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The effects of transfer from steady-state to tidally-changing salinities on plasma and branchial osmoregulatory variables in adult Mozambique tilapia

K. Keano Pavlosky[a,b], Yoko Yamaguchi[a,c], Darren T. Lerner[a,d], Andre P. Seale[a,e,*]

[a] Hawaiʻi Institute of Marine Biology, University of Hawaiʻi, Kāneʻohe, HI 96744, USA
[b] Department of Molecular Biosciences and Bioengineering, University of Hawaiʻi at Mānoa, Honolulu, HI 96822, USA
[c] Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690-8504, Japan
[d] University of Hawaiʻi Sea Grant College Program, University of Hawaiʻi at Mānoa, Honolulu, HI 96822, USA
[e] Department of Human Nutrition, Food and Animal Sciences, University of Hawaiʻi at Mānoa, Honolulu, HI 96822, USA

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ABSTRACT

The Mozambique tilapia, *Oreochromis mossambicus*, is a teleost fish native to estuarine waters that vary in salinity between fresh water (FW) and seawater (SW). The neuroendocrine system plays a key role in salinity acclimation by directing ion uptake and extrusion in osmoregulatory tissues such as gill. While most studies with *O. mossambicus* have focused on acclimation to steady-state salinities, less is known about the ability of adult fish to acclimate to dynamically-changing salinities. Plasma osmolality, prolactin (PRL) levels, and branchial gene expression of PRL receptors (PRLR1 and PRLR2), Na^+/Cl^- and $Na^+/K^+/2Cl^-$ co-transporters (NCC and NKCC), Na^+/K^+-ATPase (NKAα1a and NKAα1b), cystic fibrosis transmembrane conductance regulator (CFTR), and aquaporin 3 (AQP3) were measured in fish reared in FW and SW steady-state salinities, in a tidal regimen (TR) where salinities changed between FW and SW every six hours, and in fish transferred from FW or SW to TR. Regardless of rearing regimen, plasma osmolality was higher in fish in SW than in FW fish, while plasma PRL was lower in fish in SW. Furthermore, branchial gene expression of effectors of ion transport in TR fish showed greater similarity to those in steady-state SW fish than in FW fish. By seven days of transfer from steady-state FW or SW to TR, plasma osmolality, plasma PRL and branchial expression of effectors of ion transport were similar to those of fish reared in TR since larval stages. These findings demonstrate the ability of adult tilapia reared in steady-state salinities to successfully acclimate to dynamically-changing salinities. Moreover, the present findings suggest that early exposure to salinity changes does not significantly improve survivability in future challenge with dynamically-changing salinities.

1. Introduction

Maintaining internal osmotic homeostasis is critical to life in many organisms, including vertebrates. Most vertebrates maintain plasma osmolality within a narrow physiological range, typically through exchange of ions and water between cells and the extracellular environment. In the Mozambique tilapia, *Oreochromis mossambicus*, as in other teleost fishes, plasma osmolality is maintained near one-third the osmolality of seawater (SW; McCormick, 2001). As a euryhaline species native to estuarine waters off the southeast coast of Africa (Trewavas, 1983), the Mozambique tilapia is capable of recovering from major departures above or below their physiological range of plasma osmolality (between 305 and 443 mOsm/kg; Seale et al., 2013). This allows these fish to survive in external salinities equivalent to fresh water (FW) through double-strength SW (Fiess et al., 2007; Stickney, 1986). While the ability of Mozambique tilapia to tolerate steady-state environments of distinct salinities is well established, less is known about their osmoregulatory physiology in dynamically-changing salinities.

Fluctuations in salinity characterize some of the environments to which Mozambique tilapia are native, such as near shore estuaries. Recently, we have described the distinct osmoregulatory profile that tilapia reared under tidally-changing salinities acquire relative to fish reared in steady-state FW or SW since the yolk-sack fry stage (up to 15 days post-fertilization, until yolk-sac is fully absorbed; Moorman et al., 2014, 2015). Here, we characterize whether the unique osmoregulatory profile of tidally-reared fish may be acquired by fully developed adult fish that have been reared in steady-state salinities for at least two years prior to a transfer to tidally-changing salinities without exposure to any salinity change during early development. Generally, tilapia and other teleosts in FW hyperosmoregulate to counteract a

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[*] Corresponding author at: Department of Human Nutrition, Food and Animal Sciences, University of Hawaiʻi at Mānoa, Honolulu, HI 96822, USA.
E-mail address: seale@hawaii.edu (A.P. Seale).

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tendency to lose solutes to the environment and to become over-hydrated (McCormick, 2001). On the other hand, in SW they hypoosmoregulate to counteract a tendency to lose water to the environment and gain solutes (McCormick, 2001). Osmoregulation is conducted predominantly via gill, kidney and intestine, with gill as the site of direct contact with the external environment and major site of monovalent ion transport (Evans et al., 2005).

The pituitary hormone prolactin (PRL) is essential for hyper-osmoregulation in fish in FW (Dharmamba et al., 1967; Manzon, 2002; Pickford and Phillips, 1959). Consistent with this action, plasma PRL in the Mozambique tilapia is inversely related to external osmolality (Seale et al., 2005), and PRL has been shown in FW to increase ion uptake and decrease water permeability at the gill (Breves et al., 2014). There are two isoforms of PRL receptors reported for Mozambique tilapia, PRLR1 and PRLR2 (Fiol et al., 2009). In vitro, receptors in the gill and pituitary are differentially responsive to PRL and environmental osmolality: increases in extracellular PRL stimulate *prlr1* expression (Inokuchi et al., 2015), whereas increased extracellular osmolality stimulates *prlr2* expression (Inokuchi et al., 2015; Seale et al., 2012). Hence, mounting evidence indicates the actions of PRL on osmoregulation are likely regulated both by circulating levels of the hormone and the availability of its receptors.

Specialized ionocytes direct osmoregulation in the gill. These cells have been categorized into FW and SW types based on their primary functions in ion uptake and extrusion, respectively (Hiroi et al., 2005; Kaneko et al., 2008). Both FW and SW ionocytes express basolateral Na^+/K^+-ATPase (NKA), an ion pump critical to establishing electrochemical gradients across the cell membrane, which drives ion secretion and absorption (Hiroi et al., 2005). NKA comprises multiple subunits, and two isoforms of NKA α sub-unit, α1a and α1b, have been described in tilapia gill (Tipsmark et al., 2011). Branchial mRNA expression of *nkaα1a* is upregulated in response to a fall in extracellular osmolality and to PRL, and is the prevalent isoform in FW type ionocytes (Inokuchi et al., 2015; Tipsmark et al., 2011). On the other hand, branchial mRNA expression of *nkaα1b* has been reported to increase when fish are transferred from FW to SW (Tipsmark et al., 2011); recent results, however, were unable to fully corroborate this relationship (Inokuchi et al., 2015; Moorman et al., 2014). The presence of Na^+/Cl^- cotransporter (NCC) in the apical membrane is specific to FW ionocytes (Hiroi et al., 2005; Hiroi et al., 2008). Transcription of *ncc* is directly regulated by PRL and a fall in extracellular osmolality (Breves et al., 2010b; Inokuchi et al., 2015). Seawater ionocytes, on the other hand, are characterized by presence of basolateral $Na^+/K^+/2Cl^-$ co-transporter (NKCC1a) and apical cystic fibrosis transmembrane conductance regulator (CFTR, an ion channel responsible for Cl^- secretion by ionocytes of teleost fish in SW; Hiroi et al., 2005; Inokuchi et al., 2008). During acclimation to SW, CFTR is trafficked into the apical membrane, while NKCC is translocated to the basolateral membrane of ionocytes (Marshall et al., 2002). Expression of *nkcc1a* has been shown to be directly osmosensitive, increasing with external osmolality (Inokuchi et al., 2015). In euryhaline teleost species, mRNA expression of *cftr* is elevated in SW-acclimated fish compared with FW-acclimated fish (Moorman et al., 2014; Moorman et al., 2015; Tse et al., 2006). Additionally, *cftr* expression increases when fish are moved from FW to SW, and decreases when subject to the opposite transfer (Moorman et al., 2015; Scott and Schulte, 2005; Singer et al., 1998; Tse et al., 2006). Importantly, it has been demonstrated that an increase in *cftr* expression in SW is linked not only to the trafficking of CFTR to the apical membrane but to the actual secretion of Cl^- (Marshall et al., 1999). Lastly, aquaporin 3 (AQP3), a basolaterally-located water channel, occurs in both FW and SW ionocytes (Watanabe et al., 2005). In Mozambique tilapia and other teleost species, branchial *aqp3* expression is elevated in FW-acclimated over SW-acclimated animals (Cutler and Cramb, 2002; Jung et al., 2012; Lignot et al., 2002; Madsen et al., 2014; Moorman et al., 2015; Tipsmark et al., 2011), and has recently been shown to increase in direct response to PRL (Breves et al., 2016).

Much of the current understanding of osmoregulation in Mozambique tilapia, including ionocyte morphology and function, is based on prior studies that are largely focused on fish reared in steady-state FW or SW, or following one-way transfers between the two. Recently, we described an experimental tidal regimen (TR) rearing paradigm in which Mozambique tilapia are exposed to alternating six-hour phases of FW and SW, simulating salinity fluctuations found in their native distribution (Moorman et al., 2014; Moorman et al., 2015). Those studies characterized the osmoregulatory profile for fish reared in tidally-changing salinities from yolk-sac fry to four month old fish. In our previous study it was concluded that developing tilapia experiencing tidal-salinity oscillations could respond better to a future one-way transfer of salinity from FW to SW, compared with fish reared in steady-state salinities (Moorman et al., 2015). It is unknown, however, whether adult fish retain such physiological plasticity as observed in juveniles. In anadromous species, individuals at different life stages often exhibit distinct tolerances to environmental salinity (Jensen et al., 2015). Despite the remarkable euryhalinity of the non-anadromous Mozambique tilapia, little is known on how osmoregulatory capacity is established and maintained throughout their life history. Hence, we tested whether the ability of adult fish to acclimate to TR required pre-exposure to both FW and SW during early developmental stages and whether the key variables associated with osmoregulation paralleled those of steady-state FW and SW fish. To address these questions, the following endpoints were measured both in fish reared in FW, SW and TR for two years, and in those transferred from FW or SW steady-states to TR for up to one week: 1) plasma osmolality; 2) circulating PRL levels; and 3) branchial mRNA expression of PRL receptors and effectors of ion transport shown previously to be responsive to changes in extracellular osmolality and/or PRL.

2. Materials and methods

2.1. Experiment 1 – Salinity regimen baseline

Male and female Mozambique tilapia were reared for two years from yolk-sac fry, under natural photoperiod, at the Hawai'i Institute of Marine Biology (HIMB; Kāne'ohe, HI). Animals were kept in outdoor 700 L tanks supplied with either FW (0.1 ± 0.1‰) or SW (34 ± 1‰; Kāne'ohe Bay, Kāne'ohe, HI), or alternating FW and SW in six-hour phases simulating a tidally-changing salinity (TR), as previously described (Moorman et al., 2014). Physicochemical properties of the FW and SW employed have been recently reported elsewhere (Breves et al., 2017). In TR tanks, 95% and 100% changes in salinity were obtained by two hours and three hours, respectively, either from FW to SW or SW to FW (Fig. 1). Water temperature was kept at 25 ± 2 °C. Fish were fed trout chow pellets (Skretting, Tooele, UT) once daily to satiation. At the time of sampling, fish weighed 191.6 g – 1.1 kg. Nine fish from each rearing salinity were sampled. Fish reared in TR were collected at the end of the FW and SW phases of the cycle.

2.2. Experiment 2 – Transfer from steady-state salinities to tidal regimen

Adult male and female Mozambique tilapia were collected from broodstock maintained at HIMB, and held under natural photoperiod in outdoor 700 L tanks supplied with FW or SW, as above. Ninety-six FW-acclimated fish were allocated randomly across four replicate FW tanks (24 fish per tank), and 96 SW-acclimated fish across four replicate SW tanks (24 fish per tank). Water temperature was kept at 25 ± 2 °C. Fish were allowed an acclimation period of three weeks after seeding to the replicate tanks. Fish were fed trout chow pellets (Skretting) once daily to satiation. On Day 0 of the experiment, eight fish from each of the four FW and four SW tanks were sampled. Then, water supply to three of the FW and three of the SW tanks was adjusted to facilitate the following salinity transfers: FW to SW (one tank), FW to TR (two tanks), SW to FW

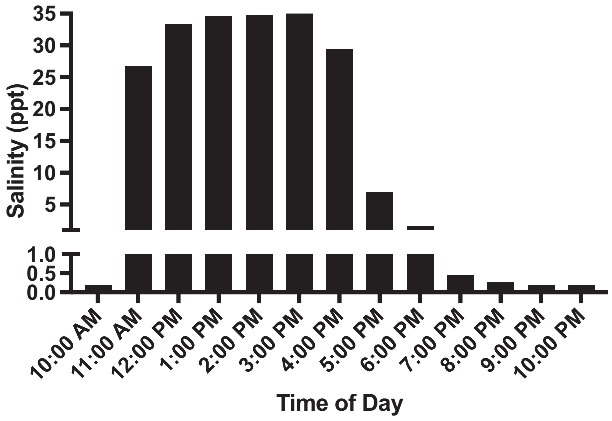


Fig. 1. Salinity (ppt) changes in a tidal regimen tank, between 10 AM and 10 PM.

(one tank), and SW to TR (two tanks). One FW tank and one SW tank were retained as parallel controls for the duration of the experiment. Fish transferred from FW to SW were first acclimated to 82–85% SW (29–30‰) over 48 h, and then the water supply was adjusted to full strength SW. From each of the eight experimental tanks, eight fish were sampled on Day 3 and Day 7. From the FW to TR and SW to TR tanks, fish from one tank were sampled at the end of the FW phase (TF) of the tidal cycle, and fish from the second tank were sampled at the end of the SW phase (TS) of the tidal cycle. The same tanks were sampled at the end of the same tidal phase for the entire experiment. Fish sampled over the seven-day period weighed 87–570 g at the time of sampling.

2.3. Sampling

At the time of sampling, fish were netted and anesthetized with 2-phenoxyethanol (0.3 mL l^{-1}; Sigma-Aldrich, St. Louis, MO). After fish were weighed, blood was collected from the caudal vasculature with a needle and syringe coated with sodium heparin (200 U/mL, Sigma-Aldrich), and fish were euthanized by rapid decapitation. Plasma was separated by centrifugation and stored at −20 °C for further analysis. Gill filaments were collected from the second gill arch on the left side of the fish, frozen in liquid nitrogen and stored at −80 °C until further analysis. All experiments and sampling were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawai‘i.

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from frozen gill samples using TRI Reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Using the High Capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA), 5 μL of total RNA (400 ng/μL) was reverse transcribed into cDNA. Quantitative real-time PCRs (qRT-PCRs) were set up as previously described (Pierce et al., 2007), using the StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA). The mRNA levels of reference and target genes were determined by absolute quantification. Standard curves for quantification were generated using serially diluted target gene cDNA fragments of known concentration (standard cDNAs). Elongation factor 1α (EF1α) was used as a reference gene to normalize the mRNA levels of target genes after it was verified that *ef1α* mRNA expression did not vary across treatments. The PCR mixture (15 uL) contained Power SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of forward and reverse primers as listed in Table 1, and 2 μL of standard cDNAs or cDNAs prepared from experimental samples. Dilution of experimental cDNA ranged from 20- to 100-fold. PCR cycling parameters were 50 °C for 2 min and 95 °C for ten minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for one minute. For both experiments, R^2 values and amplification efficiencies for standard curves varied between 0.989 and 0.999, and 67.5–96.8%, respectively. Relative mRNA abundance data are expressed as fold-change compared with FW:FW control Day 0 values, and referred to as mRNA expression throughout the manuscript.

Table 1
Primers used for qPCR.

Gene name	Primer sequence (5′-3′)		Reference
ef1α	Forward	AGCAAGTACTACGTGACCATCATTG	Breves et al., 2010b
	Reverse	AGTCAGCCTGGGAGGTACCA	
prlr1	Forward	TGGGTCAGCTACAACATCACTGT	Pierce et al., 2007
	Reverse	GGATGGGGCTTGACAATGTAGA	
prlr2	Forward	GCCCTTGGGAATACATCTTCAG	Breves et al., 2010b
	Reverse	GTGCATAGGGCTTCACAATGTC	
ncc	Forward	CCGAAAGGCACCCTAATGG	Inokuchi et al., 2008
	Reverse	CTACACTTGCACCAGAAGTGACAA	
nkcc1a	Forward	GGAGGCAAGATCAACAGGATTG	Inokuchi et al., 2008
	Reverse	AATGTCCGAAAAGTCTATCCTGAACT	
nkaα1a	Forward	AACTGATTTGGTCCCTGCAA	Tipsmark et al., 2011
	Reverse	ATGCATTTCTGGGCTGTCTC	
nkaα1b	Forward	GGAGCGTGTGCTTCATCACT	Tipsmark et al., 2011
	Reverse	ATCCATGCTTTGTGGGGTTA	
cftr	Forward	CATGCTCTTCACCGTGTTCT	Moorman et al., 2014
	Reverse	GCCACAATAATGCCAATCTG	
aqp3	Forward	CATGTACTATGATGCTTTGTTGCTC	Watanabe et al., 2005
	Reverse	CAAAGAAACCATTGACAAGTGTGA	

ef1α: elongation factor 1α; *prlr1*: prolactin receptor 1; *prlr2*: prolactin receptor 2; *ncc*: Na^+/Cl^- cotransporter; *nkcc1a*: $Na^+/K^+/2Cl^-$ cotransporter; *nkaα1a*: Na^+/K^+-ATPase α sub-unit isoform 1a; *nkaα1b*: Na^+/K^+-ATPase α sub-unit isoform 1b; *cftr*: cystic fibrosis transmembrane conductance regulator; *aqp3*: aquaporin 3.

2.5. Plasma osmolality and prolactin

Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5100C; Wescor, Logan, UT). Of the two isoforms of PRL, PRL_{177} and PRL_{188}, produced and released by the pituitary of tilapia, PRL_{188} was measured in this study based on its robust responses to changes in salinity (Seale et al., 2012), and is referred to as PRL throughout the text. Plasma PRL was measured via homologous radioimmunoassay (RIA) as previously described (Ayson et al., 1993; Yamaguchi et al., 2016).

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Main and interaction effects ($P < 0.05$) of salinity (FW or SW) and rearing regimen (tidal or steady-state) in Experiment 1, and effects of salinity treatment (eight experimental groups) and time (Day 0, 3 and 7) in Experiment 2 were analyzed by two-way analysis of variance (ANOVA). Fisher's protected Least Significant Difference (LSD) test was used to assess the effects of interactions when detected. Where applicable, individual values were log-transformed prior to the analysis, to meet assumptions of normality and equal variance. Statistical calculations were performed using a statistical software program, Prism 6.0 (GraphPad, La Jolla, CA).

3. Results

3.1. Experiment 1 – Plasma osmolality and prolactin

Plasma osmolality and PRL were compared between fish in FW and SW, reared under steady-state and tidal regimens. A two-way ANOVA revealed an effect of salinity on plasma osmolality, which was elevated in SW fish compared with FW fish; there was no effect of rearing regimen (Fig. 2A). Effects of salinity and rearing regimen on plasma PRL were observed: PRL levels were higher in FW fish than in SW fish, and

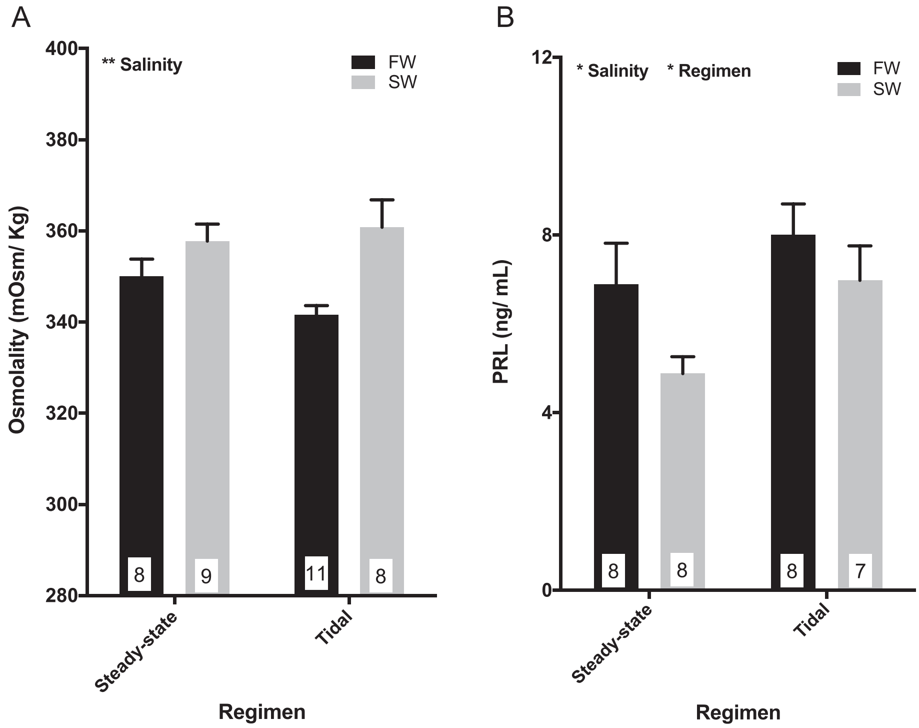


Fig. 2. Effects of rearing condition on plasma osmolality (A) and plasma prolactin (B) in fish sampled in steady-state FW and SW, and at the end of the FW and SW phase of the tidal cycle. Values are expressed as means ± S.E.M. ($n = 7–11$). Numbers within bars represent sample sizes for each group. Effects of salinity and regimen were analyzed by two-way ANOVA (*,**Significant at $P < 0.05$ and 0.01, respectively).

in tidal fish compared with steady-state fish (Fig. 2B).

3.2. Experiment 1 – Branchial gene expression of PRL receptors, ion and water transporters and ion ATPases

The branchial gene mRNA expression of PRL receptors, ion and water transporters and ion ATPases was compared between fish in FW and SW, and reared under steady-state and tidal regimens. A two-way ANOVA revealed an interaction effect of salinity and rearing regimen on branchial mRNA expression of *prlr1*; while expression of *prlr1* in steady-state was higher in fish in FW than those in SW, in a tidal regimen, expression was higher in fish in SW (Fig. 3A). By contrast, a single effect of salinity was detected on *prlr2* mRNA expression, which was elevated in SW regardless of rearing regimen (Fig. 3B). Single and interaction effects of both salinity and rearing regimen were observed on *ncc* mRNA expression; *ncc* expression in steady-state FW fish was nearly 100-fold higher than that of SW, TF, and TS fish, which were mutually similar (Fig. 3C). Single and interaction effects of salinity and rearing regimen were also observed in branchial *nkcc1a* mRNA expression; unlike *ncc* mRNA expression, however, expression was higher in steady-state SW fish than in FW fish, and highest in SW fish reared in a tidal regimen (Fig. 3D). Salinity, rearing regimen and interaction effects were observed on branchial mRNA expression of *nkaα1a*; expression in steady-state FW fish was nearly 10-fold higher compared with SW fish, and similar to that of fish reared in a tidal regimen (Fig. 3E). With a single effect of salinity, *nkaα1b* mRNA expression was higher in fish in SW compared with those in FW (Fig. 3F). Salinity, rearing regimen and interaction effects were observed on *cftr* mRNA expression; expression in both steady-state and tidal SW fish exceeded that of FW fish reared under the same regimens (Fig. 3G). By contrast, salinity and interaction effects on branchial *aqp3* mRNA expression indicated higher expression in steady-state fish in FW compared with those in SW. While expression was similar across both phases of the tidal cycle, it was higher in FW than in SW steady-state fish (Fig. 3H).

3.3. Experiment 2 – Plasma osmolality and prolactin

Plasma osmolality and PRL were compared between fish reared in FW or SW and transferred to steady-state or tidally-changing salinities over a seven-day period. A two-way ANOVA revealed salinity and interaction effects on plasma osmolality (Fig. 4A). By Day 3, plasma osmolality increased in fish transferred from FW to SW (FW:SW) and decreased in fish transferred from SW to FW (SW:FW) when compared with both parallel (FW:FW and SW:SW, respectively) and time 0 controls. Plasma osmolality was elevated in fish in TS compared with those in TF following transfers from either FW or SW to tidal salinities by Day 3 (FW:TF vs. FW:TS and SW:TF vs. SW:TS, respectively).

Salinity, time, and interaction effects were observed on plasma PRL (Fig. 4B). In FW controls (FW:FW) and SW controls (SW:SW), PRL levels remained steady and similar throughout the experiment. Plasma PRL was lower in fish transferred from FW to SW (FW:SW), than in FW controls (FW:FW) by Day 7. On the other hand, in fish transferred from SW to FW (SW:FW) PRL was significantly elevated compared to SW controls (SW:SW) by Day 3. By Day 7, plasma PRL in fish in FW:SW, FW:TF, and FW:TS groups were similar to those in SW:TF and SW:TS groups, which remained unchanged from SW controls throughout the experiment.

3.4. Experiment 2 – Branchial gene expression of PRL receptors, ion and water transporters and ion ATPases

The branchial gene expression of PRL receptors, ion and water transporters and ion ATPases were compared between fish reared in FW or SW and transferred to steady-state or tidally-changing salinities over a seven-day period. A two-way ANOVA indicated interaction effects of salinity and time for all genes analyzed (Fig. 5 A–H.) Branchial *prlr1* mRNA expression decreased following transfer from FW to SW by Day 3, and increased following transfer from SW to FW by Day 7 relative to time-matched steady-state controls (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5A). Expression of *prlr1* was elevated in fish in TF compared with those in TS following transfers from either FW or SW to tidal salinities by Day 7 (FW:TF vs. FW:TS and SW:TF vs. SW:TS, respectively). By contrast, branchial *prlr2* mRNA expression increased and decreased following transfers from FW to SW and SW to FW, respectively, by Day 3 (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively); this pattern, however, was not sustained through Day 7

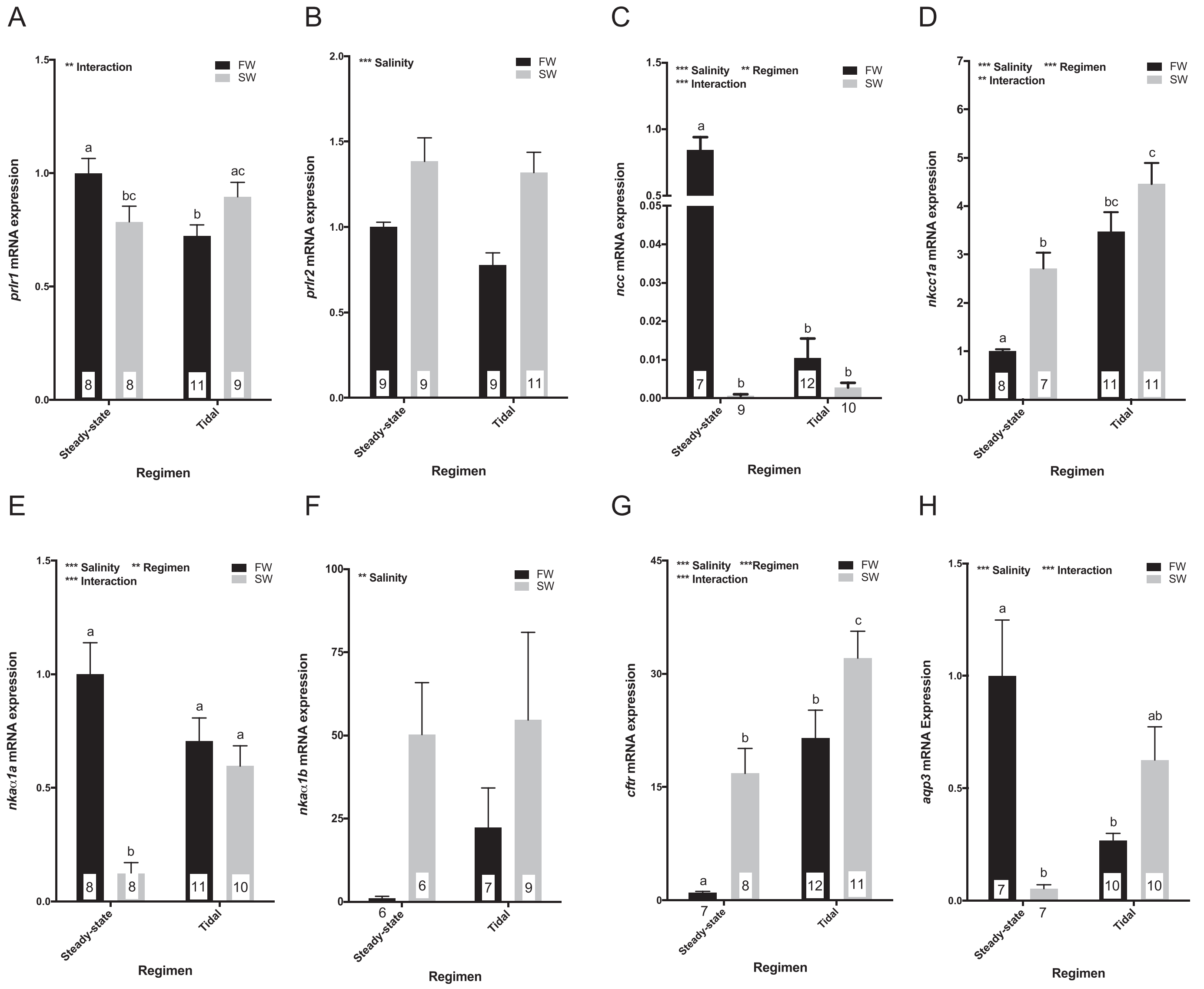


Fig. 3. Effects of rearing condition on branchial mRNA expression of *prlr1* (A), *prlr2* (B), *ncc* (C), *nkcc1a* (D), *nkaα1a* (E), *nkaα1b* (F), *cftr* (G) and *aqp3* (H) in fish sampled in steady-state FW and SW, and at the end of the FW and SW phase of the tidal cycle. Values are expressed as means ± S.E.M. (n = 6–12). Numbers within bars represent sample sizes for each group. Effects of salinity and regimen were analyzed by two-way ANOVA (*,**,***Significant at $P < 0.05$, 0.01 and 0.001, respectively). Interaction effects were followed up by Fisher's LSD test. Means not sharing the same letter are significantly different at $P < 0.05$.

(Fig. 5B). Moreover, by Day 7, expression of *prlr2* was elevated in fish in TS compared with those in TF following transfers from either FW or SW to tidal salinities (FW:TS vs. FW:TF and SW:TS vs. SW:TF, respectively).

Branchial *ncc* mRNA expression decreased following transfer from FW to SW, and increased following SW to FW transfer by Day 3, as compared with time-matched, steady-state controls (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5C). Also by Day 3, *ncc* expression in TF fish was elevated over TS fish transferred from SW, but in those transferred from FW, *ncc* expression in TS fish was elevated over TF fish, converging by Day 7 (SW:TS vs. SW:TF and FW:TS vs. FW:TF, respectively). Conversely, branchial *nkcc1a* mRNA expression increased after FW to SW transfer, and decreased after SW to FW transfer by Day 3, compared with steady-state controls (Fig. 5D). Following transfer from FW, *nkcc1a* mRNA expression in TS fish was nearly double that of TF fish on Day 3; this difference, however, was no longer observed by Day 7 (FW:TF vs. FW:TS). By contrast, following transfer from SW, there was no difference in expression on Day 3 between TF and TS fish; on Day 7, however, expression in TF fish exceeded that of TS fish (SW:TF vs. SW:TS).

Branchial mRNA expression of *nkaα1a* decreased and increased following transfer from FW to SW and SW to FW, respectively, compared with time-matched, steady-state controls (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5E). These differences were observed by Day 3 and were further enhanced by Day 7. Although there was a difference in *nkaα1a* mRNA expression between TF and TS in fish transferred from FW by Day 3, by Day 7 there was no difference in expression between TF and TS-sampled fish, regardless of transfer from FW or SW. There was no difference in branchial *nkaα1b* mRNA expression following transfer from FW to SW or vice versa, compared to time-matched, steady-state controls (Fig. 5F). The same pattern was observed for fish transferred from FW and sampled during TF and TS. In fish transferred from SW, expression in TF fish was higher than in TS fish by Day 3, but this pattern was not sustained through Day 7.

Branchial mRNA expression of *aqp3* was decreased in fish transferred from FW to SW, and increased in fish subject to the opposite transfer by Day 3, as compared with steady-state controls (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5G). In TF fish transferred from FW, *aqp3* mRNA expression remained unchanged,

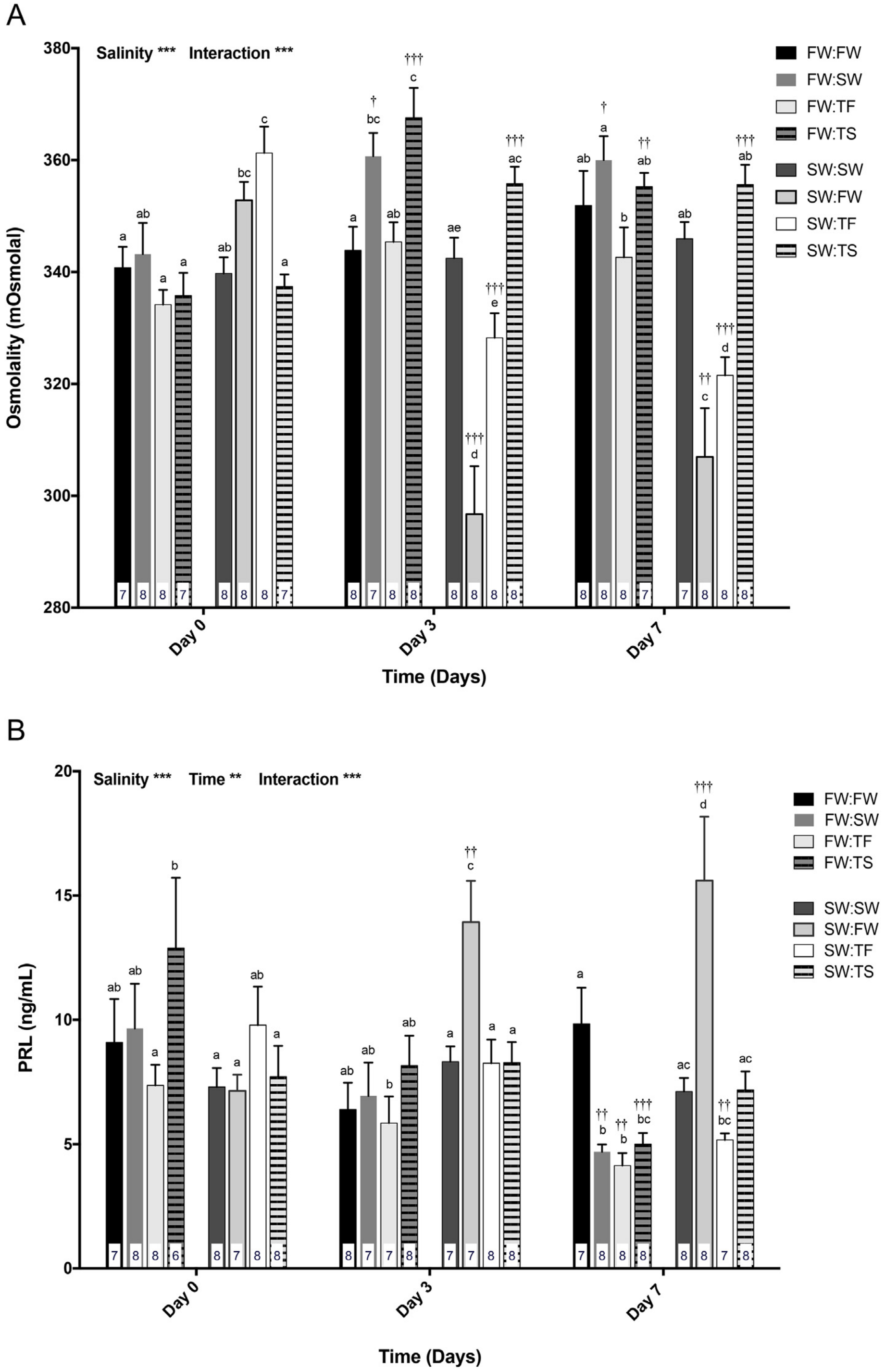


Fig. 4. Plasma osmolality (A), and plasma PRL (B) in fish sampled in FW, SW, and following transfer from FW or SW to FW, SW or to TR. TR fish were sampled at the end of the either FW or SW phases of the tidal cycle (TF or TS, respectively). Values are expressed as means ± S.E.M. (n = 6–8). Numbers within bars represent sample sizes for each group. Effects of salinity and time were analyzed by two-way ANOVA (*,**,***Significant at $P < 0.05$, 0.01, and 0.001, respectively). Within each time point, means not sharing the same letter are significantly different at $P < 0.05$. Daggers indicate difference from Day 0 within salinity treatments (†,††,†††Significant at $P < 0.05$, 0.01 and 0.001, respectively; Fisher's LSD test).

whereas in TS fish expression decreased by Day 3, with TF and TS expression at similar levels by Day 7 (FW:TF and FW:TS; Fig. 5G). Following transfer from SW, expression in TF and TS fish increased over the seven-day period, reaching mutually similar levels by Day 3, but with TF exceeding TS by Day 7 (SW:TF and SW:TS; Fig. 5G). Branchial *cftr* mRNA expression increased in fish transferred from FW to SW and decreased in those transferred from SW to FW by Day 3, compared to steady-state controls (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5H). Branchial *cftr* expression in TF fish was lower than in TS fish by Day 3 regardless of transfer from FW or SW; this difference was absent by Day 7 (FW:TF vs. FW:TS and SW:TF vs. SW:TS, respectively; Fig. 5H).

4. Discussion

The objective of this experiment was to determine the capacity of adult fish reared in steady-state FW or SW to acclimate to TR, by characterizing plasma osmolality, PRL, and branchial gene expression of PRLRs, ion and water transporters, and ion ATPases. This is the first study to both describe an osmoregulatory profile for adult Mozambique tilapia reared for two years under cyclically changing salinity, which is similar to the species' native habitat, and to investigate in adult fish the osmoregulatory effects of transfer from FW and SW steady-state rearing conditions to a tidal environment. In light of recent findings suggesting that tilapia exposed to changing salinities during early stages of larval development may better respond to subsequent salinity challenges

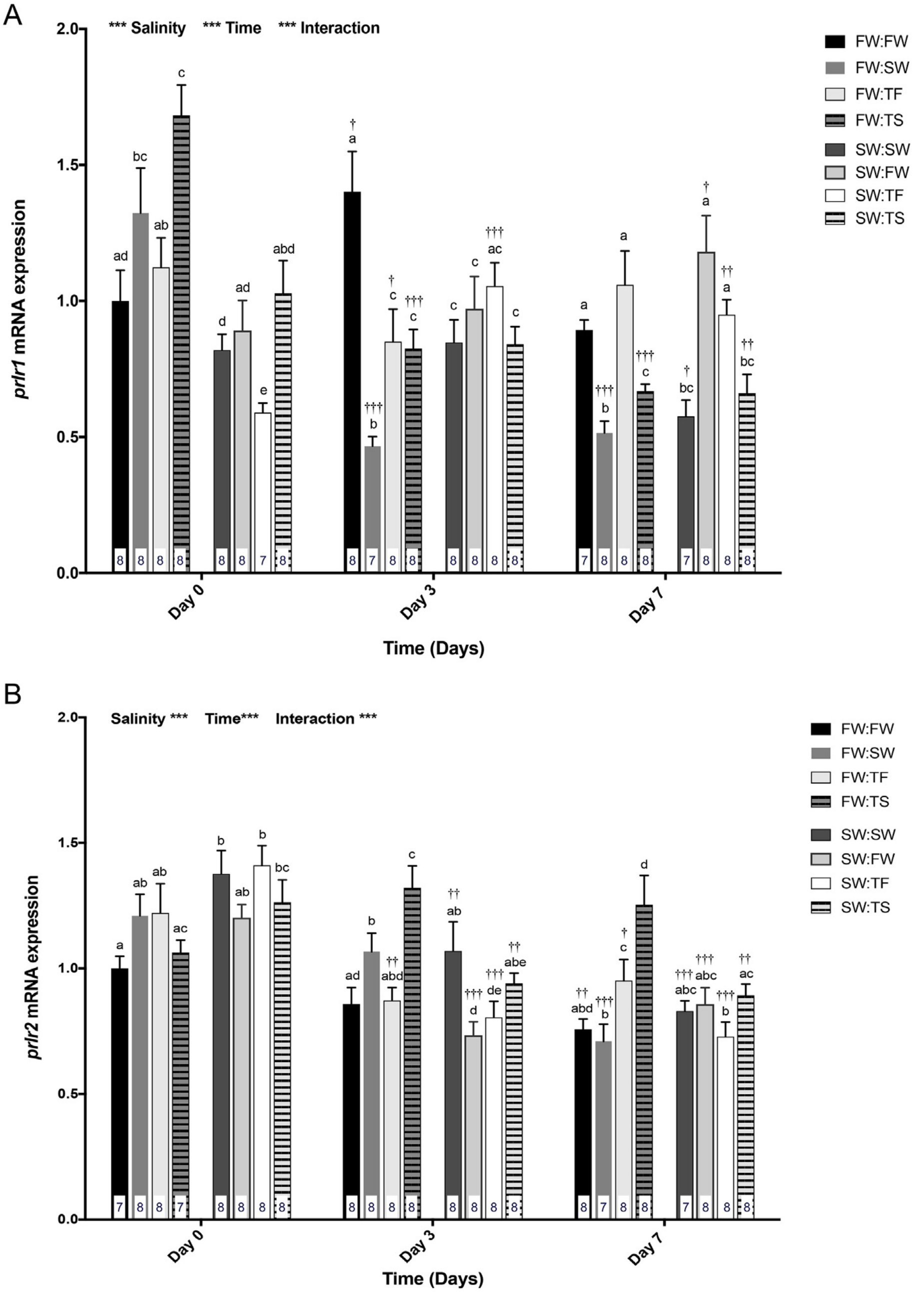
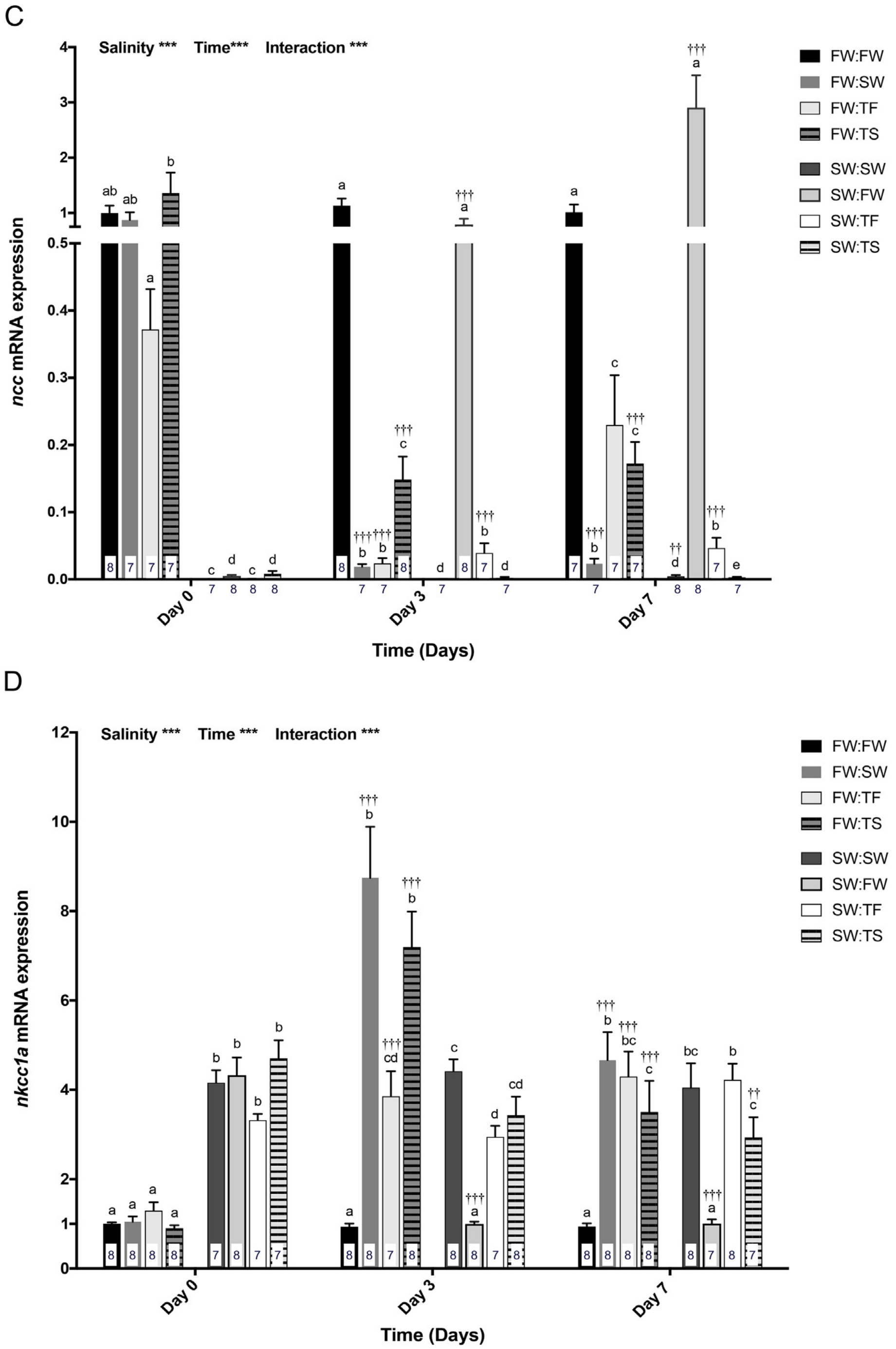


Fig. 5. Branchial mRNA expression of *prlr1* (A), *prlr2* (B), *ncc* (C), *nkcc1a* (D), *nkaa1a* (E), *nkaa1b* (F*)*, *cftr* (G) and *aqp3* (H) in fish FW, SW, and following transfer from FW or SW to FW, SW or to TR. TR fish were sampled at the end of the either FW or SW phases of the tidal cycle (TF or TS, respectively). Values are expressed as means ± S.E.M. ($n = 7$–8). Numbers within bars represent sample sizes for each group. Effects of salinity and time were analyzed by two-way ANOVA (*,**,***Significant at $P < 0.05$, 0.01 and 0.001, respectively). Within each time point, means not sharing the same letter are significantly different at $P < 0.05$. Daggers indicate difference from Day 0 within salinity treatments (†,††,†††Significant at $P < 0.05$, 0.01 and 0.001, respectively; Fisher's LSD test).

(Moorman et al., 2015), we tested the central concept of whether there is an adaptive advantage of rearing fish in changing salinities from the yolk-sac fry stage. By comparing two year old adult tilapia reared in steady-state FW and SW with fish reared under TR, our findings support the notion that the physiological experience of dynamically-changing salinities during early life history does not significantly improve survivability or osmoregulatory responses compared with fish that were exposed to TR for the first time as adults.

Specifically, the findings of this study were: (1) adult Mozambique tilapia acclimated to TR maintain a distinct osmoregulatory profile, which neither coincides fully with that of FW- nor SW-acclimated counterparts; (2) fish reared since yolk-sac fry for two years in steady-state salinities (either FW or SW) can rapidly acclimate to a tidal regimen, a finding that previously had only been observed in young fish (four months of age); (3) by seven days post-transfer, the osmoregulatory profile of fish reared in steady-state salinity and transferred to TR is similar to that of fish reared in TR since yolk-sac fry.

Upon conducting the salinity transfer experiments of this study, we found that adult tilapia reared in both steady-state FW and SW could withstand a direct transfer to TR, with 100% survival at seven days. Specifically, FW fish transferred to TR suffered no mortalities despite their initial exposure to full-strength SW within two hours of the first TS phase. This was suggestive of an ability of two year old adult fish to survive exposure to dynamic salinity changes, regardless of acclimation history. It is well established that FW-acclimated tilapia cannot survive direct transfer to SW, but are able to survive when first transferred to an



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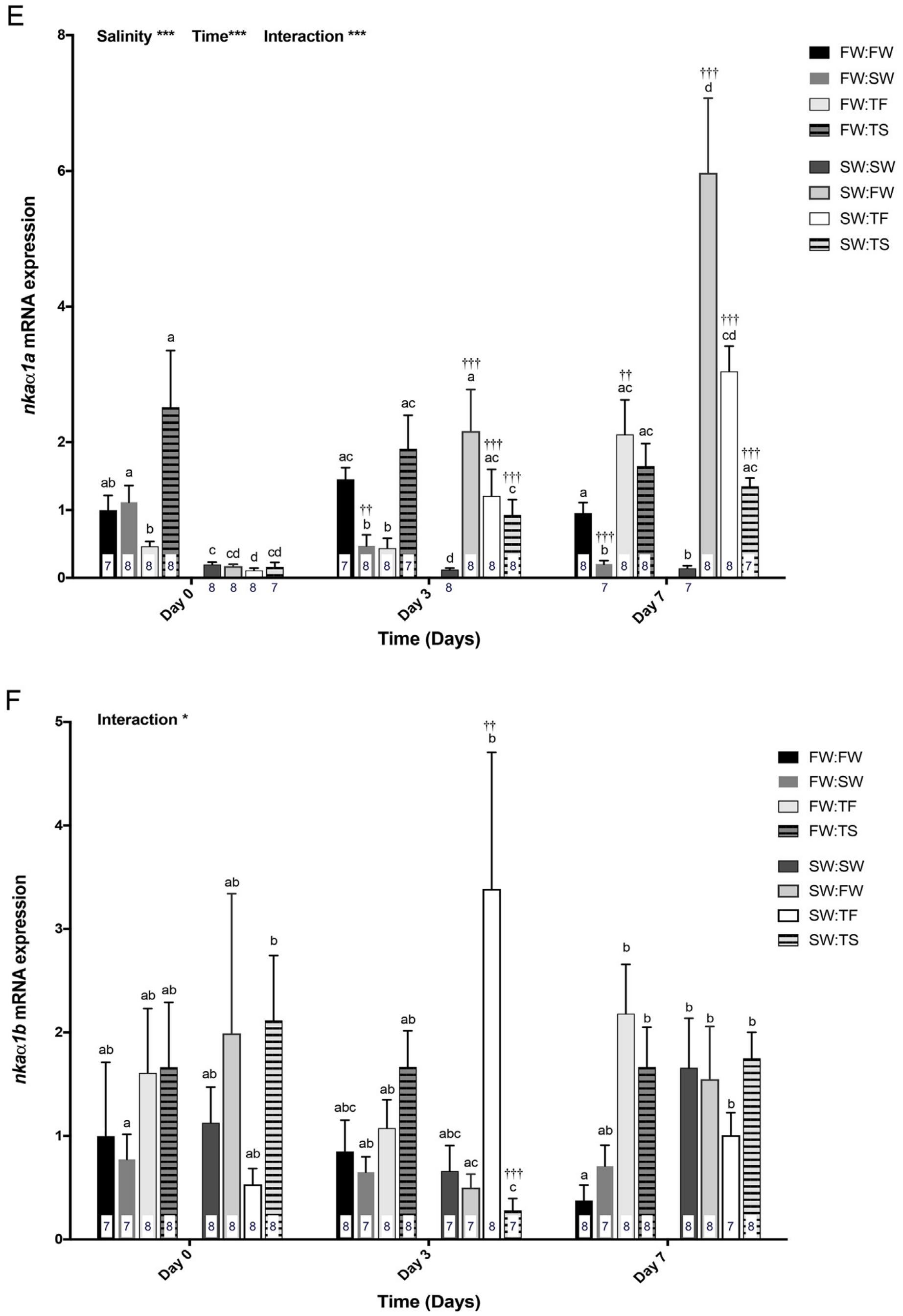
intermediate salinity (Breves et al., 2010a; Moorman et al., 2015; Seale et al., 2012; Seale et al., 2002; Yada et al., 1994). Consistent with these findings, the present protocol involved transfer to 80% SW for 48 hours, then to full strength SW. The current observations indicate that transition of FW fish to cyclically-changing salinity is less challenging than to steady-state SW.

In Mozambique tilapia, plasma osmolality is higher in fish acclimated to steady-state SW than in those acclimated to FW (Grau and Borski, 1994; Seale et al., 2002; Seale et al., 2006). Moreover, the inverse relation between plasma osmolality and PRL release has been well established (Grau et al., 1981; Nagahama et al., 1975; Seale et al., 2002; Seale et al., 2006; Seale et al., 2012). This relation is consistent with the potent hyperosmoregulatory action of PRL in gill and other osmoregulatory epithelia (Manzon, 2002). Consistent with previous reports, in Experiment 1 plasma osmolality was higher in fish in SW than those in FW, whether fish were kept in a steady-state or tidal regimen (Moorman et al., 2014; Moorman et al., 2015; Seale et al., 2006; Seale et al., 2002; Yada et al., 1994). In the same experiment, plasma PRL was higher in fish in FW compared with those in SW, regardless of rearing regimen, which is also consistent with the expected release of PRL in response to a reduction in plasma osmolality. The similarity in plasma PRL levels in TF and TS fish observed in Experiments 1 and 2 was consistent with previous reports (Moorman et al., 2014; Moorman et al., 2015), suggesting that the fish reared in or transferred to a tidal cycle are not as physiologically dependent on osmotically-driven variations in circulating PRL as fish that are acclimated to steady-state salinities.

Environmental salinity has been shown to modulate the actions of



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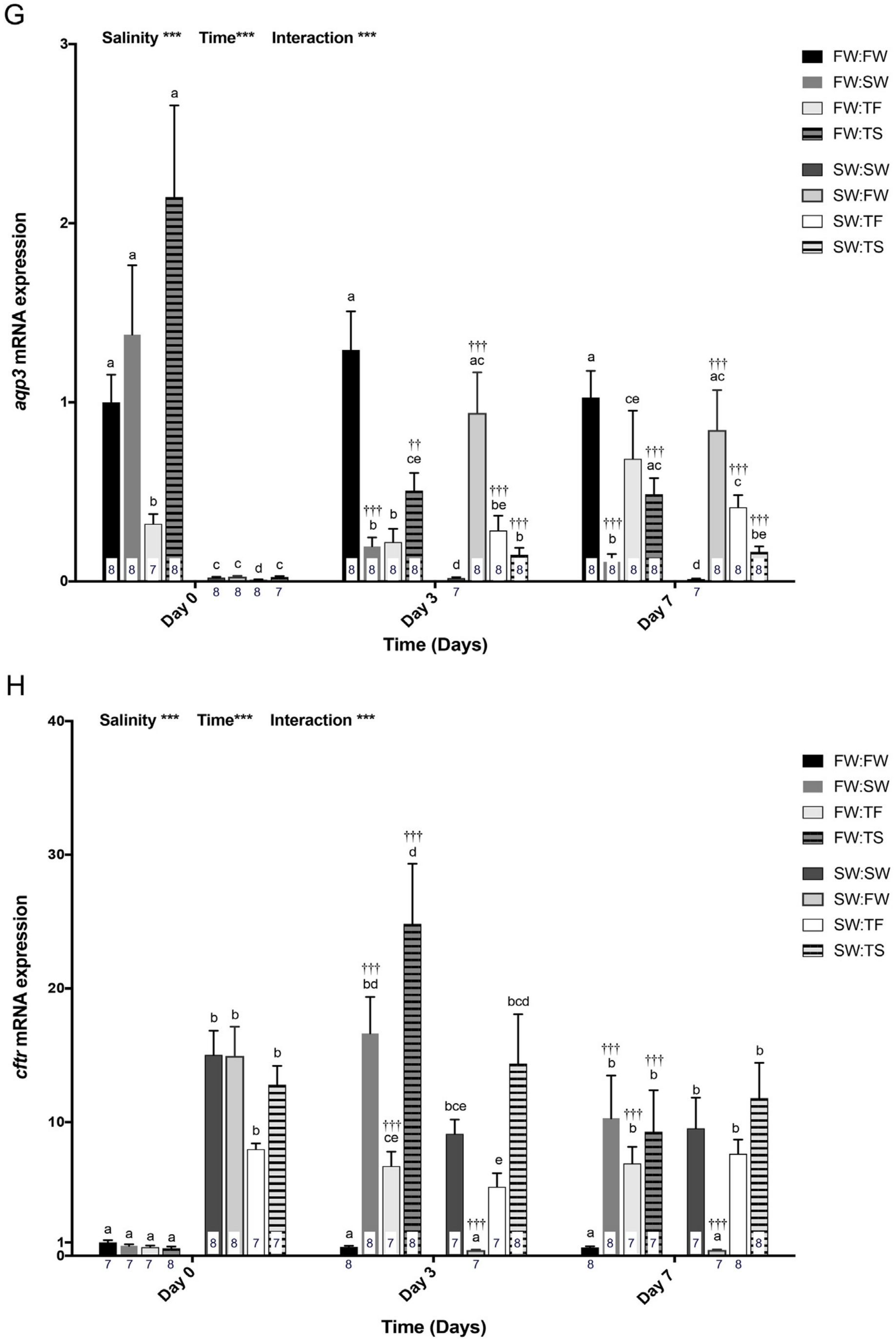
PRL not only by regulating its release from the pituitary, but also by directing the expression of its receptors in osmoregulatory epithelia (Breves et al., 2011; Inokuchi et al., 2015). Additionally, mRNA expression of *prlr1* in gill is stimulated in a dose-dependent manner by PRL (Inokuchi et al., 2015). In Experiment 1 and in three of the four comparisons in Experiment 2, *prlr1* expression in FW steady-state fish was elevated over that in SW fish. Elevated branchial expression of *prlr1* in FW relative to SW is consistent with previous reports where fish were sampled in either FW or SW steady-states, or in FW and SW phases of a tidal regimen, or following transfer from SW to FW (Breves et al., 2011; Fiol et al., 2009; Moorman et al., 2014; Moorman et al., 2015). Moreover, branchial *prlr1* expression in TR fish varied between fish in TF and TS. Moorman et al. (2014) suggested that the differential regulation of branchial *prlr1* expression between the two phases of TR may be attributable to direct regulation of transcription at the tissue level by environmental salinity. It is also possible that nuances in *prlr1* expression in TR-acclimated fish are associated with variables other than salinity and age, such as sex or size, which may be elucidated with additional studies using this tidal paradigm.

Branchial mRNA expression of *prlr2* has also been reported to vary with extracellular osmolality, in vivo and in vitro; unlike *prlr1*, however, its expression increases in hyperosmotic conditions (Fiol et al., 2009; Inokuchi et al., 2015; Seale et al., 2012). In the present study, branchial *prlr2* expression was higher in fish in SW than those in FW in both tidal and steady-state rearing regimens, whether they had been exposed to TR for two years or seven days. This finding is consistent with our recent results employing four month old tilapia (Moorman et al., 2014 and Moorman et al., 2015), suggesting that tight regulation of PRLR2 by salinity is independent of acclimation history. Moreover, binding of PRL to PRLR2 may not elicit the same hyperosmoregulatory



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response as binding to PRLR1 (Fiol et al., 2009). It has been postulated that increased *prlr2* expression in hyperosmotic conditions may facilitate acclimation of tilapia to SW (Seale et al., 2012; Moorman et al., 2014; Inokuchi et al., 2015; Yamaguchi et al., 2018). The molecular mechanism underlying this outcome may be associated with PRL binding either the regular length or short form of PRLR2. While the former has been hypothesized to activate a different pathway than PRLR1 upon binding PRL, the latter is thought to reduce the formation of functional receptors, thereby preventing PRL's actions (Fiol et al., 2009). In the present study, primers that detect regular length *prlr2* were employed. It is tenable, therefore, that salinity driven changes in *prlr2* in tidally-acclimated fish facilitate the attenuation of PRL's effects by diverting downstream signaling from hyperosmoregulatory outcomes. Moreover, the observed dynamic changes in *prlr2* transcription with environmental salinity, regardless of rearing regimen, strongly suggest that this isoform is highly osmosensitive.

In previous studies of salinity acclimation in euryhaline teleosts, including the Mozambique tilapia, it has been shown that NCC and NKAα1a are involved in ion uptake in gill and highly expressed in FW, whereas NKCC1a, CFTR and NKAα1b are involved in ion extrusion and predominantly expressed in SW (Hiroi et al., 2005; Hiroi et al., 2008; Kaneko et al., 2008; Tipsmark et al., 2011). In tilapia, AQP3 has been implicated in FW-acclimation as it is highly expressed in response to both hyposmotic stimuli and PRL (Breves et al., 2016). The overall similarity in branchial *ncc*, *nkcc1a*, and *cftr* mRNA expression between SW and TR fish in both Experiments 1 and 2 was consistent with a previous report on TR-acclimated, four month old fish (Moorman et al., 2014), as was the finding that branchial *aqp3* expression in TR fish was intermediate to levels in FW and SW controls. The intermediate expression of *aqp3* in TR is likely a reflection of the shifting need for water transport in a dynamically-changing environment. By contrast, the mRNA expression of ion transporters, *ncc*, *nkcc1a* and *cftr* in dynamically-changing environments were either strongly suppressed (*ncc*) or elevated (*nkcc1a and cftr*). The expression patterns of these three ion transporters in TR follows those observed in SW-type ionocytes (Breves et al., 2010b; Inokuchi et al., 2015). Moreover, the strong suppression of *ncc* in fish reared in TR or transferred to TR, is consistent with the previously reported immunohistochemistry results indicating suppression of NCC protein in the apical region of branchial ionocytes of tilapia reared in TR (Moorman et al., 2014). Conversely, in the same study, signal intensities for NKCC and CFTR were consistently high in TR fish. Together, the mRNA results of this and other studies suggest that transcriptional regulation of these three key ion transporters in steady-state and dynamically-changing conditions are paralleled by changes in protein abundance. In both experiments, branchial mRNA expression of *nkaα1a* was higher in FW fish than in SW fish. This pattern is consistent with that previously reported in fish acclimated to steady-state salinities (Tipsmark et al., 2011; Moorman et al., 2014), although in the current study, the *nkaα1a* expression pattern in TR was variabl—in some instances similar to that of FW fish, and in others, intermediate to that of FW and SW controls. Likewise, branchial expression of *nkaα1b* observed in TR-acclimated fish (Experiment 1) was consistent with previous studies reporting up-regulation in response to increased extracellular osmolality (Inokuchi et al., 2015; Tipsmark et al., 2011). Overall, these findings suggest that the osmoregulatory profile of adult fish reared in TR for two years is more similar to that of SW fish than that of FW fish.

By Day 7 of transfer from steady-state salinities to TR, patterns in plasma variables and branchial gene expression of PRLRs, ion transporters, and AQP3 were largely similar across both phases of the tidal cycle, regardless of whether fish were initially reared in FW or SW. Moreover, by Day 7, these osmoregulatory parameters were largely similar to those in the TR-acclimated fish sampled in Experiment 1. Together, these observations indicate that tilapia retain, even after being reared in a steady-state salinity for two years, the remarkable osmoregulatory capacity to overcome fluctuations in environmental salinity regardless of the salinity in which they were reared.

In the current study, we have provided novel insights into osmoregulation of tilapia under TR rearing conditions at a life stage not previously examined under this paradigm. It is worth mentioning that in our previous study, fish reared in TR for four months grew faster than those reared in steady-state FW or SW (Moorman et al., 2016). Such findings may lead to applications in aquaculture production, and bear particular importance to tilapia in general, which rank second among the most widely aquacultured fishes in the world (FAO, 2015). The use of the TR rearing paradigm can foster the elucidation of novel and comprehensive physiological insights, including providing a potential means to develop optimal rearing conditions for Mozambique tilapia and other euryhaline fish.

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