above. Fish were fasted for the duration of the experiment. Fish sampled over the experimental period weighed 362.7 ± 23.1 g (mean ± S.E.M.) at time of sampling.

2.5. RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues by the TRI Reagent procedure according to the manufacturer's protocol. RNA concentration and purity were assessed by spectrophotometric absorbance (Nanodrop 1000, Thermo Scientific, Wilmington, DE). First strand cDNA was synthesized by reverse transcribing 100–150 ng total RNA with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Relative levels of mRNA were determined by qRT-PCR using the StepOnePlus real-time PCR system (Life Technologies). We employed previously validated primer sets for all target and normalization genes aside from *ncc1*. Primers for *ncc1* (GenBank accession no. XM_003439377.5) were designed using NCBI Primer-BLAST to amplify a product of 94 bp. The forward primer spans a predicted exon-exon junction. Non-specific product amplification and primer-dimer formation were assessed by melt curve analyses. The amplification efficiency of the *ncc1* primers was 93%. The sequences of all primer sets are provided in Table 1. qRT-PCR reactions were performed in a 15 μl volume containing 2× Power SYBR Green PCR Master Mix (Life Technologies), 200 nmol/l of each primer, nuclease free water, and 1 μl cDNA template. The following cycling parameters were employed: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After verification that *elongation factor 1α* (*ef1α*) mRNA levels did not vary across treatments, *ef1α* levels were used to normalize target genes. Reference and target genes were calculated by the relative quantification method with PCR efficiency correction (Pfaffl, 2001). Standard curves were prepared from serial dilutions of urinary bladder, kidney, or gill cDNA and included on each plate to calculate the PCR efficiencies for target and normalization gene assays. Relative gene expression ratios between groups are reported as a fold-change from controls.

2.6. Statistics

Multiple group comparisons for the tissue expression profiles (Fig. 2) were performed by one-way ANOVA followed by Tukey's HSD test. Significance was set at $P < 0.05$. For a single comparison, a Student's *t*-test was employed (Figs. 3-4) and significant differences are indicated in figures: $^{**}P < 0.01$, and $^{***}P < 0.001$. The transfer experiment (Figs. 5-7) was analyzed by two-way ANOVA. Significant effects of treatment, time, or an interaction ($P < 0.05$) are indicated in figures: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. When a main effect of treatment, or an interaction between treatment and time was detected, post hoc comparisons (Bonferroni's multiple comparisons test) were employed at each time point. Significant differences between groups at a given time point are also indicated in figures: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

3. Results

3.1. Transmission electron microscopy

Elongated columnar cells (>15 μm in height) were clearly evident in the mucosal epithelium of tilapia urinary bladders (Fig. 1). The apical surface of these cells exhibited numerous microvilli that extended into the lumen of the urinary bladder. Aside from the cytoplasmic region directly adjacent to the apical surface, abundant mitochondria were distributed throughout the cells. Regions of attachment (tight-junctions) between adjacent columnar cells were observed in the intercellular spaces near the lumen.

3.2. Tissue distribution of ncc1, ncc2, nhe3, clc2c, and clc3 gene expression

In FW-acclimated tilapia, *ncc1* was highly expressed in the kidney with markedly lower expression in all other examined tissues. By contrast, *ncc2* expression was negligible in all tissues aside from the gill (Fig. 2A, B). The highest expression levels of *nhe3* were detected in the gill, kidney, and urinary bladder (Fig. 2C). *clc2c* expression was highest in the gill and kidney (Fig. 2D); *clc2c* data were reported in our prior study (Breves et al., 2017). *clc3* expression levels were highest in the brain and consistently low across all other tissues (Fig. 2E).

3.3. Steady-state plasma osmolality and ncc1, nhe3, clc2c, and clc3 gene expression

Plasma osmolality was lower in long-term FW- versus SW-acclimated tilapia (Fig. 3A). In the urinary bladder and kidney, *ncc1* and *clc2c* levels were higher in FW- versus SW-acclimated tilapia (Figs. 3B, D and 4A, C) whereas *nhe3* and *clc3* levels were not impacted by salinity (Figs. 3C, E and 4B, D).

Table 1
Specific primer sequences for quantitative real-time PCR.

Gene	Primer sequence (5′-3′)	Amplicon (bp)	Efficiency (%)	Reference/Acc. No.
cftr1	F: CATGCTCTTCACCGTGTTCT R: GCCACAATAATGCCAATCTG	90	109	Moorman et al., 2014
clc2c	F: AGAAGGTCAGTCAGCCAAGC R: AGCGAAATGGGCCGAACTT	72	96	Breves et al., 2017
clc3	F: CCCTGTGATCGTGTCTAAGGA R: TAGCGATTGTGATGTCTCTGC	71	92	Tang and Lee, 2011
ef1α	F: AGCAAGTACTACGTGACCATCATTG R: AGTCAGCCTGGGAGGTACCA	85	109	Breves et al., 2010b
ncc1	F: GAGCAGAAGCAGGAGGTGTT R: GCTGAGGAGGCTGGTTGATT	94	93	XM_003439377.5
ncc2	F: CCGAAAGGCACCCTAATGG R: CTACACTTGCACCAGAAGTGACAA	79	96	Inokuchi et al., 2008
nhe3	F: ATGGCGTGTGGAGGCTTG R: CCTGTCCCAGTTTCTGTTTGTG	74	100	Inokuchi et al., 2008
nkcc1a	F: GGAGGCAAGATCAACAGGATTG R: AATGTCCGAAAAGTCTATCCTGAACT	84	94	Inokuchi et al., 2008

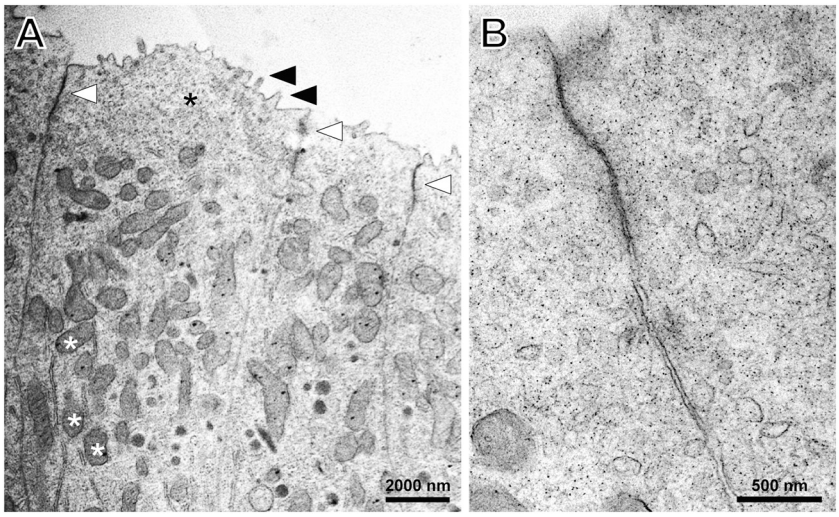

Fig. 1. Ultrastructure of columnar cells within the urinary bladder of freshwater-acclimated tilapia. Apical microvilli (black arrowheads), tight-junctions within intracellular spaces (white arrowheads), mitochondria (white asterisks), and the cytoplasmic area adjacent to the microvilli (black asterisk) are indicated in the micrograph (A). Magnified view of a tight-junction between columnar cells adjacent to the lumen of the urinary bladder (B).

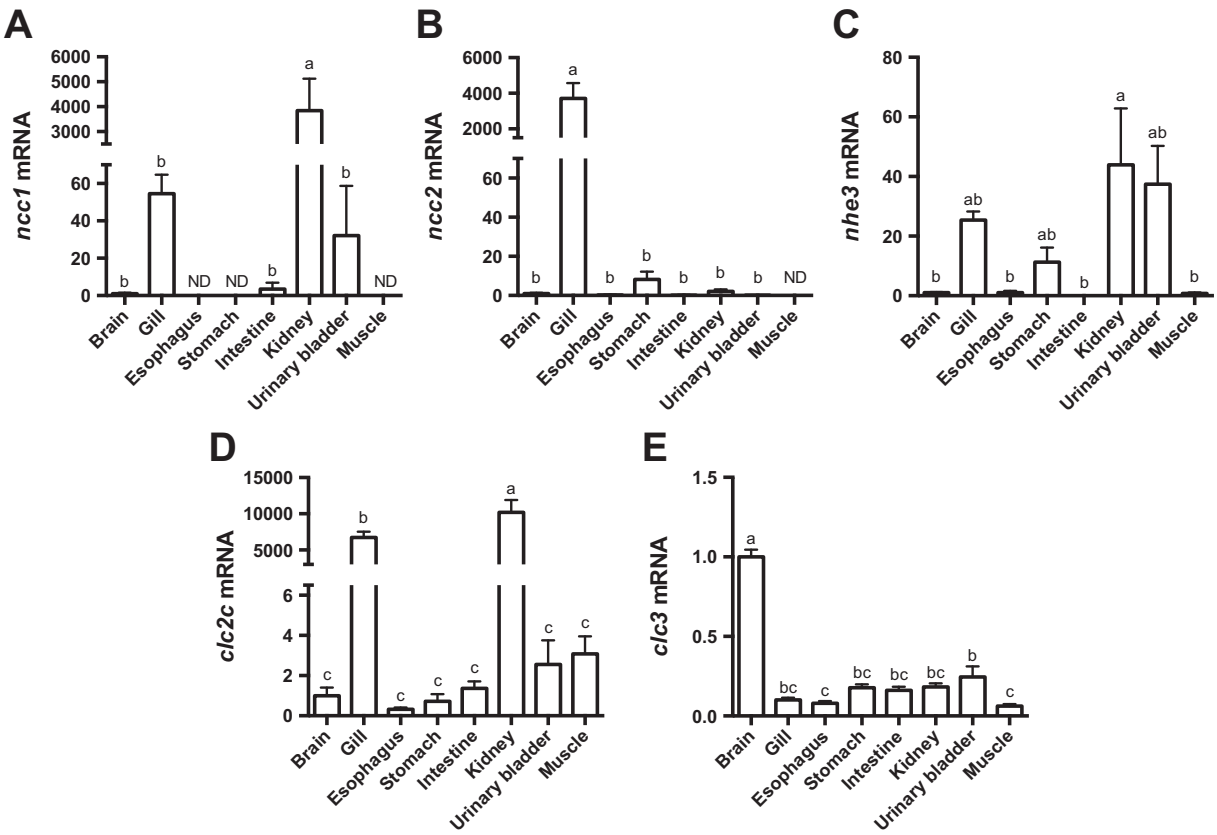

Fig. 2. Tissue expression of *ncc1* (A), *ncc2* (B), *nhe3* (C), *clc2c (D)*, and *clc3* (E) mRNA in freshwater-acclimated tilapia. Means ± S.E.M. ($n = 6$). Data were normalized to *ef1α* as a reference gene and are presented relative to brain expression levels. ND = no detection. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD, $P < 0.05$).

3.4. Effect of transfer from SW to FW on plasma osmolality and branchial gene expression

There were significant main effects of treatment, time, and an interaction on plasma osmolality (Fig. 5A). At 24 and 48 h, plasma osmolality was reduced in tilapia transferred from SW to FW compared with time-matched (SW-SW) controls. Following transfer from SW to FW, there were significant effects of treatment, time, and an interaction on branchial *ncc2* levels (Fig. 5B). In tilapia transferred from SW to FW, *ncc2* levels were elevated above SW-SW controls at 6, 24, and 48 h after transfer. For *$Na^+/K^+/2Cl^-$ cotransporter 1a* (*nkcc1a*), there were significant effects of treatment and time (Fig. 5C), and for *cystic fibrosis transmembrane conductance regulator 1* (*cftr1*), there was a significant effect of treatment (Fig. 5D). *nkcc1a* and *cftr1* levels in tilapia transferred

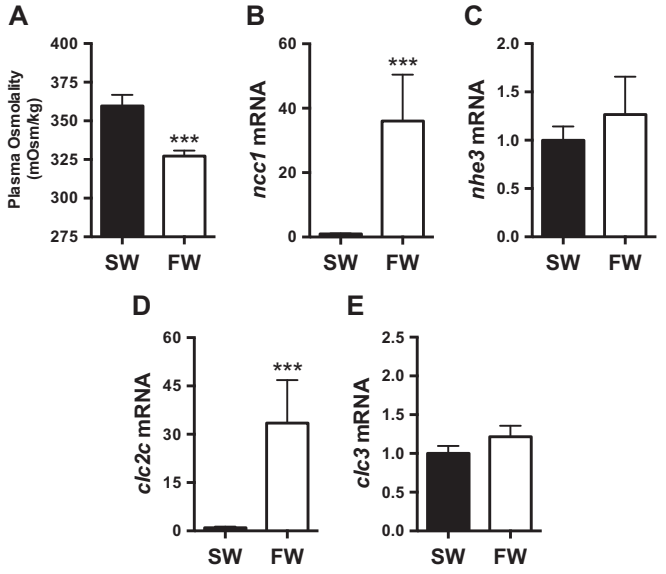

Fig. 3. Plasma osmolality (A) and urinary bladder *ncc1* (B), *nhe3* (C), *clc2c* (D), and *clc3* (E) mRNA levels in seawater- (SW; solid bars) and freshwater (FW; open bars)-acclimated tilapia. Means ± S.E.M. ($n = 16$). mRNA levels in FW are presented as a fold-change from SW. Asterisks indicate significant differences between salinities (***$P < 0.001$) by Student's *t*-test.

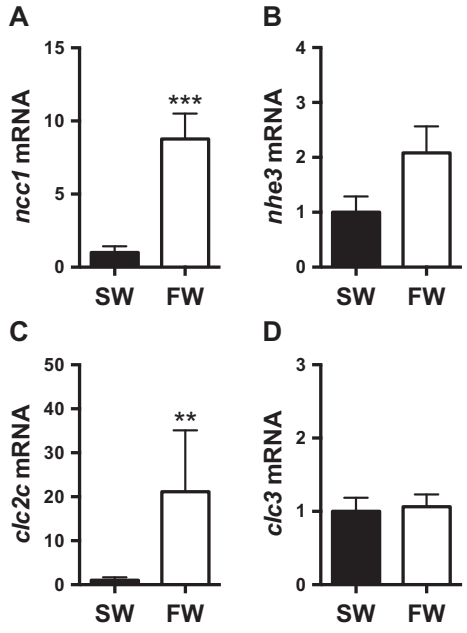

Fig. 4. Renal *ncc1* (A), *nhe3* (B), *clc2c* (C), and *clc3* (D) mRNA levels in seawater- (SW; solid bars) and freshwater (FW; open bars)-acclimated tilapia. Means ± S.E.M. ($n = 16$). mRNA levels in FW are presented as a fold-change from SW. Asterisks indicate significant differences between salinities (**$P < 0.01$ and ***$P < 0.001$) by Student's t-test.

from SW to FW were diminished from SW-SW controls at 24 and 48 h.

3.5. Effect of transfer from SW to FW on urinary bladder gene expression

In the urinary bladder, there was a significant main effect of treatment on *ncc1* with increased levels in tilapia transferred from SW to FW compared with SW-SW controls at 6, 24, and 48 h (Fig. 6A). There were no significant main or interaction effects on *nhe3* and *clc3* levels (Fig. 6B, D). Transfer from SW to FW resulted in a significant effect of time, and an interaction with treatment, on *clc2c*; *clc2c* levels were elevated above SW-SW controls at 48 h (Fig. 6C).

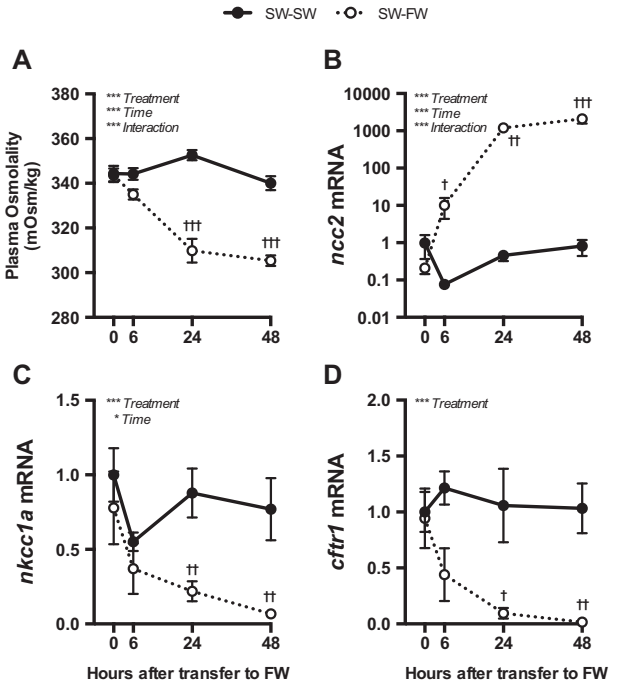

Fig. 5. Plasma osmolality (A) and branchial *ncc2* (B), *nkcc1a* (C), and *cftr1* (D) mRNA levels at 6, 24, and 48 h after transfer of tilapia from seawater (SW) to fresh water (FW; open symbols). Time-matched control fish were maintained in SW (solid symbols). Means ± S.E.M. ($n = 8$). Gene expression is presented as a fold-change from the SW-acclimated group at time 0. Differences among groups were evaluated by two-way ANOVA. Significant effects of treatment, time, or an interaction are indicated in respective panels (*$P < 0.05$ and ***$P < 0.001$). When there was a significant treatment effect, post hoc comparisons (Bonferroni's multiple comparisons test of time-matched groups) were made at each time point ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$).

3.6. Effect of transfer from SW to FW on renal gene expression

There were significant main effects of treatment, time, and an interaction on renal *ncc1* levels (Fig. 7A). As in the urinary bladder (Fig. 6A), renal *ncc1* was elevated in tilapia transferred from SW to FW at 6, 24, and 48 h. There was a significant effect of treatment on *nhe3* levels, with elevations above SW-SW controls occurring at 24 and 48 h after transfer to FW (Fig. 7B). There were significant main effects of treatment, time, and an interaction on *clc2c*; *clc2c* levels in tilapia transferred from SW to FW were elevated above SW-SW controls at 24 and 48 h (Fig. 5C). No significant main or interaction effects were detected for *clc3* levels (Fig. 7D).

4. Discussion

In the current study, we characterized the expression patterns of multiple ion transporter/channel-encoding genes during FW-acclimation in tilapia. Most notably, *ncc1* and *clc2c* showed attendant increases in the kidney and urinary bladder during the acute phase of FW acclimation; moreover, their expression remained elevated in steady-state FW-acclimated tilapia. While Ncc1 was previously linked with adaptive responses to FW (Cutler and Cramb, 2008; Kato et al., 2011; Teranishi et al., 2013), to our knowledge, this is the first description of salinity-responsive *clc2c* expression in the kidney or urinary bladder. Therefore, we relate patterns of *ncc1* and *clc2c* expression in tilapia to previously established ionoregulatory responses to FW. We

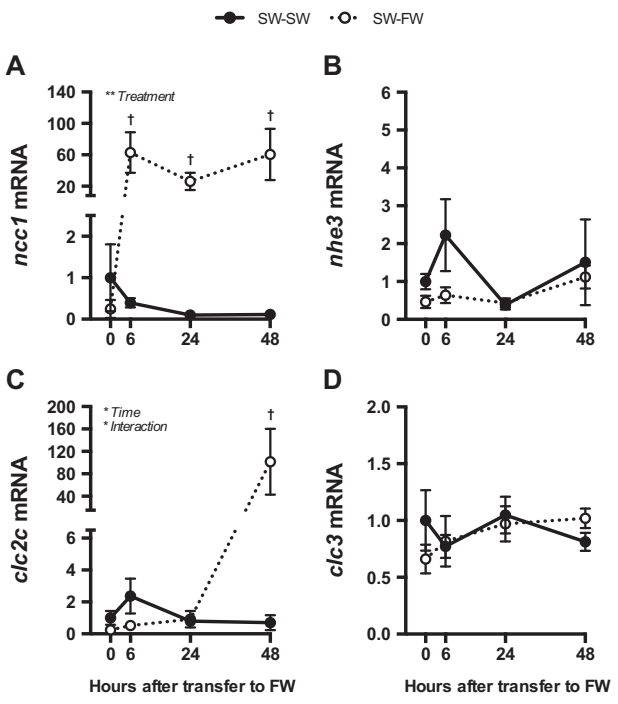

Fig. 6. Urinary bladder *ncc1* (A), *nhe3* (B), *clc2c* (C), and *clc3* (D) mRNA levels at 6, 24, and 48 h after transfer of tilapia from seawater (SW) to fresh water (FW; open symbols). Time-matched control fish were maintained in SW (solid symbols). Means ± S.E.M. ($n = 8$). Gene expression is presented as a fold-change from the SW-acclimated group at time 0. Differences among groups were evaluated by two-way ANOVA. Significant effects of treatment, time, or an interaction are indicated in respective panels ($^*P < 0.05$ and $^{**}P < 0.01$). When there was a significant treatment or interaction effect, post hoc comparisons (Bonferroni's multiple comparisons test of time-matched groups) were made at each time point ($^{\dagger}P < 0.05$).

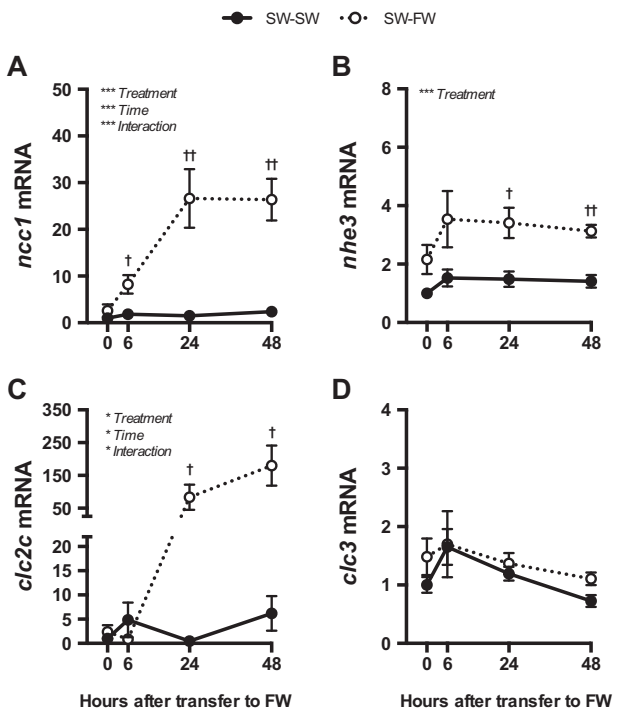

Fig. 7. Renal *ncc1* (A), *nhe3* (B), *clc2c* (C), and *clc3* (D) mRNA levels at 6, 24, and 48 h after transfer of tilapia from seawater (SW) to fresh water (FW; open symbols). Time-matched control fish were maintained in SW (solid symbols). Means ± S.E.M. ($n = 8$). Gene expression is presented as a fold-change from the SW-acclimated group at time 0. Differences among groups were evaluated by two-way ANOVA. Significant effects of treatment, time, or an interaction are indicated in respective panels ($^*P < 0.05$ and $^{***}P < 0.001$). When there was a significant treatment effect, post hoc comparisons (Bonferroni's multiple comparisons test of time-matched groups) were made at each time point ($^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$).

selected the transcriptional changes that occur within branchial ionocytes to provide a temporal context for how *ncc1* and *clc2c* contribute to FW acclimation.

Given that anatomical and functional assessments of tilapia urinary bladder are scant, we first determined whether epithelial cells of the tilapia bladder exhibit ultrastructural features indicative of ion and/or water transport capacities. The luminal epithelia of mudsucker and European sea bass (*Dicentrarchus labrax*) urinary bladders are composed of columnar cells that express Na^+/K^+-ATPase to energize Na^+ and Cl^- transport (Loretz and Bern, 1980; Nagahama et al., 1975; Nebel et al., 2005). In tilapia, we located columnar cells with features remarkably similar to those described in mudsucker acclimated to 5% SW (Nagahama et al., 1975). These features included: apical microvilli, abundant mitochondria, and tight-junctions between adjoining cells (Fig. 1). Columnar cells operate within the urinary bladders of mudsucker acclimated to both hypo- and hyperosmotic environments (Nagahama et al., 1975); thus, the occurrence of these cells suggests similar capacities for ion and/or water transport in tilapia urinary bladder. To date, the net movements of Na^+ and Cl^- in the urinary bladder have not been determined for either FW- or SW-acclimated tilapia. With respect to water transport, however, tilapia urinary bladders are relatively impermeable (compared with other euryhaline species) regardless of whether they are sampled from FW- or SW-acclimated fish (Hirano et al., 1973). Therefore, we propose that the salinity-responsive pathways for Na^+ and Cl^- absorption described in the current study do not facilitate, or operate in parallel with, substantial water absorption by the tilapia urinary bladder.

Our initial step toward identifying molecular responses associated with FW acclimation was to survey the distribution of *ncc1*, *ncc2*, *nhe3*, *clc2c*, and *clc3*. Consistent with findings in eel, mefugu, and Atlantic salmon (*Salmo salar*) (Cutler and Cramb, 2008; Kato et al., 2011; Madsen et al., 2020; Teranishi et al., 2013), *ncc1* was highly expressed in the kidney and urinary bladder where *ncc2* levels were negligible (Fig. 2A, B). Robust *ncc2* levels in the gill reflect Ncc2-mediated Na^+ and Cl^- absorption by 'Type-II' ionocytes (Fig. 2B) (Hiroi et al., 2008). Nhe3 also supports Na^+ absorption by ionocytes (Watanabe et al., 2008), but in a distinct sub-type coined 'Type-III' ionocytes (Hiroi et al., 2008). Accordingly, *nhe3* was highly expressed in the gill, in addition to the kidney and urinary bladder (Fig. 2C). The robust expression of *nhe3* in the tilapia urinary bladder contrasts with the eel urinary bladder where *nhe3* was not detected (Teranishi et al., 2013). The gill, kidney, and urinary bladder were among the organs with the highest *clc2c* levels (Fig. 2D). This is noteworthy because *clc2c* is not highly expressed in the kidney of zebrafish (Pérez-Ruis et al., 2015; Wang et al., 2015), and suggests broader roles for Clc2c in tilapia given its wider distribution. The low *clc3* levels across osmoregulatory organs (Fig. 2E) align with previous data that were equivocal regarding a role for Clc3 in supporting organismal Cl^- balance (Breves et al., 2017; Tang and Lee, 2011). Given that our tissue expression analyses only included males, we cannot rule out that female tilapia exhibit different gene expression patterns in the urinary bladder. Indeed, there are sex-specific roles for urine secretion that underlie interspecific chemical communication that may impact urine-producing pathways (Barata et al., 2007).

After determining that particular genes associated with Na^+ and Cl^- transport were expressed in the kidney and urinary bladder, we next considered their responses to FW conditions. Our central hypothesis was that genes encoding ion transporters/channels with complementary roles (i.e., putatively mediate apical and basolateral Cl^- transport within

the same cell) in the kidney and urinary bladder would show attendant patterns of expression. *ncc1* and *clc2c* were highest in FW- versus SW-acclimated fish (Figs. 3B, D and 4A, C) and this difference was set into motion within 48 h of FW-acclimation (Figs. 6A, C and 7A, C). In congeneric pufferfishes, Ncc1 expression in the distal tubule was correlated with the adaptability of particular species to FW conditions (Kato et al., 2011). In an analogous fashion, the salinity-dependent expression of *ncc1* in tilapia aligns with their strong FW-adaptability and capacity to produce dilute urine (Furukawa et al., 2014). The temporal relationship between FW-induced changes in *ncc1* and *clc2c* is particularly interesting given the uncertainty in how Cl^- exits Ncc1-expressing cells. The first described teleost Clc-K was localized to the basolateral tubular system of putative Nkcc2-expressing cells in the tilapia distal tubule (Miyazaki et al., 2002). Kato et al. (2011) proposed that Clc-K is also expressed in teleost Ncc1-expressing cells. This link between Ncc1 and Clc-K was supported by the overlapping expression of their gene transcripts in the distal pronephros of zebrafish (Wingert et al., 2007). More recently, however, zebrafish Clc-K was localized to the apical membrane of distal tubule cells (Pérez-Rius et al., 2019), and thus, does not mediate the basolateral exit of Cl^-. Because *clc2c* was strongly induced by FW in tilapia kidney and urinary bladder, and *clc-k* is not expressed in the urinary bladder (Miyazaki et al., 2002), Clc2c emerges as a candidate mediator of Cl^- transport in Ncc1-expressing cells of tilapia, and perhaps other teleosts. This working model is consistent with how serosal application of diphenylamine-2-carboxylic acid (DPC) inhibited transepithelial Cl^- uptake by mudsucker urinary bladder (Chang and Loretz, 1993). Accordingly, Chang and Loretz (1993) assigned the DPC-sensitive Cl^- channel to the basolateral membrane of columnar cells. Immunohistological studies are now warranted to determine whether Clc2c is expressed in the basolateral membrane of Ncc1-expressing cells.

In rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon, and Japanese eel, Nhe3 supports Na^+ and water reabsorption in the proximal renal tubule (Ivanis et al., 2008; Madsen et al., 2020; Teranishi et al., 2013). *nhe3* expression was thus higher in the proximal nephron of SW- versus FW-acclimated salmon and eel (Madsen et al., 2020; Teranishi et al., 2013). We therefore predicted that *nhe3* expression would be modulated in an opposite fashion as *ncc1* and *clc2c* during FW-acclimation. *nhe3* levels, however, were not salinity-responsive in the urinary bladder and only showed an increase in the kidney above SW-SW controls at 24 and 48 h after FW-transfer (Fig. 7B), a pattern that was not sustained under steady-state conditions (Fig. 4B). Because renal (and presumably urinary bladder) Nhe3 plays a role in supporting acid-base balance (Ivanis et al., 2008), *nhe3* expression may not be solely determined by environmental salinity.

In the current study, plasma osmolality declined in tilapia transferred from SW to FW during the initial 24 h of the experimental time course (Fig. 5A). The stabilization of plasma osmolality between 24 and 48 h suggested a transition, at least at the organismal level, to osmoregulatory processes befitting FW conditions. Because the branchial epithelium plays a central role in ion balance (Marshall and Grosell, 2006), the expression of ionocyte-related genes provided a temporal context for the *ncc1* and *clc2c* responses in the kidney and urinary bladder. 'Type-II' ionocytes express Ncc2 to absorb Na^+ and Cl^- from FW and there is strong agreement between *ncc2* levels and the densities of Type-II ionocytes (Hiroi et al., 2008; Inokuchi et al., 2008). Accordingly, *ncc2* was markedly increased within 6 h after exposure to FW (Fig. 5B). An interesting pattern observed in our study was the remarkable difference in *ncc2* levels between treatments at 24 and 48 h. This seemingly reflected the use of FW-naïve tilapia that were spawned and continuously reared in SW for >2 years. A similar *ncc2* response was observed in tilapia transferred to FW following their development in SW since the fry stage (Moorman et al., 2015). Consistent with the paradigm for ion secretion by teleost ionocytes, tilapia 'SW-type/Type-IV' ionocytes express Nkcc1 and Cftr1 in the basolateral and apical membranes, respectively (Hiroi et al., 2008; Kaneko et al., 2008). Here, *nkcc1a* and *cftr1* levels were markedly diminished within 24 h of FW exposure in agreement with the disappearance of SW-type ionocytes that occurs during FW acclimation (Fig. 5C, D) (Hiroi et al., 2008). The transition from ion secretion to absorption in the gill, as indicated by *ncc2*, *nkcc1a*, and *cftr1*, was coincident with increased *ncc1* in the kidney and urinary bladder. Thus, our study provides new insight into how these three ionoregulatory organs work simultaneously during the acute phase of FW acclimation to mitigate perturbations to osmoregulatory balance.

In conclusion, the capacity to modulate ionoregulatory processes via the regulated expression of genes that encode effectors of ion transport is a critical aspect of euryhalinity (Breves et al., 2010a; Fiol and Kültz, 2007). Our collective results suggest that tilapia employ Ncc1 and Clc2c to reabsorb Na^+ and Cl^- from insipient urine when residing in FW, and furthermore, the expression of their associated gene transcripts is highly plastic. The insertion of Ncc1 into the apical membrane of cells lining the collecting duct underlies the FW-adaptability of mefugu (Kato et al., 2011). In turn, it will be particularly interesting to learn whether tilapia Clc2c is trafficked to the basolateral membrane of Ncc1-expressing cells in response to FW conditions. Lastly, by describing the salinity-dependent nature of *ncc1* and *clc2c* expression in the urinary bladder, we are now better positioned to resolve how hormones regulate this key teleost osmoregulatory organ at the molecular level (Bern, 1975; Foster, 1975; Hirano, 1975; Hirano et al., 1971; Utida et al., 1972).

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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References

Barata, E.N., Hubbard, P.C., Almeida, O.G., Miranda, A., Canario, A.V.M., 2007. Male urine signals social rank in the Mozambique tilapia (*Oreochromis mossambicus*). BMC Biol. 5, 54.

Bern, H.A., 1975. Prolactin and osmoregulation. Am. Zool. 15, 937–949.

Beyenbach, K.W., 1995. Secretory electrolyte transport in renal proximal tubules of fish. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Cellular and Molecular Approaches to Fish Ionic Regulation, Vol. 14. Academic Press, San Diego, pp. 85–105.

Breves, J.P., Hasegawa, S., Yoshioka, M., Fox, B.K., Davis, L.K., Lerner, D.T., Takei, Y., Hirano, T., Grau, E.G., 2010a. Acute salinity challenges in Mozambique and Nile tilapia: differential responses of plasma prolactin, growth hormone and branchial expression of ion transporters. Gen. Comp. Endocrinol. 167, 135–142.

Breves, J.P., Hirano, T., Grau, E.G., 2010b. Ionoregulatory and endocrine responses to disturbed salt and water balance in Mozambique tilapia exposed to confinement and handling stress. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 155, 294–300.

Breves, J.P., Keith, P.L.K., Hunt, B.L., Pavlosky, K.K., Inokuchi, M., Yamaguchi, Y., Lerner, D.T., Seale, A.P., Grau, E.G., 2017. *clc-2c* is regulated by salinity, prolactin and extracellular osmolality in tilapia gill. J. Mol. Endocrinol. 59, 391–402.

Chang, W., Loretz, C.A., 1993. DPC blockade of transepithelial chloride absorption and single anion channels in teleost urinary bladder. Am. J. Phys. 265, R66–R75.

Curtis, B.J., Wood, C.M., 1991. The function of the urinary bladder *in vivo* in the freshwater rainbow trout. J. Exp. Biol. 155, 567–583.

Cutler, C.P., Cramb, G., 2008. Differential expression of absorptive cation-chloride-cotransporters in the intestinal and renal tissues of the European eel (*Anguilla anguilla*). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 149, 63–73.

Dantzler, W.H., 2003. Regulation of renal proximal and distal tubule transport: sodium, chloride and organic anions. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 136, 453–478.

Demarest, J.R., Machen, T.E., 1984. Passive and active ion transport by the urinary bladder of a euryhaline flounder. Am. J. Phys. 246 (4 Pt 2), F402–F408.
Evans, D.H., Claiborne, J.B., 2008. Osmotic and ionic regulation in fishes. In: Evans, D.H. (Ed.), Osmotic and Ionic Regulation: Cells and Animals. CRC Press, Boca Raton, pp. 295–366.
Fiess, J.C., Kunkel-Patterson, A., Mathias, L., Riley, L.G., Yancey, P.H., Hirano, T., Grau, E.G., 2007. Effects of environmental salinity and temperature on osmoregulatory ability, organic osmolytes, and plasma hormone profiles in the Mozambique tilapia (*Oreochromis mossambicus*). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 146, 252–264.
Fiol, D.F., Kültz, D., 2007. Osmotic stress sensing and signaling in fishes. FEBS J. 274 (22), 5790–5798.
Flik, G., Klaren, P.H.M., Schoenmakers, T.J.M., Bijvelds, M.J.C., Verbost, P.M., Wendelaar Bonga, S.E., 1996. Cellular calcium transport in fish: unique and universal mechanisms. Physiol. Biochem. Zool. 69, 403–417.
Foster, R.C., 1975. Changes in urinary bladder and kidney function in the starry flounder (*Platichthys stellatus*) in response to prolactin and to freshwater transfer. Gen. Comp. Endocrinol. 27, 153–161.
Furukawa, F., Watanabe, S., Kakumura, K., Hiroi, J., Kaneko, T., 2014. Gene expression and cellular localization of ROMKs in the gills and kidney of Mozambique tilapia acclimated to fresh water with high potassium concentration. Am. J. Phys. Regul. Integr. Comp. Phys. 307 (11), R1303–R1312.
Gamba, G., Saltzberg, S.N., Lombardi, M., Miyanoshita, A., Lytton, J., Hediger, M.A., Brenner, B.M., Hebert, S.C., 1993. Primary structure and functional expression of a cDNA encoding the thiazide-sensitive, electroneutral sodium-chloride cotransporter. Proc. Natl. Acad. Sci. U. S. A. 90, 2749–2753.
Grosell, M., 2014. Intestinal transport. In: Evans, D.H., Claiborne, J.B., Currie, S. (Eds.), The Physiology of Fishes. CRC Press, Boca Raton, pp. 175–203.
Guh, Y.J., Lin, C.H., Hwang, P.P., 2015. Osmoregulation in zebrafish: ion transport mechanisms and functional regulation. EXCLI J. 14, 627–659.
Hickman, C.P., Trump, B.F., 1969. The kidney. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology. Academic Press, New York, pp. 91–239.
Hirano, T., 1975. Effects of prolactin on osmotic and diffusion permeability of the urinary bladder of the flounder, *Platichthys flesus*. Gen. Comp. Endocrinol. 27, 88–94.
Hirano, T., Mayer-Gostan, N., 1976. Eel esophagus as an osmoregulatory organ. Proc. Natl. Acad. Sci. U. S. A. 73, 1348–1350.
Hirano, T., Johnson, D.W., Bern, H.A., 1971. Control of water movement in flounder urinary bladder by prolactin. Nature 230 (5294), 469–470.
Hirano, T., Johnson, D.W., Bern, H.A., Utida, S., 1973. Studies on water and ion movements in the isolated urinary bladder of selected freshwater, marine and euryhaline teleosts. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 45, 529–540.
Hiroi, J., Yasumasu, S., McCormick, S.D., Hwang, P.P., Kaneko, T., 2008. Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. J. Exp. Biol. 211, 2584–2599.
Howe, D., Gutknecht, J., 1978. Role of urinary bladder in osmoregulation in marine teleost, *Opsanus tau*. Am. J. Phys. 235 (1), R48–R54.
Inokuchi, M., Hiroi, J., Watanabe, S., Lee, K.M., Kaneko, T., 2008. Gene expression and morphological localization of NHE3, NCC and NKCC1a in branchial mitochondria-rich cells of Mozambique tilapia (*Oreochromis mossambicus*) acclimated to a wide range of salinities. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 151, 151–158.
Ivanis, G., Esbaugh, A.J., Perry, S.F., 2008. Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 211, 2467–2477.
Johnson, D.W., Hirano, T., Bern, H.A., Conte, F.P., 1972. Hormonal control of water and sodium movements in the urinary bladder of the starry flounder, *Platichthys stellatus*. Gen. Comp. Endocrinol. 19, 115–128.
Kaneko, T., Watanabe, S., Lee, K.M., 2008. Functional morphology of mitochondrion-rich cells in euryhaline and stenohaline teleosts. Aqua-BioSci. Monogr. 1, 1–62.
Kato, A., Muro, T., Kimura, Y., Li, S., Islam, Z., Ogoshi, M., Doi, H., Hirose, S., 2011. Differential expression of Na^+-Cl^- cotransporter and Na^+-K^+-Cl^- cotransporter 2 in the distal nephrons of euryhaline and seawater pufferfishes. Am. J. Phys. Regul. Integr. Comp. Phys. 300, R284–R297.
Loretz, C.A., Bern, H.A., 1980. Ion transport by the urinary bladder of the gobiid teleost, *Gillichthys mirabilis*. Am. J. Phys. 239 (5), R415–R423.
Madsen, S.S., Bollinger, R.J., Brauckhoff, M., Engelund, M.B., 2020. Gene expression profiling of proximal and distal renal tubules in Atlantic salmon (*Salmo salar*) acclimated to fresh water and seawater. Am. J. Physiol. Ren. Physiol. 319 (3), F380–F393.
Marshall, W.S., 1995. Transport processes in isolated teleost epithelia: Opercular epithelium and urinary bladder. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Cellular and Molecular Approaches to Fish Ionic Regulation, Vol. 14. Academic Press, San Diego, pp. 1–23.
Marshall, W.S., Grosell, M., 2006. Ion transport, osmoregulation and acid-base balance. In: Evans, D.H., Claiborne, J.B. (Eds.), The Physiology of Fishes. CRC Press, Boca Raton, pp. 177–230.
Miyazaki, H., Kaneko, T., Uchida, S., Sasaki, S., Takei, Y., 2002. Kidney-specific chloride channel, OmClC-K, predominantly expressed in the diluting segment of freshwater-adapted tilapia kidney. Proc. Natl. Acad. Sci. U. S. A. 99 (24), 15782–15787.
Moorman, B.P., Inokuchi, M., Yamaguchi, Y., Lerner, D.T., Grau, E.G., Seale, A.P., 2014. The osmoregulatory effects of rearing Mozambique tilapia in a tidally changing salinity. Gen. Comp. Endocrinol. 207, 94–102.
Moorman, B.P., Lerner, D.T., Grau, E.G., Seale, A.P., 2015. The effects of acute salinity challenges on osmoregulation in Mozambique tilapia reared in a tidally changing salinity. J. Exp. Biol. 218, 731–739.
Nagahama, Y., Bern, H.A., Doneen, B.A., Nishioka, R.S., 1975. Cellular differentiation in the urinary bladder of a euryhaline marine fish, *Gillichthys mirabilis*, in response to environmental salinity change. Develop. Growth Differ. 17 (4), 367–381.
Nebel, C., Nègre-Sadargues, G., Blasco, C., Charmantier, G., 2005. Morphofunctional ontogeny of the urinary system of the European sea bass *Dicentrarchus labrax*. Anat. Embryol. 209 (3), 193–206.
Nishimura, H., Fan, Z., 2003. Regulation of water movement across vertebrate renal tubules. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 136 (3), 479–498.
Nishimura, H., Imai, M., Ogawa, M., 1983. Sodium chloride and water transport in the renal distal tubule of the rainbow trout. Am. J. Physiol. Ren. Physiol. 244 (3), F247–F254.
Pérez-Rius, C., Castellanos, A., Gaitán-Peñas, H., Navarro, A., Artuch, R., Barrallo-Gimeno, A., Estévez, R., 2019. Role of zebrafish ClC-K/barttin channels in apical kidney chloride reabsorption. J. Physiol. 597 (15), 3969–3983.
Pérez-Ruis, C., Gaitán-Peñas, H., Estévez, R., Barrallo-Gimeno, A., 2015. Identification and characterization of the zebrafish ClC-2 chloride channel orthologs. Pflugers Arch. 467, 1769–1781.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), e45.
Renfro, J.L., 1975. Water and ion transport by the urinary bladder of the teleost *Pseudopleuronectes americanus*. Am. J. Phys. 228 (1), 52–61.
Schmidt-Nielsen, B., Renfro, J.L., 1975. Kidney function of the American eel *Anguilla rostrata*. Am. J. Phys. 228 (2), 420–431.
Takei, Y., Hiroi, J., Takahashi, H., Sakamoto, T., 2014. Diverse mechanisms for body fluid regulation in teleost fishes. Am. J. Phys. Regul. Integr. Comp. Phys. 307, R778–R792.
Takvam, M., Wood, C.M., Kryvi, H., Nilsen, T.O., 2021. Ion transporters and osmoregulation in the kidney of teleost fish as a function of salinity. Front. Physiol. 12, 664588.
Tang, C.H., Lee, T.H., 2011. Ion-deficient environment induces the expression of basolateral chloride channel, ClC-3-like protein, in gill mitochondrion-rich cells for chloride uptake of the tilapia *Oreochromis mossambicus*. Physiol. Biochem. Zool. 84, 54–67.
Teranishi, K., Mekuchi, M., Kaneko, T., 2013. Expression of sodium/hydrogen exchanger 3 and cation-chloride cotransporters in the kidney of Japanese eel acclimated to a wide range of salinities. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 164, 333–343.
Utida, S., Hirano, T., Oide, H., Ando, M., Johnson, D.W., Bern, H.A., 1972. Hormonal control of the intestine and urinary bladder in teleost osmoregulation. Gen. Comp. Endocrinol. 3 (Suppl), 317–327.
Wang, Y.F., Yan, J.J., Tseng, Y.C., Chen, R.D., Hwang, P.P., 2015. Molecular physiology of an extra-renal Cl^- uptake mechanism for body fluid Cl^- homeostasis. Int. J. Biol. Sci. 11, 1190–1203.
Watanabe, S., Niida, M., Maruyama, T., Kaneko, T., 2008. Na^+/H^+ exchanger isoform 3 expressed in apical membrane of gill mitochondrion-rich cells in Mozambique tilapia *Oreochromis mossambicus*. Fish. Sci. 74, 813–821.
Wingert, R.A., Selleck, R., Yu, J., Song, H.D., Chen, Z., Song, A., Zhou, Y., Thisse, B., Thisse, C., McMahon, A.P., Davidson, A.J., 2007. The *cdx* genes and retinoic acid control the positioning and segmentation of the zebrafish pronephros. PLoS Genet. 3, 1922–1938.