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

Jason P. Breves[1,*], Nastasia N. Nelson[1], Victor Koltenyuk[1], Cody K. Petro-Sakuma[2], Fritzie T. Celino-Brady[2] and Andre P. Seale[2]

[1] *Department of Biology, Skidmore College, Saratoga Springs, NY 12866, USA*
[2] *Department of Human Nutrition, Food and Animal Sciences, University of Hawai'i at Mānoa, 1955 East-West Road, Honolulu, HI 96822, USA*

*** Corresponding author:**
J.P. Breves
Department of Biology
Skidmore College
815 N. Broadway
Saratoga Springs, NY 12866 USA
Tel.: +1 518 580-5079
Email: jbreves@skidmore.edu

J.P.B. ORCID: 0000-0003-1193-4389
A.P.S. ORCID: 0000-0003-2398-4201

## 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.

**Highlights:** ► Columnar cells are located within the tilapia urinary bladder. ► *ncc1* and *clc2c* gene transcripts are expressed in the kidney and urinary bladder. ► Exposure to freshwater stimulates *ncc1* and *clc2c* expression. ► Exposure to freshwater induced branchial responses linked with ion absorption.

**Keywords:** Clc family $Cl^-$ channel; electron microscopy; euryhaline; gill; ionocytes; kidney; $Na^+/Cl^-$ cotransporter; osmoregulation; urinary bladder

## 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-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) is linked with $Na^+$ and water reabsorption in the proximal renal tubule, 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

171 (MRC, Cincinnati, OH) at -80°C until RNA isolation. To compare plasma osmolality and
172 *ncc1*, *nhe3*, *clc2c*, and *clc3* mRNA levels between SW- and FW-acclimated animals
173 (mixed sex; $n = 16$), blood plasma, urinary bladder, and kidney were collected from
174 animals acclimated to the two environmental salinities for >1 month. Blood was collected
175 from the caudal vasculature by a needle and syringe treated with heparin ammonium
176 salt (200 U/ml, Sigma-Aldrich). Plasma was separated by centrifugation for
177 measurement of plasma osmolality using a vapor pressure osmometer (Wescor 5100C,
178 Logan, UT, USA).
179
180 *2.4. Effect of salinity transfer (SW to FW) on plasma osmolality and gene expression in*
181 *gill, urinary bladder, and kidney*
182 Sixty-four tilapia maintained in SW for >2 years (males) were distributed across
183 eight 700-l tanks supplied with SW (8 fish/tank). Fish were fed daily to satiation and
184 acclimated to the experimental tanks for >2 weeks prior to salinity transfer. On day 0, all
185 fish were sampled from two tanks. Then, water supplies to three tanks were changed to
186 FW (SW-FW treatment) while three tanks were maintained as time-matched controls
187 (SW-SW treatment). FW conditions were reached after 60 min. At the time of sampling
188 (6, 24, and 48 h after transfer), all fish housed in one SW-SW and one SW-FW tank
189 were quickly netted and lethally anesthetized with 2-PE. Blood plasma, gill filaments,
190 urinary bladder, and body kidney were collected and stored as described above. Fish
191 were fasted for the duration of the experiment. Fish sampled over the experimental
192 period weighed 362.7 ± 23.1 g (mean ± S.E.M.) at time of sampling.
193
194 *2.5. RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)*
195 Total RNA was extracted from tissues by the TRI Reagent procedure according
196 to the manufacturer's protocol. RNA concentration and purity were assessed by
197 spectrophotometric absorbance (Nanodrop 1000, Thermo Scientific, Wilmington, DE).
198 First strand cDNA was synthesized by reverse transcribing 100-150 ng total RNA with a
199 High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA).
200 Relative levels of mRNA were determined by qRT-PCR using the StepOnePlus real-time
201 PCR system (Life Technologies). We employed previously validated primer sets for all
202 target and normalization genes aside from *ncc1*. Primers for *ncc1* (GenBank accession
203 no. **XM_003439377.5**) were designed using NCBI Primer-BLAST to amplify a product of
204 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 2X 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 sec 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

239 distributed throughout the cells. Regions of attachment (tight-junctions) between
240 adjacent columnar cells were observed in the intercellular spaces near the lumen.
241
242 *3.2. Tissue distribution of ncc1, ncc2, nhe3, clc2c, and clc3 gene expression*
243 In FW-acclimated tilapia, *ncc1* was highly expressed in the kidney with markedly
244 lower expression in all other examined tissues. By contrast, *ncc2* expression was
245 negligible in all tissues aside from the gill (Fig. 2A, B). The highest expression levels of
246 *nhe3* were detected in the gill, kidney, and urinary bladder (Fig. 2C). *clc2c* expression
247 was highest in the gill and kidney (Fig. 2D); *clc2c* data were reported in our prior study
248 (Breves et al., 2017). *clc3* expression levels were highest in the brain and consistently
249 low across all other tissues (Fig. 2E).
250
251 *3.3. Steady-state plasma osmolality and ncc1, nhe3, clc2c, and clc3 gene expression*
252 Plasma osmolality was lower in long-term FW- versus SW-acclimated tilapia (Fig.
253 3A). In the urinary bladder and kidney, *ncc1* and *clc2c* levels were higher in FW- versus
254 SW-acclimated tilapia (Figs. 3B, D and 4A, C) whereas *nhe3* and *clc3* levels were not
255 impacted by salinity (Figs. 3C, E and 4B, D).
256
257 *3.4. Effect of transfer from SW to FW on plasma osmolality and branchial gene*
258 *expression*
259 There were significant main effects of treatment, time, and an interaction on
260 plasma osmolality (Fig. 5A). At 24 and 48 h, plasma osmolality was reduced in tilapia
261 transferred from SW to FW compared with time-matched (SW-SW) controls. Following
262 transfer from SW to FW, there were significant effects of treatment, time, and an
263 interaction on branchial *ncc2* levels (Fig. 5B). In tilapia transferred from SW to FW, *ncc2*
264 levels were elevated above SW-SW controls at 6, 24, and 48 h after transfer. For
265 *$Na^+/K^+/2Cl^-$ cotransporter 1a* (*nkcc1a*), there were significant effects of treatment and
266 time (Fig. 5C), and for *cystic fibrosis transmembrane conductance regulator 1* (*cftr1*),
267 there was a significant effect of treatment (Fig. 5D). *nkcc1a* and *cftr1* levels in tilapia
268 transferred from SW to FW were diminished from SW-SW controls at 24 and 48 h.
269
270 *3.5. Effect of transfer from SW to FW on urinary bladder gene expression*
271 In the urinary bladder, there was a significant main effect of treatment on *ncc1*
272 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).

*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 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

307 mudsucker acclimated to 5% SW (Nagahama et al., 1975). These features included:
308 apical microvilli, abundant mitochondria, and the presence of tight-junctions between
309 adjoining cells (Fig. 1). Columnar cells operate within the urinary bladders of mudsucker
310 acclimated to both hypo- and hyperosmotic environments (Nagahama et al., 1975); thus,
311 the occurrence of these cells suggests similar capacities for ion and/or water transport in
312 tilapia urinary bladder. To date, the net movements of $Na^+$ and $Cl^-$ in the urinary bladder
313 have not been determined for either FW- or SW-acclimated tilapia. With respect to water
314 transport, however, tilapia urinary bladders are relatively impermeable (compared with
315 other euryhaline species) regardless of whether they are sampled from FW- or SW-
316 acclimated fish (Hirano et al., 1973). Therefore, we propose that the salinity-responsive
317 pathways for $Na^+$ and $Cl^-$ absorption described below do not facilitate, or operate in
318 parallel with, substantial water absorption by the tilapia urinary bladder.
319 Our initial step toward identifying molecular responses associated with FW
320 acclimation was to survey the distribution of *ncc1*, *ncc2*, *nhe3*, *clc2c*, and *clc3*.
321 Consistent with findings in eel, mefugu, and Atlantic salmon (*Salmo salar*) (Cutler and
322 Cramb, 2008; Kato et al., 2011; Madsen et al., 2020; Teranishi et al., 2013), *ncc1* was
323 highly expressed in the kidney and urinary bladder where *ncc2* levels were negligible
324 (Fig. 2A, B). Robust *ncc2* levels in the gill reflect Ncc2-mediated $Na^+$ and $Cl^-$ absorption
325 by 'Type-II' ionocytes (Fig. 2B) (Hiroi et al., 2008). Nhe3 also supports $Na^+$ absorption by
326 ionocytes (Watanabe et al., 2008), but in a distinct sub-type coined 'Type-III' ionocytes
327 (Hiroi et al., 2008). Accordingly, *nhe3* was highly expressed in the gill, in addition to the
328 kidney and urinary bladder (Fig. 2C). The robust expression of *nhe3* in the tilapia urinary
329 bladder contrasts with the eel urinary bladder where *nhe3* was not detected (Teranishi et
330 al., 2013). The gill, kidney, and urinary bladder were among the organs with the highest
331 *clc2c* levels (Fig. 2D). This is noteworthy because *clc2c* is not highly expressed in the
332 kidney of zebrafish (Pérez-Ruis et al., 2015; Wang et al., 2015), and suggests broader
333 roles for Clc2c in tilapia given its wider distribution. The low *clc3* levels across
334 osmoregulatory organs (Fig. 2E) align with previous data that were equivocal regarding
335 a role for Clc3 in supporting organismal $Cl^-$ balance (Breves et al., 2017; Tang and Lee,
336 2011). Given that our tissue expression analyses only included males, we cannot rule
337 out that female tilapia exhibit different gene expression patterns in the urinary bladder.
338 Indeed, there are sex-specific roles for urine secretion that underlie interspecific
339 chemical communication that may impact urine-producing pathways (Barata et al.,
340 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

375 modulated in an opposite fashion as *ncc1* and *clc2c* during FW-acclimation. *nhe3* levels,
376 however, were not salinity-responsive in the urinary bladder and only showed an
377 increase in the kidney above SW-SW controls at 24 and 48 h after FW-transfer (Fig. 7B),
378 a pattern that was not sustained under steady-state conditions (Fig. 4B). Because renal
379 (and presumably urinary bladder) Nhe3 plays a role in supporting acid-base balance
380 (Ivanis et al., 2008), *nhe3* expression may not be solely determined by environmental
381 salinity.
382 In the current study, plasma osmolality declined in tilapia transferred from SW to
383 FW during the initial 24 h of the experimental time course (Fig. 5A). The stabilization of
384 plasma osmolality between 24 and 48 h suggested a transition, at least at the
385 organismal level, to osmoregulatory processes befitting FW conditions. Because the
386 branchial epithelium plays a central role in ion balance (Marshall and Grosell, 2006), the
387 expression of ionocyte-related genes provided a temporal context for the *ncc1* and *clc2c*
388 responses in the kidney and urinary bladder. 'Type-II' ionocytes express Ncc2 to absorb
389 $Na^+$ and $Cl^-$ from FW and there is strong agreement between *ncc2* levels and the
390 densities of Type-II ionocytes (Hiroi et al. 2008, Inokuchi et al. 2008). Accordingly, *ncc2*
391 was markedly increased within 6 h after exposure to FW (Fig. 5B). An interesting pattern
392 observed in our study was the remarkable difference in *ncc2* levels between treatments
393 at 24 and 48 h. This seemingly reflected the use of FW-naïve tilapia that were spawned
394 and continuously reared in SW for >2 years. A similar *ncc2* response was observed in
395 tilapia transferred to FW following their development in SW since the fry stage (Moorman
396 et al., 2015). Consistent with the paradigm for ion secretion by teleost ionocytes, tilapia
397 'SW-type/Type-IV' ionocytes express Nkcc1 and Cftr1 in the basolateral and apical
398 membranes, respectively (Hiroi et al., 2008; Kaneko et al., 2008). Here, *nkcc1a* and *cftr1*
399 levels were markedly diminished within 24 h of FW exposure in agreement with the
400 disappearance of SW-type ionocytes that occurs during FW acclimation (Fig. 5C, D)
401 (Hiroi et al., 2008). The transition from ion secretion to absorption in the gill, as indicated
402 by *ncc2*, *nkcc1a*, and *cftr1*, was coincident with increased *ncc1* in the kidney and urinary
403 bladder. Thus, our study provides new insight into how these three ionoregulatory
404 organs work simultaneously during the acute phase of FW acclimation to mitigate
405 perturbations to osmoregulatory balance.
406 In conclusion, the capacity to modulate ionoregulatory processes via the
407 regulated expression of genes that encode effectors of ion transport is a critical aspect of
408 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).

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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640

641 **Figure legends**

642 **Fig. 1.** Ultrastructure of columnar cells within the urinary bladder of freshwater-
643 acclimated tilapia. Apical microvilli (black arrowheads), tight-junctions within intracellular
644 spaces (white arrowheads), mitochondria (white asterisks), and the cytoplasmic area
645 adjacent to the microvilli (black asterisk) are indicated in the micrograph (A). Magnified

646 view of a tight-junction between columnar cells adjacent to the lumen of the urinary
647 bladder (B).
648
649 **Fig. 2.** Tissue expression of *ncc1* (A), *ncc2* (B), *nhe3* (C), *clc2c (D)*, and *clc3* (E) mRNA
650 in freshwater-acclimated tilapia. Means ± S.E.M. ($n$ = 6). Data were normalized to *ef1α*
651 as a reference gene and are presented relative to brain expression levels. ND = no
652 detection. Means not sharing the same letter are significantly different (one-way ANOVA,
653 Tukey's HSD, $P < 0.05$).
654
655 **Fig. 3.** Plasma osmolality (A) and urinary bladder *ncc1* (B), *nhe3* (C), *clc2c* (D), and *clc3*
656 (E) mRNA levels in seawater- (SW; solid bars) and freshwater (FW; open bars)-
657 acclimated tilapia. Means ± S.E.M. ($n$ = 16). mRNA levels in FW are presented as a fold-
658 change from SW. Asterisks indicate significant differences between salinities (***$P <$
659 0.001) by Student's t-test.
660
661 **Fig. 4.** Renal *ncc1* (A), *nhe3* (B), *clc2c* (C), and *clc3* (D) mRNA levels in seawater- (SW;
662 solid bars) and freshwater (FW; open bars)-acclimated tilapia. Means ± S.E.M. ($n$ = 16).
663 mRNA levels in FW are presented as a fold-change from SW. Asterisks indicate
664 significant differences between salinities (**$P < 0.01$ and ***$P < 0.001$) by Student's t-
665 test.
666
667 **Fig. 5.** Plasma osmolality (A) and branchial *ncc2* (B), *nkcc1a* (C), and *cftr1* (D) mRNA
668 levels at 6, 24, and 48 h after transfer of tilapia from seawater (SW) to fresh water (FW;
669 open symbols). Time-matched control fish were maintained in SW (solid symbols).
670 Means ± S.E.M. ($n$ = 8). Gene expression is presented as a fold-change from the SW-
671 acclimated group at time 0. Differences among groups were evaluated by two-way
672 ANOVA. Significant effects of treatment, time, or an interaction are indicated in
673 respective panels (*$P < 0.05$ and ***$P < 0.001$). When there was a significant treatment
674 effect, *post hoc* comparisons (Bonferroni's multiple comparisons test of time-matched
675 groups) were made at each time point ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$).
676
677 **Fig. 6.** Urinary bladder *ncc1* (A), *nhe3* (B), *clc2c* (C), and *clc3* (D) mRNA levels at 6, 24,
678 and 48 h after transfer of tilapia from seawater (SW) to fresh water (FW; open symbols).
679 Time-matched control fish were maintained in SW (solid symbols). Means ± S.E.M. ($n$ =

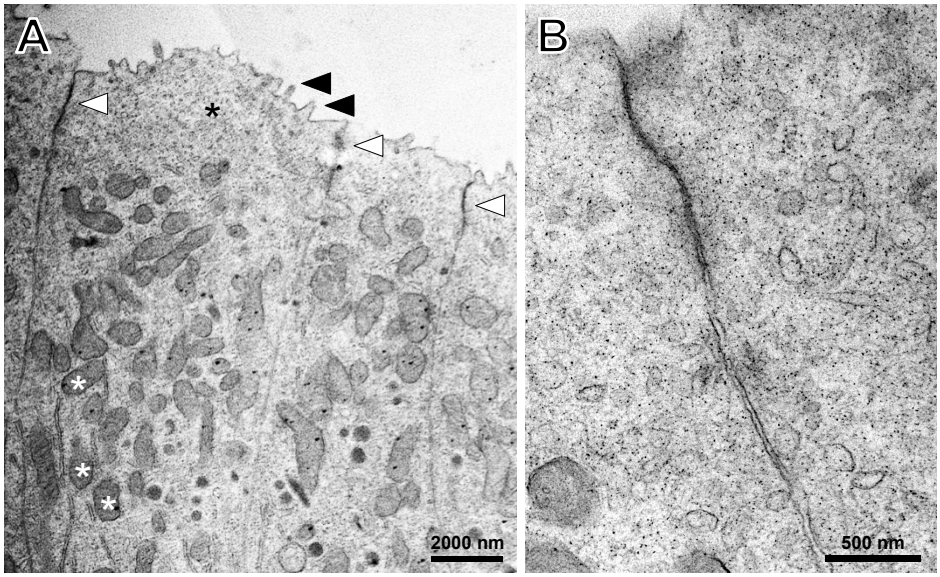
8). Gene expression is presented as a fold-change from the SW-acclimated group at time 0. Differences among groups were evaluated by two-way ANOVA. Significant effects of treatment, time, or an interaction are indicated in respective panels (*$P < 0.05$ and **$P < 0.01$). When there was a significant 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 $^{\dagger\dagger}P < 0.01$).

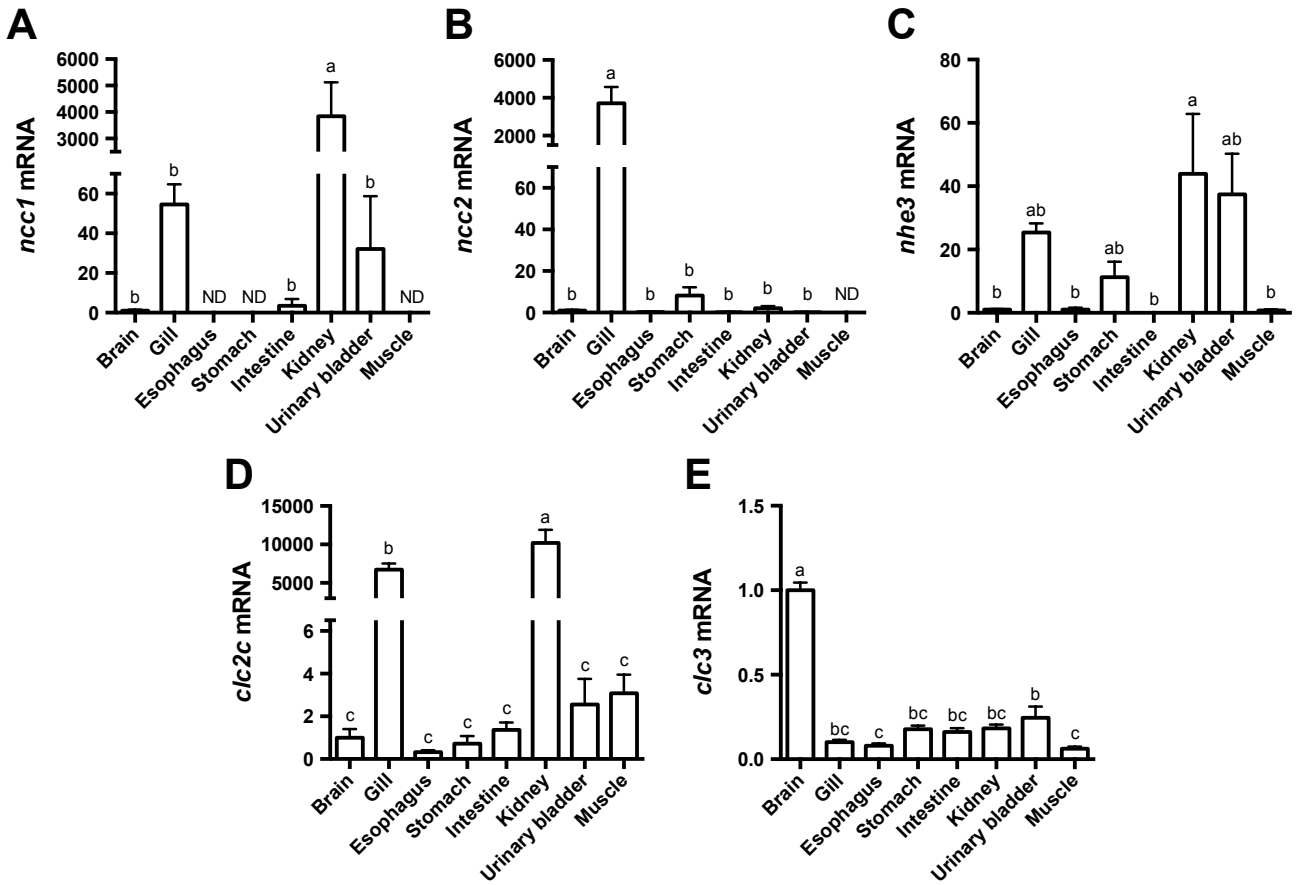
**Table 1.** Specific primer sequences for quantitative real-time PCR.

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

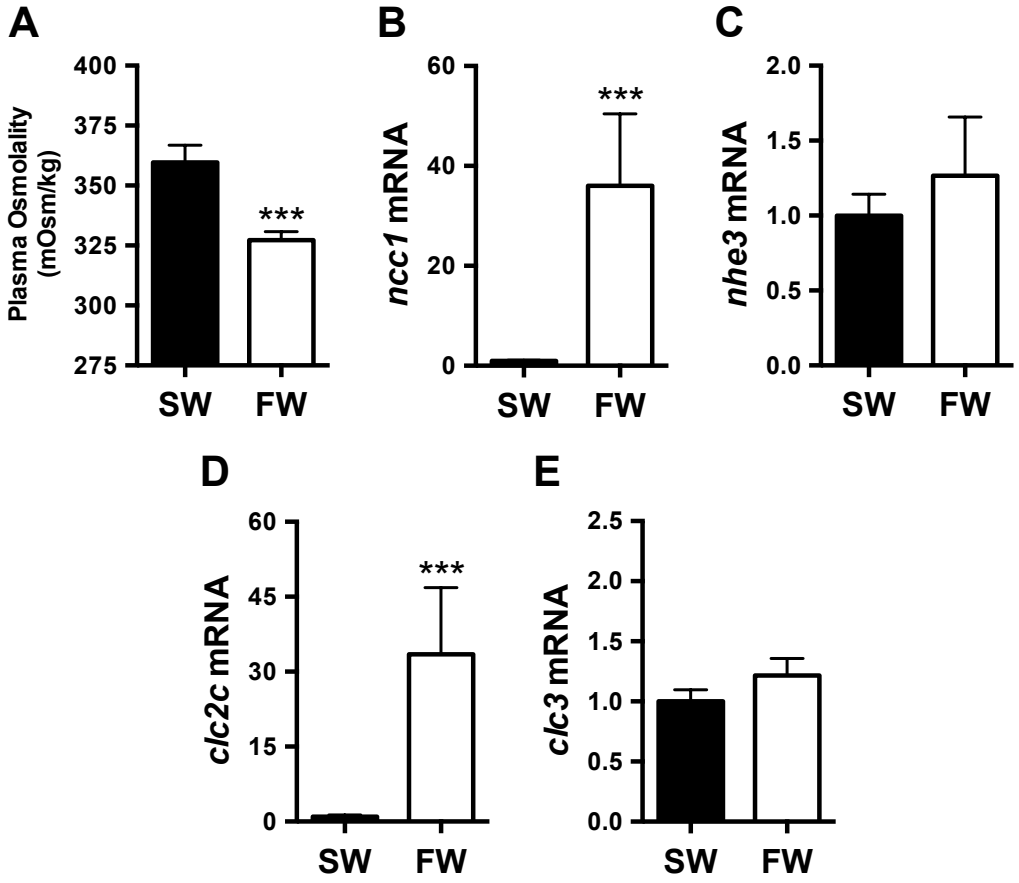
# Fig. 1



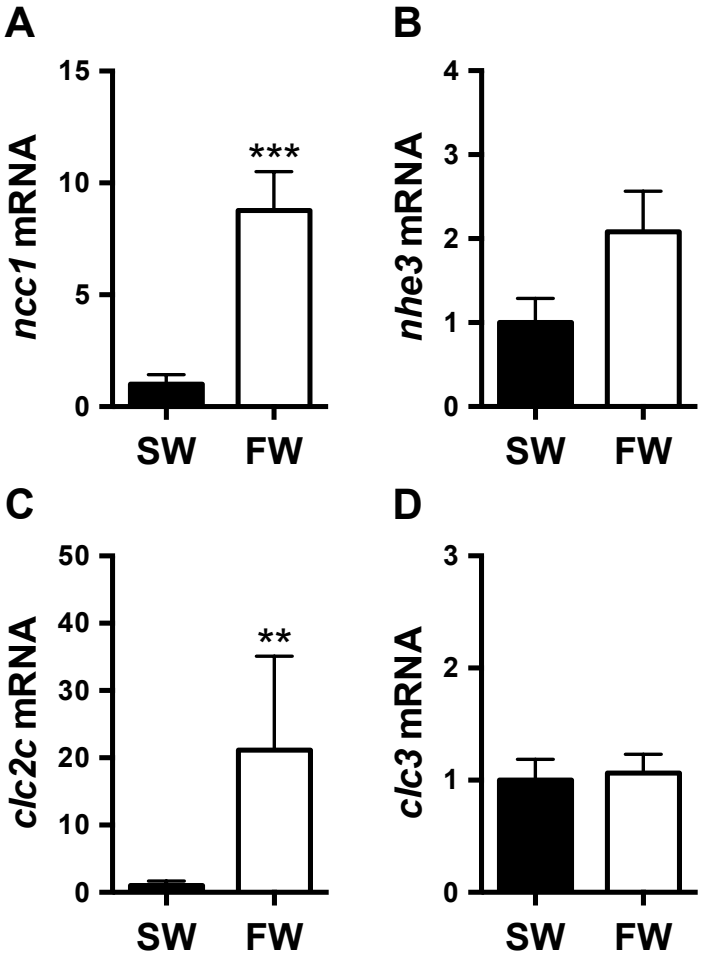
# Fig. 2


A
ncc1 mRNA
6000
5000
4000
3000
60
40
20
0
b
b
ND
ND
b
a
b
ND
Brain
Gill
Esophagus
Stomach
Intestine
Kidney
Urinary bladder
Muscle
B
ncc2 mRNA
6000
4000
2000
60
40
20
0
b
a
b
b
b
b
b
ND
Brain
Gill
Esophagus
Stomach
Intestine
Kidney
Urinary bladder
Muscle
C
nhe3 mRNA
80
60
40
20
0
b
ab
b
ab
b
a
ab
b
Brain
Gill
Esophagus
Stomach
Intestine
Kidney
Urinary bladder
Muscle
D
clc2c mRNA
15000
10000
5000
6
4
2
0
c
b
c
c
c
a
c
c
Brain
Gill
Esophagus
Stomach
Intestine
Kidney
Urinary bladder
Muscle
E
clc3 mRNA
1.5
1.0
0.5
0.0
a
bc
c
bc
bc
bc
b
c
Brain
Gill
Esophagus
Stomach
Intestine
Kidney
Urinary bladder
Muscle

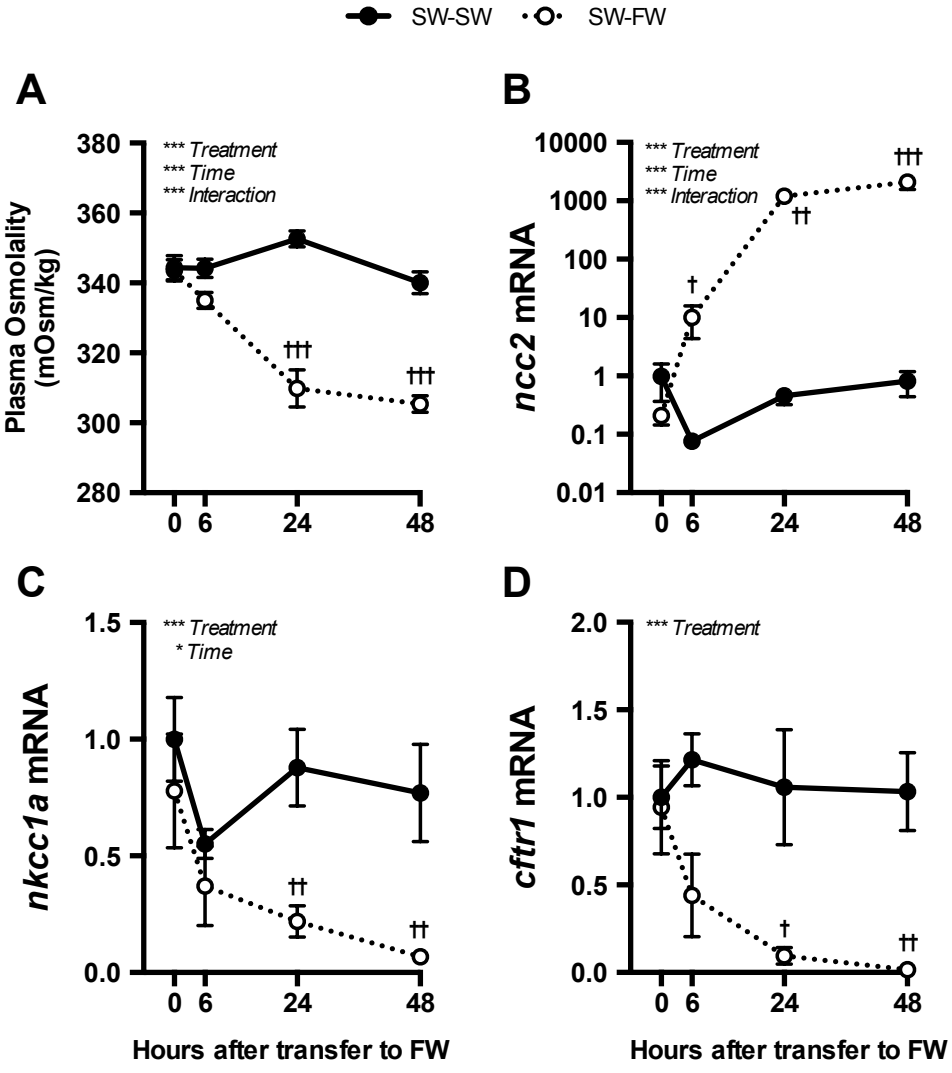
# Fig. 3


A
Plasma Osmolality (mOsm/kg)
400
375
350
325
300
275
***
SW
FW
B
ncc1 mRNA
60
40
20
0
***
SW
FW
C
nhe3 mRNA
2.0
1.5
1.0
0.5
0.0
SW
FW
D
clc2c mRNA
60
45
30
15
0
***
SW
FW
E
clc3 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
SW
FW

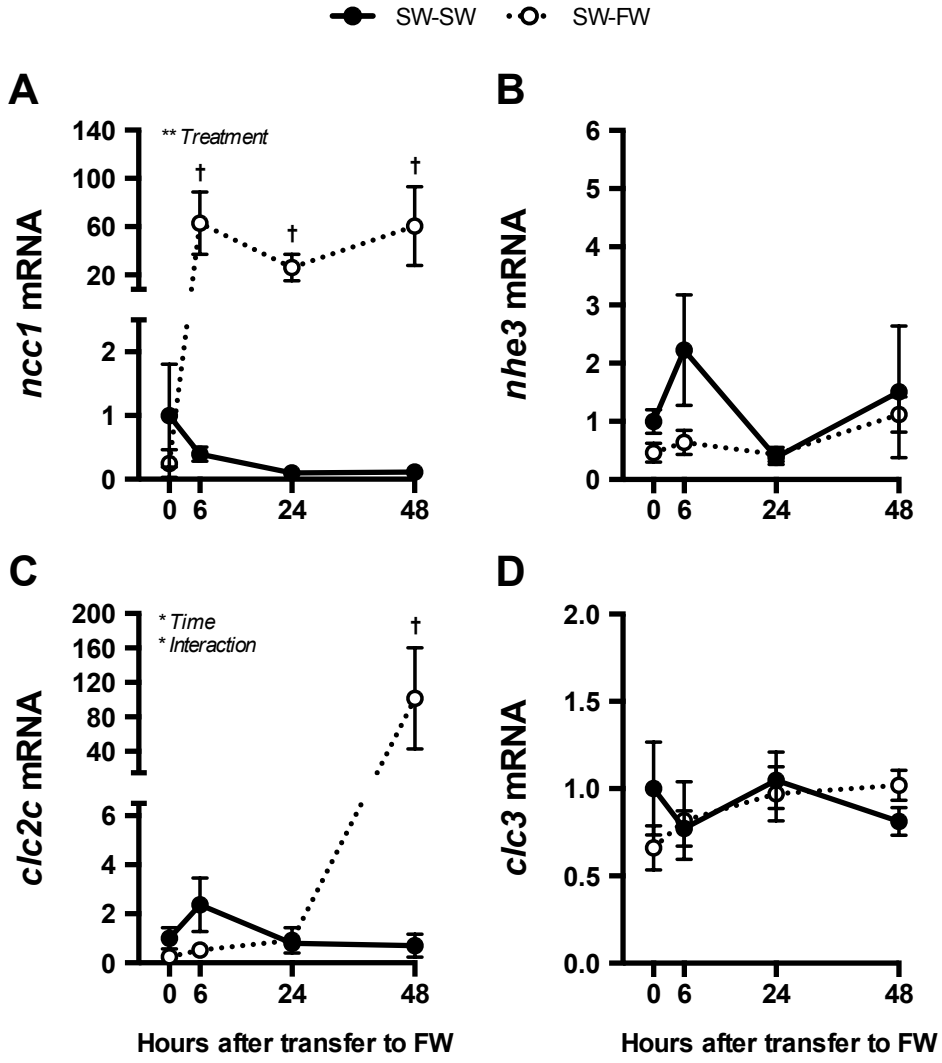
# Fig. 4


A
ncc1 mRNA
15
10
5
0
***
SW
FW
B
nhe3 mRNA
4
3
2
1
0
SW
FW
C
clc2c mRNA
50
40
30
20
10
0
**
SW
FW
D
clc3 mRNA
3
2
1
0
SW
FW

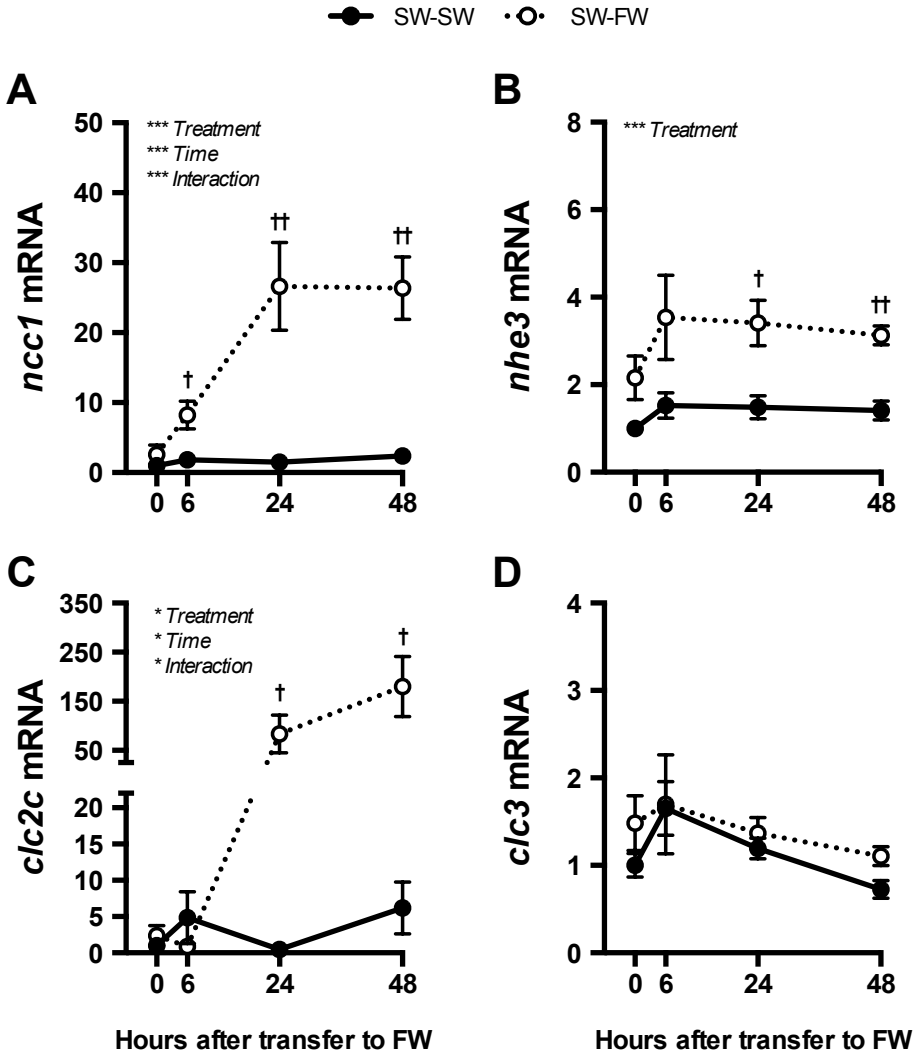
# Fig. 5

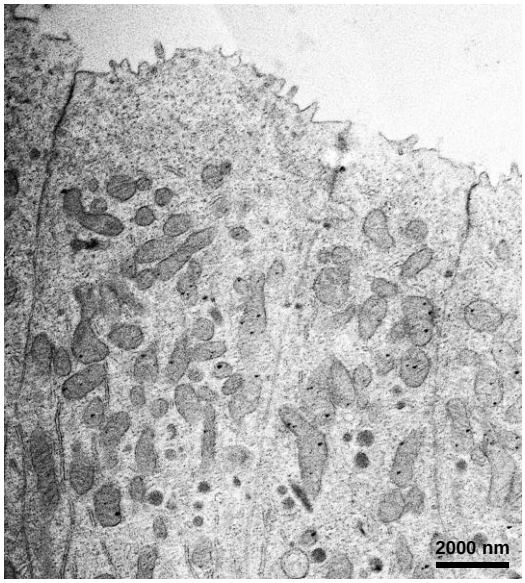

SW-SW
SW-FW
A
Plasma Osmolality (mOsm/kg)
*** Treatment
*** Time
*** Interaction
380
360
340
320
300
280
0 6 24 48
B
ncc2 mRNA
*** Treatment
*** Time
*** Interaction
10000
1000
100
10
1
0.1
0.01
0 6 24 48
C
nkcc1a mRNA
*** Treatment
* Time
1.5
1.0
0.5
0.0
0 6 24 48
Hours after transfer to FW
D
cftr1 mRNA
*** Treatment
2.0
1.5
1.0
0.5
0.0
0 6 24 48
Hours after transfer to FW

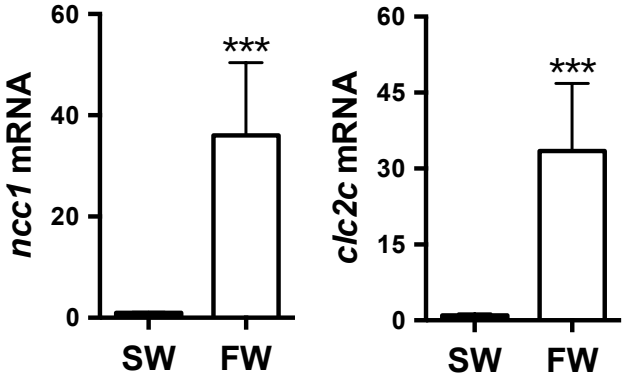
# Fig. 6


SW-SW
SW-FW
A
** Treatment
ncc1 mRNA
140
100
60
20
2
1
0
0 6 24 48
B
nhe3 mRNA
6
5
4
3
2
1
0
0 6 24 48
C
* Time
* Interaction
clc2c mRNA
200
160
120
80
40
6
4
2
0
0 6 24 48
Hours after transfer to FW
D
clc3 mRNA
2.0
1.5
1.0
0.5
0.0
0 6 24 48
Hours after transfer to FW

# Fig. 7


SW-SW
SW-FW
A
*** Treatment
*** Time
*** Interaction
ncc1 mRNA
50
40
30
20
10
0
0 6 24 48
B
*** Treatment
nhe3 mRNA
8
6
4
2
0
0 6 24 48
C
* Treatment
* Time
* Interaction
clc2c mRNA
350
250
150
50
20
15
10
5
0
0 6 24 48
Hours after transfer to FW
D
clc3 mRNA
4
3
2
1
0
0 6 24 48
Hours after transfer to FW


2000 nm


ncc1 mRNA
60
40
20
0
***
SW
FW
clc2c mRNA
60
45
30
15
0
***
SW
FW