EFFECTS OF CYCLICAL CHANGES IN ENVIRONMENTAL SALINITY ON OSMOREGULATORY PARAMETERS IN THE MOZAMBIQUE TILAPIA

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ABSTRACT

Many euryhaline teleost fish, including the Mozambique tilapia, *Oreochromis mossambicus*, are native to waters in which salinity varies tidally between that of freshwater (FW) and seawater (SW). Acclimation to changes in environmental salinity is regulated largely by the neuroendocrine system, which directs ion extrusion or ion uptake response via the gill and other osmoregulatory tissues. In teleosts, prolactin (PRL) is critical to FW acclimation, stimulating ion uptake mechanisms. Osmoregulation in euryhaline teleosts has been studied extensively in steady state FW and SW, and after one-way transfers between FW and SW. It is unclear, however, how euryhaline fish respond to cyclical salinity changes. Mozambique tilapia were reared in FW, SW, and under a tidal regimen (TR), characterized by salinity changes between FW (TF) and SW (TS) every 6 hours (h), and transferred from FW or SW to TR to investigate adaptive ability to TR. TR fish were also sampled every 3 h in a 24-hour period to observe osmoregulatory parameters throughout the TR cycle. Regardless of the rearing regimen, plasma osmolality changed in direct relation to salinity, rising in SW and falling in FW, while plasma PRL was inversely related to salinity. In fish reared in TR, branchial gene expression of effectors of ion transport and PRL receptors was more similar to those of fish reared in SW than to those in FW. When fish were transferred from either FW or SW to TR, all measured osmoregulatory parameters were identical to those of fish reared in TR by 7 days. In TR fish sampled multiple times over a 24-hour period the greater resolution in sampling revealed several nuances within the overall patterns of salinity and regimen-dependent changes in osmoregulatory parameters. These findings indicate that life-long acclimation to SW and FW does not preclude adaptation to TR at the adult stage. The results also show that throughout a 24-hour period, TR fish are able to compensate for broad and frequent changes in external salinity while maintaining osmoregulatory parameters within a narrow range. These are the first known studies to investigate environmental adaptation of adult Mozambique tilapia to TR, and to characterize osmoregulatory parameters in TR fish over multiple iterations of TF & TS during a full diurnal cycle. As such, these studies have further characterized the effects of TR-acclimation at a more advanced life stage than addressed by previous reports, and

have yielded greater insights to rearing in an environment that approximates the native habitat of the Mozambique tilapia.

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SYMBOLS AND ABBREVIATIONS

AQP3 = Aquaporin 3

CFTR = Cystic fibrosis transmembrane conductance regulator

EF1a = Elongation factor 1a

FW = Fresh water

NCC = Na^+/Cl^- cotransporter

NHE3 = Na^+/H^+ exchanger 3

NKA = Na^+/K^+ ATPase

NKCC = $Na^+/K^+/2Cl^-$ cotransporter

PRL = Prolactin

PRLR = Prolactin receptor

qRT-PCR or qPCR = quantitative real-time PCR

RIA = Radioimmunoassay

RPD =Rostral pars distalis

SW = Seawater

TR = Tidal regimen

TF = Fish sampled at that end of the FW phase of the tidal cycle

TS = Fish sampled at the end of the SW phase of the tidal cycle

CHAPTER I
INTRODUCTION

Maintaining osmotic homeostasis is critical to life. The function of macromolecules that enable necessary biological processes is dependent on their structure, which in turn is maintained by weak intramolecular forces, such as hydrogen bonds and hydrophobic interactions. These weak bonds are sensitive to osmotic changes, which affect ion concentrations, in the surrounding environment. As a result, organisms osmoregulate to preserve macromolecular structure and function. Where osmoregulation is vital to survival, the metabolic cost of this process underscores its necessity. For example, in marine and estuarine fish the demands of osmoregulation can range from 25% to over 50% of metabolic output (Bushnell & Brill, 1992; see review by Bœuf & Payan, 2001).

In spite of the importance of osmoregulation in many organisms, the extent and interaction of the complex mechanisms underlying this process are not fully understood. Disturbances in osmotic homeostasis are associated with many disease and injury pathologies, and maintaining optimal blood ion concentration in critically ill patients remains a prevalent issue for physicians currently. Though it is known that the central nervous and endocrine systems interact with bodily tissues to maintain osmotic homeostasis, physical manipulation of plasma osmolality by fluid withholding, or by fluid administration intravenously—a technique first described over 80 years ago (Hirshfeld, et al., 1931)—continues to serve as a key management strategy (Braun, et al., 2015; Gonzalez & Vincent, 2011). As the clinical needs requiring additional scientific study persist, furthering our understanding of osmoregulation remains reliant upon identification of suitable model organisms (see Woodhead, 1989).

Teleost fish, with gills as a primary site of water and ion exchange with the environment, have long served as model organisms for studying osmoregulation (see Woodhead, 1989). The Mozambique tilapia (*Oreochromis mossambicus*) is a euryhaline, teleost fish native to the Lower Zambezi River and estuarine waters along the southeastern coast of Africa (Trewavas, 1983). These and other euryhaline teleosts are able to survive in a range of environmental salinities by modifying the morphology and function of cells and tissues active in osmoregulation, including gill, kidney and gut. The

functional and morphological plasticity of these osmoregulatory cells and tissues facilitates the ability of Mozambique tilapia to survive when faced with major departures from the physiological osmotic range. By contrast, in humans and other mammalian species, plasma osmolality is tightly maintained within a narrow range of 285 - 295 mOsm/kg (Bradshaw & Smith, 2008). Uncorrected excursions above or below this range may yield symptoms as severe as coma or death (Bradshaw & Smith, 2008). In Mozambique tilapia, baseline plasma osmolality has been observed to remain between 305 - 360 mOsm/Kg, in the upper half of this range for SW acclimated fish, and in the lower half for FW acclimated fish (Breves, et al. 2010a, 2011; Fiess et al., 2007; Moorman, et al., 2015; Seale et al., 2002, 2006a; Yada, et al., 1994). However, these fish are capable of withstanding fluctuations in plasma osmolality below 300 mOsm/Kg, to well over 400 mOsm/Kg when transferred from SW to FW and FW to SW, respectively (Breves et al., 2010a; Moorman et al., 2015; Seale et al., 2002, 2006a; Yada et al., 1994). The ability to tolerate and recover from osmotic challenge is an important characteristic that makes the Mozambique tilapia an ideal model organism for studying vertebrate osmoregulation.

Teleost Osmoregulation

In tilapia and other teleosts, it is widely accepted that to maintain optimal ionic and osmotic conditions internally, fish in freshwater (FW) retain Na^+ and Cl^- ions and generate dilute urine (see Evans, 2008). By contrast, fish in seawater (SW) generate concentrated urine, ingest large volumes of water, and extrude ions via branchial, renal and intestinal tissues (see Evans, 2008). The neuroendocrine system plays a key role in regulating osmoregulatory processes in vertebrates (see McCormick & Bradshaw, 2006); in teleosts, the pituitary hormone prolactin (PRL) is necessary to survival in FW (Pickford & Phillips, 1959; see review by Manzon, 2002). PRL acts on gill, kidney and gut epithelia to stimulate both ion uptake and water extrusion processes in FW (see Manzon, 2002; see McCormick, 2001; Seale, et al., 2006a). Consistent with this role, circulating PRL levels in the Mozambique tilapia increase when the fish are exposed to hypoosmotic conditions (see Manzon, 2002). Two isoforms of PRL, PRL_{177} and PRL_{188},

are produced in the anterior pituitary (*rostral pars distalis*, or RPD) of the tilapia (Specker, et al., 1985; Yamaguchi, et al., 1988). PRL_{177} and PRL_{188} release from cultured RPDs and dispersed PRL cells have shown similar trends in response to changes in osmolality, but the PRL_{188} response is more robust (Seale, et al., 2012a). PRL signaling occurs via prolactin receptors 1 and 2 (PRLRs 1 and 2), which have been found in the main osmoregulatory tissues in Mozambique tilapia, including gill, kidney and gut (Fiol et al., 2009). In the gill and pituitary *in vitro*, these receptors appear to be differentially responsive to PRL and environmental osmolality, with increases in PRLR1 expression primarily stimulated by PRL (Inokuchi, et al., 2015). On the other hand, PRLR2 expression increases in response to a rise in extracellular osmolality (Seale et al. 2012a; Inokuchi, et al., 2015).

Specialized epithelial cells in the gill called mitochondria-rich cells (MRCs), or ionocytes play a central role in osmoregulation. Ionocytes facilitate uptake and extrusion of monovalent ions, including Na^+ and Cl^-, across the gill and maintain characteristic morphologies and functions to support ion uptake and extrusion in fish adapted to FW and SW, respectively. Four types of ionocytes are known (Hiroi et al., 2005; Kaneko et al., 2008) and can be generally categorized as FW-type, specialized for ion uptake, or SW-type, specialized for ion extrusion. Both FW and SW type ionocytes possess basolateral Na^+/K^+ ATPase (NKA) (Hiroi, et al., 2008). However, FW-type ionocytes express Na^+/Cl^- cotransporter (NCC) apically, in contrast with SW-type ionocytes which express $Na^+/K^+/2Cl^-$ cotransporter (NKCC) basolaterally, and cystic fibrosis transmembrane conductance regulator (CFTR; a Cl^- channel) apically (Hiroi et al., 2008). Both FW and SW ionocytes express basolateral membrane proteins Na^+/H^+ exchanger-3 (NHE3) (Watanabe, et al., 2008) and aquaporin-3 (AQP3) (Watanabe et al., 2005), which respectively couple Na^+ uptake with acid extrusion (as reviewed in Choe, K. P., et al. 2005) and transport water (see Connolly, et al., 1998).

Prior reports characterizing osmoregulatory parameters in both FW and SW, including plasma osmolality, plasma PRL, and branchial gene expression of PRLRs and the effectors of ion transport described above, have provided the groundwork for further investigation into how Mozambique tilapia acclimates to dynamically changing salinities.

The Importance of Tilapia in Aquaculture

Beyond their value as a model organism for osmoregulation, tilapia are the most widely farmed fish in aquaculture globally according to the United Nations Food and Agriculture Organization (UNFAO; FAO, 2012). Tilapiine fishes' ability to thrive and reproduce in captivity and to survive under a variety of environmental conditions, including over broad ranges of temperature and salinity, makes them ideal species for aquaculture (Trewavas, 1983). The UNFAO's Global Aquaculture Production database indicates that tilapia accounted for just under 5.3 million tons of aquaculture products in 2014, valued at an estimated USD 8.8 billion (FAO, 2016). By 2010, aquaculture yielded 47% of food fish for human consumption globally, and has continued to rise (FAO, 2012). As the importance of aquaculture and of tilapiine species as nutritional resources continues to grow, understanding ways in which various rearing conditions affects endocrine parameters associated with osmoregulation and other biological processes which are relevant to maintaining fish stocks, such as growth and reproduction, may stand to add value for fish farmers by informing aquaculture best practices.

Goals and Objectives

Mozambique tilapia have been studied extensively in freshwater (FW) and seawater (SW) steady state conditions, as well as when subjected to one-way transfers from FW to SW and vice versa. However, little is known about how Mozambique tilapia and other euryhaline teleosts respond to dynamic salinity changes that mimic the tidal environment. Moorman and colleagues (Moorman, et al., 2014; Moorman, et al., 2015) describe the results of the first known studies observing the osmoregulatory effects of acclimation to a tidal-simulating rearing regime (TR), in which fish are exposed to FW for six hours and then SW for six hours on a continuous cycle. These studies have provided insights to osmoregulation in four month-old fish reared under conditions characterized by dynamic changes in environmental salinity, and when transferred from TR to steady-state FW or SW. The proposed study seeks to provide new insights to the osmoregulatory effects of tidal acclimation by examining the ability of adult fish at a

more advanced life stage to adapt to TR, and by measuring endocrine and physiological parameters associated with osmoregulation in TR fish over a 24-hour period.

Aside from the differences in FW and SW branchial ionocytes described above, Moorman and colleagues observed key differences in osmoregulatory endpoints in Mozambique tilapia reared under the tidal paradigm (TR), versus those reared in FW or SW only. In TR fish, plasma osmolality fluctuated between values measured in FW and SW fish, while circulating PRL levels remained stable over FW and SW phases of the tidal cycle. Additionally, TR rearing appeared to confer an advantage to SW acclimation when compared with FW fish. This advantage was corroborated by increased expression in TR of genes that are upregulated in SW, such as CFTR. Such findings regarding osmoregulation of Mozambique tilapia in conditions that more closely resemble that of their native environment have not previously been published. Thus, this area of research is novel in relation to *O. mossambicus* and euryhaline teleosts generally. My thesis builds on this body of knowledge by further examining osmoregulation in TR fish.

Specifically, my first objective (Chapter II) was to characterize plasma osmolality, PRL and branchial expression of ion transporters, pumps, and channels in adult fish reared under TR, and to determine the capacity of fish, reared to adulthood in steady-state FW or SW, to acclimate to TR. In the second objective (Chapter III), I characterized the time course of changes in osmoregulatory parameters during a 24-hour period in fish acclimated to TR. Moorman and colleagues examined these parameters at the end of a single FW and SW phase of the TR cycle (TF and TS, respectively). Contrastingly, my study expanded the examination of these parameters through a full diurnal period, including a contiguous second set of TF and TS phases, and intermediate time points during the salinity transition. This work has yielded key insight to temporal osmoregulatory hormone profiles and changes in gene expression in relation to the TR salinity changes and associated fluctuations in plasma osmolality.

CHAPTER II

The effects of transfer from steady-state to tidally-changing salinities on plasma and gill osmoregulatory parameters in Mozambique tilapia

Abstract

The Mozambique tilapia, *Oreochromis mossambicus*, is a teleost fish native to estuarine waters that vary in salinity between freshwater (FW) and seawater (SW). The neuroendocrine system directs ion uptake and extrusion processes that are critical to environmental salinity acclimation, in the gill and other osmoregulatory tissues. Most studies with *O. mossambicus* have focused on fish in steady-state FW and SW, and after one-way transfers between the two; little is known about their response to a cyclically changing salinity that simulates their native habitat. Plasma osmolality, PRL, and branchial expression of PRL receptors (PRLR1 and PRLR2), Na^+/Cl^- and $Na^+/K^+/2Cl^-$ co-transporters (NCC and NKCC), Na^+/K^+ ATPase (NKAa1a and NKA a1b), and Cl^- and water channels (CFTR and AQP3) were measured in fish reared in steady-state salinities, FW and SW, and a tidal regimen (TR)—where salinities changed between FW and SW every 6 h—and in fish transferred from FW or SW to TR. Regardless of rearing regimen, plasma osmolality was higher in fish in SW than those in FW; by contrast, plasma PRL was lower in fish in SW. Furthermore, branchial gene expression of effectors of ion transport in TR fish was more similar to those in steady-state SW fish than in FW fish. By 7 days of transfer from steady state FW or SW to TR, plasma osmolality, PRL and branchial gene 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, suggesting that the capacity of tilapia to acclimate to distinct salinity regimens is not restricted by developmental stage.

1. Introduction

Maintaining internal osmotic homeostasis is critical to life in many organisms, including vertebrates. Osmoregulation occurs through exchange of ions and water between cells and the extracellular environment, and is reliant upon the function of membrane transport proteins, pumps and channels to facilitate the exchange. Most vertebrates tightly control osmoregulatory mechanisms to maintain plasma osmolality within a narrow physiological range. In the Mozambique tilapia, as in other teleost fishes, plasma osmolality is maintained near one-third the osmolality of SW (see McCormick, 2001). As a euryhaline species native to estuarine waters off the Southeast coast of Africa (Trewavas, 1983), Mozambique tilapia are capable of recovering from major departures above or below their physiological range of plasma osmolality (between 305 and 360 mOsm/ Kg; see Seale et al., 2013). This allows these fish to survive in external salinities equivalent to FW through double-strength SW (Fiess et al., 2007; Stickney, 1986). These and other teleosts in FW hyperosmoregulate to counteract a tendency to lose solutes to the environment and to become over-hydrated (see McCormick, 2001). On the other hand, in SW they hypoosmoregulate to counteract a tendency to lose water to the environment and gain solutes (see McCormick, 2001). Osmoregulation is conducted predominantly via gill, kidney and intestine, with gill as the site of direct contact with the external environment (Evans, 2008). In FW fish, active uptake of ions occurs across the gill and gut, and dilute urine is excreted via the kidney (Evans, et al., 2005). In SW, ions are extruded at the gill, and water is reabsorbed in the intestine (Marshall & Grosell, 2005).

The pituitary hormone prolactin (PRL) is essential for hyperosmoregulation in FW in euryhaline teleost species (Dharmamba, et al., 1967; see Manzon, 2002; Pickford, & Phillips, 1959). Consistent with this action, plasma PRL in the Mozambique tilapia is inversely related to external osmolality, and PRL has been shown in FW fish to increase ion uptake and decrease water permeability at the gill (see Manzon, 2002). Two isoforms of PRL, PRL_{177} and PRL_{188}, are produced in the anterior pituitary (*rostral pars distalis*, or RPD) of the tilapia. PRL_{177} and PRL_{188} release from cultured RPDs and dispersed PRL cells have shown similar trends in response to changes in osmolality, however, the

PRL_{188} response is more robust (Seale, et al., 2012a). For this reason, only PRL_{188} was measured, and is referred to as PRL in this study. The effects of PRL are mediated by downstream signaling subsequent to binding of receptors 1 and 2 (PRLRs 1 and 2), which are present in the gill, kidney and gut in Mozambique tilapia (Fiol et al., 2009). In the gill and pituitary *in vitro*, these receptors have been shown to be 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 (Seale et al. 2012a; Inokuchi, et al., 2015).

Specialized cells called ionocytes facilitate osmoregulation processes at the gill, and can be 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; Hiroi, et al., 2008), an ion pump critical to establishing electrochemical gradients across the cell membrane, which in turn drives ion secretion and absorption (McCormick, 1995). Two isoforms of NKA, a1a and a1b, have been described in tilapia gill (Tipsmark et al., 2011). Branchial expression of NKAα1a is upregulated in response to a drop 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 expression of the NKAα1b isoform has been reported to increase when fish are transferred from FW to SW (Tipsmark et al., 2011), however, more recent results were unable to corroborate this relationship (Inokuchi, et al., 2015). The presence of Na^+/Cl^- cotransporter (NCC) in the apical membrane is specific to FW ionocytes (Choe, 2005; Hiroi et al., 2005, 2008; Watanabe, et al., 2008). Transcription of NCC is directly regulated by PRL (Breves, et al., 2010b; Inokuchi, et al., 2015). SW ionocytes, on the other hand, are characterized by expression of basolateral Na^+/K^+/2Cl^- cotransporter (NKCC1a) and apical cystic fibrosis transmembrane conductance regulator (CFTR; a Cl^- channel) (Hiroi et al., 2005). Expression of NKCC1a has been shown to be directly osmosensitive, increasing with external osmolality (Inokuchi, et al., 2015). In euryhaline teleost species, expression of CFTR is elevated in SW-acclimated fish compared with FW-acclimated fish (Moorman et al., 2015; Moorman et al., 2014; 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 & Schulte, 2005; Singer, et al., 1998; Tse et al., 2006). In both FW and SW ionocytes, the basolaterally-located water channel AQP3 facilitates fluid exchange with the extracellular milieu (Connolly, et al., 1998; Watanabe et al., 2005). In Mozambique tilapia and other teleost species, AQP3 expression in gill is elevated in FW-acclimated over SW-acclimated animals (Cutler & Cramb, 2002; Jung, et al., 2012; Lignot, et al., 2002; Madsen, et al., 2014; Moorman et al., 2015; Tipsmark, et al., 2010; Tse, et al., 2006), and has recently been shown to increase in direct response to PRL (Breves, et al., 2016).

Much of our current understanding of osmoregulation in Mozambique tilapia, including ionocyte morphology and function, is based on prior study that is largely focused on fish reared entirely in steady-state FW or SW, or after one-way transfers between the two. Little is known about how they respond when faced with a cyclically changing salinity, which more closely resembles their native habitat. Recently, Moorman and colleagues (Moorman et al., 2015; Moorman et al., 2014) have described a tidal rearing (TR) paradigm in which fish are exposed to alternating six-hour phases of FW and SW, simulating the salinity fluctuations of the tidal cycle. Those studies characterized an osmoregulatory profile for four-month old fish reared in TR, and investigated the ability of TR-acclimated fish to adapt to FW and SW steady-states upon acute salinity transfer. By contrast, in the current study, I sought to characterize baseline osmoregulatory parameters in adult fish reared in steady-state FW and SW, and TR, for at least two years, and tested their capacity to acclimate from steady-state FW or SW salinities to TR.

I hypothesized that fish reared in FW and SW would be able to adapt to TR within seven days of transfer, and would exhibit osmoregulatory parameters similar to those of fish that had been reared from fry in TR. To investigate this hypothesis, I measured the following endpoints in fish reared in FW, SW and TR, and in fish transferred from FW or SW: (1) plasma osmolality and circulating PRL levels; and (2) branchial mRNA expression of PRL receptors and effectors of ion transport shown previously to be responsive to changes in extracellular osmolality and /or PRL, including PRLR1, PRLR2, NCC, NKCC1a, NKAα1a, NKAα1b, CFTR and AQP3.

2. Materials and Methods

2.1 Fish Rearing and Treatments

Experiment 1 - Baseline

Male and female Mozambique tilapia, *O. mossambicus*, were reared from yolk-sac fry for at least two years under natural photoperiod at the Hawaii Institute of Marine Biology (Kaneohe, HI, USA) in outdoor, 700L tanks supplied with FW (0.1±0.1‰) or SW (34±1 ‰; Kaneohe Bay, Kaneohe, HI, USA) or alternating FW and SW in 6-hour phases simulating a tidally-changing salinity (TR) as previously described (Moorman et al., 2014). Water temperature was kept at 25±2°C. Fish were fed trout chow pellets (Skretting, Tooele, UT) to satiation once daily. At the time of sampling, fish weighed 191.6g - 1.1kg. Animals were maintained in steady-state FW, SW or a tidally-changing salinity until sampling, at which time nine fish were sampled from each rearing salinity. Fish reared in a tidally-changing salinity were collected at the end of the FW and SW phases of the cycle.

Experiment 2 – Salinity Transfers

Adult male and female Mozambique tilapia, *O. mossambicus*, were collected from broodstocks maintained at the Hawaii Institute of Marine Biology, and held under natural photoperiod in outdoor, 700L tanks supplied with FW or SW, as above. FW fish were allocated randomly across four replicate FW tanks, and SW fish across four replicate SW tanks. 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, Tooele, UT) once daily to satiation. On Day 0 of the experiment, 8 fish from each of the four FW and four SW tanks were sampled. Then, water sources to three of the FW and three of the SW tanks were adjusted to facilitate the following salinity transfers: FW to SW (one tank), FW to TR (two tanks), SW to FW (one tank), and SW to TR (two tanks). One FW and one SW control tank were retained. As fish previously transferred from FW to full-strength SW died within 12 hours (Moorman, 2015), fish transferred from FW to SW in the current study were first acclimated to 82-85% SW (29 - 30‰)

over 48 hours (h), at which point the water supply was adjusted to full strength SW. This yielded a full SW conversion prior to sampling on Day 3, which was maintained through Day 7. Eight fish from each of the eight tanks 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 87-570g at the time of sampling.

All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

2.2 Sampling

At the time of sampling, fish were netted and anesthetized with 2-phenoxyethanol (0.3mL/L; Sigma-Aldrich, St. Louis, MO). After fish were weighed, blood was drawn with a needle and syringe coated with sodium heparin (200 U/ml, Sigma-Aldrich, St. Louis, MO), and fish were euthanized by rapid decapitation. Plasma was separated by centrifugation and stored at -20°C for further analysis. Gill filaments from the second gill arch on the left side of the fish were collected. Gill samples were frozen in liquid nitrogen and stored at -80°C, pending further analysis.

2.3 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), 400 ng of total RNA 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). The mRNA levels of reference and target genes were determined by an absolute quantification standard curve. Elongation factor 1a (EF1a) was used as a reference gene to normalize the mRNA levels of target genes. The

PCR mixture (15 uL) contained Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA), 200 nM of each primer, and 2 µl of standard cDNAs or cDNAs prepared from experimental samples. PCR cycling parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For Experiment 1 – Baseline, R^2 values and amplification efficiencies for standard curves were as follows, respectively: 0.998 and 83.0% (EF1a), 0.995 and 83.1% (PRLR1), 0.989 and 78.4% (PRLR2), 0.997 and 85.5% (NCC), 0.999 and 89.0% (NKCC1a), 0.998 and 94.6% (NKA a1a), 0.997 and 94.8% (NKA a1b), 0.999 and 88.3% (CFTR), and 0.999 and 89.8% (AQP3). For Experiment 2 – Salinity Transfers, R^2 values and amplification efficiencies for standard curves were as follows, respectively: 0.996-0.997 and 91.0-91.2% (EF1a), 0.993-0.999 and 88.6-88.9% (PRLR1), 0.989-0.993 and 67.8-70.1% (PRLR2), 0.998-0.999 and 94.2-94.7% (NCC), 0.999-1 and 82.7-83.6% (NKCC1a), 0.999-1 and 67.5-96.8% (NKA a1a), 1 and 85.2-86.3% (NKA a1b), 0.998-1 and 87.7-88.2% (CFTR), and 1 and 87.0-87.2% (AQP3). All primer pairs have been previously described: NCC (Inokuchi, et al., 2008), NKCC1a (Inokuchi et al., 2008), NKA a1a (Tipsmark et al., 2011), NKA a1b (Tipsmark et al., 2011), EF1a (Breves et al., 2010a), PRLR1 (Pierce et al., 2007), PRLR2 (Breves, et al., 2010c), AQP3 (Watanabe et al., 2005), and CFTR (Moorman et al., 2014).

2.4 Plasma Parameters

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 secreted by the pituitary of tilapia, PRL_{188} was measured in this study, based on its robust responses to changes in salinity, and referred as PRL throughout the text. Plasma PRL was measured via homologous radioimmunoassay (RIA) as described by Ayson and colleagues (Ayson et al., 1993).

2.5 Statistical Analysis

Statistical analyses were conducted using a two-way analysis of variance (ANOVA). In Experiment 1, salinity (FW or SW) and regimen (tidal or non-tidal) were the independent variables. Significant main effects are indicated in the figures. Significant interaction effects of salinity and regimen ($P<0.05$) were followed up by post-hoc analysis employing the Fisher's Least Significant Difference (LSD) test. Data are expressed as means ± S.E.M. In Experiment 2, salinity (FW:FW; FW:SW; FW:TR, sampled at TF or TS; SW:SW; SW:FW; and SW:TR, sampled at TF or TS) and time (Day 0, Day 3, Day 7) were the independent variables. Significant interaction effects of salinity and time ($P<0.05$) were followed up by one-way ANOVA of single main effects. Post-hoc Fisher's LSD comparisons within each time point and salinity treatment were used to (1) compare salinity means within each day; and (2) compare Day 3 and 7 means to the Day 0 mean within each salinity. Where applicable, individual values were log-transformed 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 Plasma parameters

Experiment 1

Plasma osmolality was significantly elevated in SW fish compared with those in FW; there was no significant effect of regimen (Fig. 1A). Plasma prolactin levels were higher in FW fish than in SW fish, and in tidal fish compared to steady-state fish; there was no difference between FW and SW PRL levels among non-tidal fish, nor among tidal fish (Fig. 1B).

Experiment 2

Prior to transfers on Day 0, initial plasma osmolality levels were similar across FW and SW steady-state fish (Fig. 3A). However, plasma osmolality in FW control fish

(FW:FW) remained steady over 7 days, while levels in fish transferred from FW to SW (FW:SW) were elevated over initial levels by Day 3 (Fig. 3A). FW fish transferred to tidally-changing salinities and sampled at the end of the TF phase (FW:TF) also maintained stable plasma osmolality similar to Day 0 values and to FW controls at each time point. By contrast, in FW fish transferred to tidally-changing salinities and sampled at the end of the TS phase (FW:TS), plasma osmolality increased over initial values by Day 3. Plasma osmolality measured in SW controls (SW:SW) remained unchanged by Day 7, where as in SW fish transferred to FW (SW:FW), levels had significantly decreased by Day 3. SW fish transferred to a tidally changing salinity and sampled at the end of the TF phase (SW:TF) had plasma osmolality lower than initial levels by Day 3. On the other hand, fish transferred from SW and sampled at the end of the TS phase (SW:TS) increased plasma osmolality over initial values by Day 3 (Fig. 3A).

Plasma PRL levels on Day 0 were similar across FW and SW steady-state fish prior to transfers (Fig. 3B). In FW controls (FW:FW) and SW controls (SW:SW), levels remained steady and similar throughout the experiment. By contrast, FW:SW levels decreased from initial levels within seven days, while SW:FW levels were significantly elevated by Day 3. By Day 7, PRL levels were still highest in SW:FW fish, whereas plasma PRL in FW:SW and FW:TS fish had decreased from Day 0 and were similar to levels in FW:TF fish; collectively, these levels were lower than those in FW controls. Additionally, by Day 7, FW:TF and FW:TS PRL levels did not differ from FW:SW, SW:TF, and SW:TS levels. Throughout the experiment, plasma PRL levels in SW:TF and SW:TS fish remained similar to those in SW controls (SW:SW) throughout the experiment (Fig. 3B).

3.2 Branchial gene expression of PRL receptors; ion transporters and pumps; and ion and water channels

Experiment 1

Branchial gene expression of PRLR1 was elevated in FW versus SW steady-state fish, and higher in TF than in TS. In tidal fish, PRLR1 was expressed at levels more similar to SW steady-state fish than FW fish (Fig. 2A). PRLR2 expression was elevated

in SW fish sampled in both steady-state and tidal rearing environments (Fig. 2B). 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. 2C). Branchial NKCC1a expression was significantly higher in steady-state SW fish than FW fish. However, expression in steady-state was lower than in tidal fish, with no difference in expression between TF and TS fish (Fig. 2D). Branchial expression of NKAα1a was nearly 10-fold higher in steady-state FW fish compared with SW fish, with levels in TF and TS fish equivalent to those in FW fish (Fig. 2E). NKAα1b expression was elevated in SW fish over FW fish in both rearing regimens (Fig. 2F). Gene expression of CFTR in both steady-state and tidal SW fish exceeded those of FW fish reared under the same regimen; expression in tidal fish, however, was higher than in steady-state fish, with levels in TF fish no different than those in steady-state SW fish (Fig. 2G). AQP3 expression was higher in FW steady-state fish relative to SW fish; expression in TF and TS fish was mutually similar and intermediate to that of FW and SW fish (Fig. 2H).

Experiment 2

Branchial PRLR1 gene expression in FW control fish remained at or above that of Day 0 over the course of sampling, while expression in fish transferred from FW:SW was similar to SW controls by Day 7 (Fig. 4A). In FW:TF fish, expression was reduced by Day 3, but returned to initial levels, coinciding with FW controls, by Day 7. However, in FW:TS fish, branchial PRLR1 expression dropped to levels measured in SW controls by Day 3, which were maintained through Day 7. Branchial PRLR1 expression in SW controls remained at or below Day 0 levels through Day 7 (Fig. 4A). By contrast, PRLR1 expression in SW:FW fish was comparable to that of SW controls; by Day 7, expression exceeded that of FW controls. In SW:TF fish, PRLR1 expression increased by Day 3 and remained comparable to that of FW controls through Day 7. Expression in SW:TS fish remained similar to that of SW controls throughout the experiment (Fig. 4A).

Branchial PRLR2 gene expression on Day 0 was higher in SW than in FW controls (Fig. 4B). In FW:SW fish, PRLR2 expression was elevated over FW control fish and SW control fish by Day 3, but returned to levels similar to those in both controls by Day 7. By Days 3 and 7, PRLR2 expression in FW:TF fish was similar to that of FW and

SW controls, but lower than that of FW:TS fish. PRLR2 expression in both SW:TF and SW:TS fish decreased by Day 3 relative to Day 0, and remained low throughout the experiment (Fig. 4B).

Branchial NCC gene expression on Day 0 was higher in FW than in SW fish; this pattern was maintained over the course of the experiment (Fig. 4C). By Day 3 there was a steep drop in NCC expression in FW:SW fish that was maintained through Day 7. Expression in FW:TF and FW:TS fish also dropped to well below that of FW controls by Day 3, with TS higher than TF. By Day 7, however, NCC expression was similar between TF and TS, and intermediate between FW and SW. By contrast, NCC expression in SW:FW fish increased significantly by Day 3 and remained elevated through Day 7 (Fig. 4C). Expression of NCC in SW:TF fish was higher than in SW:TS fish by Day 3 and Day 7; in both groups, however, NCC expression was closer to that of SW than FW controls (Fig. 4C).

Day 0 branchial NKCC1a gene expression was elevated in SW fish compared with FW fish; expression in FW and SW control groups remained similar to Day 0 levels over the course of the experiment (Fig. 4D). In FW:SW fish, expression increased to double that of SW controls by Day 3; by Day 7, expression was similar to that of SW controls. NKCC1a expression in FW:TF fish was similar to that of SW controls by Day 3. Expression in FW:TS fish spiked by Day 3, coinciding with that of FW:SW fish. By Day 7, however, NKCC1a expression in FW:SW fish and FW:TS fish was similar to that of both SW controls and FW:TF fish. On the other hand, in SW:FW fish, branchial NKCC1a expression dropped four-fold relative to Day 0 control and was similar to that of FW control fish by Day 3. In SW fish transferred to TF and TS, expression remained similar to SW controls at all time points, with TS significantly lower than TF by Day 7 (Fig. 4D).

Branchial gene expression of NKAα1a was elevated in FW fish over SW fish on Day 0 (Fig. 4E). Expression in FW:SW fish was reduced by Day 3, and further lowered to that of SW controls by Day 7. In FW fish transferred to TR, NKAα1a expression in TF fish was elevated by Day 7, relative to Day 0, whereas expression in TS fish did not change, remaining similar to that of FW controls throughout the experiment. By Day 3, NKAα1a expression in SW:FW fish was similar to FW controls and significantly

elevated over both Day 0 and SW controls. By Day 7, expression in SW:FW was six-fold higher than FW controls. In SW fish transferred to TR, NKAα1a expression in TF and TS fish was elevated over both Day 0 and SW controls by Day 3, remaining elevated through Day 7 (Fig. 4E).

Branchial NKAα1b gene expression was similar between FW and SW fish on Day 0 (Fig. 4F). By Day 3, FW transfers to TF and TS were no different than FW and SW controls. By contrast, NKAα1b expression in SW:TF fish was higher than that of Day 0 controls and three-fold higher than FW and SW controls by Day 3. Expression in SW:TS fish was similar to FW and SW controls by Day 3, but significantly lower compared with Day 0. NKAα1b expression in FW:TR (TF and TS) fish was similar to SW:TR (TF and TS) transferred fish by Day 7; both had higher expression relative to FW controls (Fig. 4F).

Day 0 branchial CFTR gene expression was elevated in SW fish compared with FW fish (Fig. 4G). In FW:SW fish, expression increased to nearly 20-fold higher than that of FW controls and was similar to that of SW controls by Day 7. Branchial CFTR expression in FW:TF fish was similar to that of SW controls by Day 3 and 7. Expression of CFTR in FW:TS fish was nearly 3-fold higher than in SW controls and FW:TF fish by Day 3; expression was similar to that of FW:SW fish. By Day 7, however, branchial CFTR expression in FW:TS fish was similar to that of SW controls and FW:TF fish. On the other hand, CFTR expression in SW:FW fish decreased to that of FW controls by days 3 and 7. In SW:TF and SW:TS fish, expression remained mostly similar to SW controls at all time points, with no difference between TF and TS expression by Day 7. Also by Day 7, CFTR expression in FW:TF was similar to that of FW:TS fish and no different than expression in SW:TF and SW:TS fish (Fig. 4G).

Branchial gene expression of AQP3 was elevated in FW fish over SW fish on Day 0 (Fig. 4H). Expression in FW:SW fish was significantly reduced compared with FW controls by Day 3, and remained low through Day 7. In FW:TF fish, AQP3 expression was lower than in FW controls by Day 3, and remained lower through Day 7. Expression in FW:TF fish was also lower than in FW:TS fish by Day 3, but was similar by Day 7. By Day 3 and continuing through Day 7, expression of AQP3 in SW:FW fish was elevated relative to SW controls, and similar to that of FW controls. In SW:TF fish and SW:TS

fish, AQP3 expression was elevated relative to SW controls by Day 3 and through Day 7. By Day 3, AQP3 expression in FW:TF and FW:TS fish was similar to that in SW:TF and SW:TS fish, remaining so through Day 7 (Fig. 4H).

Fig. 1. Effects of rearing condition on plasma osmolality (A) and plasma prolactin (B) in Mozambique tilapia (*Oreochromis mossambicus*) sampled in fresh water (FW), seawater (SW), at the end of the FW phase of the tidal cycle (TF), and at the end of the SW phase of the tidal cycle (TS). Values are expressed as means ± S.E.M. (n = 7-11). Means not sharing the same letter are significantly different (two-way ANOVA, followed by Fisher's Least Significant Difference test when an interaction effect was observed, **P*<0.05, ***P*<0.01, ****P*<0.001).

Figure 1

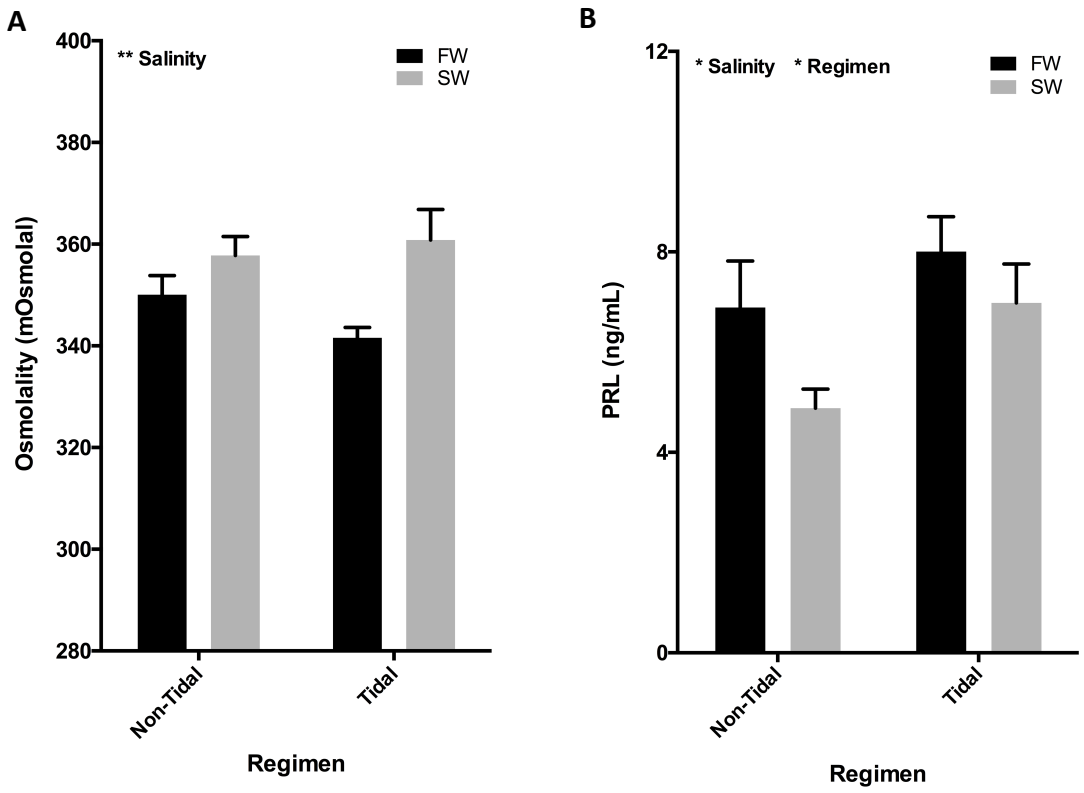

A
** Salinity
FW
SW
Osmolality (mOsmolal)
400
380
360
340
320
300
280
Non-Tidal
Tidal
Regimen
B
* Salinity * Regimen
FW
SW
PRL (ng/mL)
12
8
4
0
Non-Tidal
Tidal
Regimen

Fig. 2. Effects of rearing condition on branchial gene expression of PRLR1 (A), PRLR2 (B), NCC (C), NKCC1a (D), NKAα1a (E), NKAα1b (F), CFTR (G) and AQP3 (J) in Mozambique tilapia (*Oreochromis mossambicus*) sampled in fresh water (FW), seawater (SW), at the end of the FW phase of the tidal cycle (TF), and at the end of the SW phase of the tidal cycle (TS). Values are expressed as means ± S.E.M. (n = 7-12). Means not sharing the same letter are significantly different (two-way ANOVA, followed by Fisher's Least Significant Difference test when an interaction effect was observed, **P*<0.05, ***P*<0.01, ****P*<0.001).

Figure 2

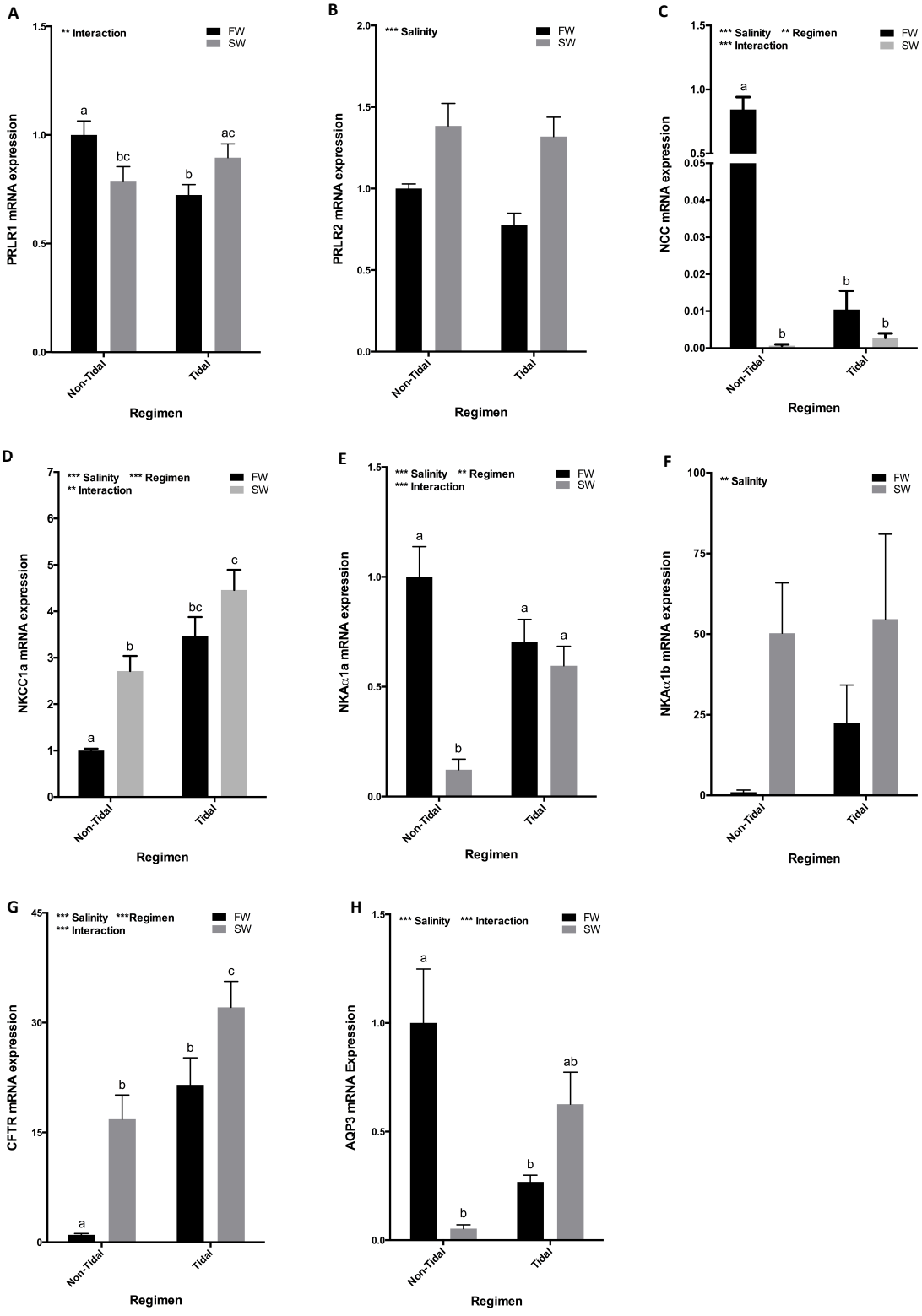

A
** Interaction
FW
SW
PRLR1 mRNA expression
a
bc
b
ac
Non-Tidal
Tidal
Regimen
B
*** Salinity
FW
SW
PRLR2 mRNA expression
Non-Tidal
Tidal
Regimen
C
*** Salinity ** Regimen
*** Interaction
FW
SW
NCC mRNA expression
a
b
b
b
Non-Tidal
Tidal
Regimen
D
*** Salinity *** Regimen
** Interaction
FW
SW
NKCC1a mRNA expression
a
b
bc
c
Non-Tidal
Tidal
Regimen
E
*** Salinity ** Regimen
*** Interaction
FW
SW
NKAα1a mRNA expression
a
b
a
a
Non-Tidal
Tidal
Regimen
F
** Salinity
FW
SW
NKAα1b mRNA expression
Non-Tidal
Tidal
Regimen
G
*** Salinity ***Regimen
*** Interaction
FW
SW
CFTR mRNA expression
a
b
b
c
Non-Tidal
Tidal
Regimen
H
*** Salinity *** Interaction
FW
SW
AQP3 mRNA Expression
a
b
b
ab
Non-Tidal
Tidal
Regimen

Fig. 3. Plasma osmolality (A), and plasma PRL (B) in Mozambique tilapia (*Oreochromis Mossambicus*) sampled in fresh water (FW), seawater (SW), after transfer from FW to SW, SW to FW, and after FW or SW transfers to a tidally-changing salinity (TR), sampled at the end of the FW phase of the tidal cycle (TF), and at the end of the SW phase of the tidal cycle (TS). Bars represent means ± SEM (n= 6-8). Data were analyzed by two-way ANOVA. Where an interaction effect was observed (**P*<0.05, ***P*<0.01, ****P*<0.001), post-hoc comparisons were made between salinity treatments within each time point, and over time within each salinity treatment. Means not sharing the same letter are different ($p < 0.05$; one-way ANOVA with salinity as independent variable, followed by Fisher's LSD test). Daggers (†) indicate difference from Day 0 mean within salinity treatments (**P*<0.05, ***P*<0.01, ****P*<0.001; one-way ANOVA with time as the independent variable, followed by Fisher's LSD test).

Figure 3

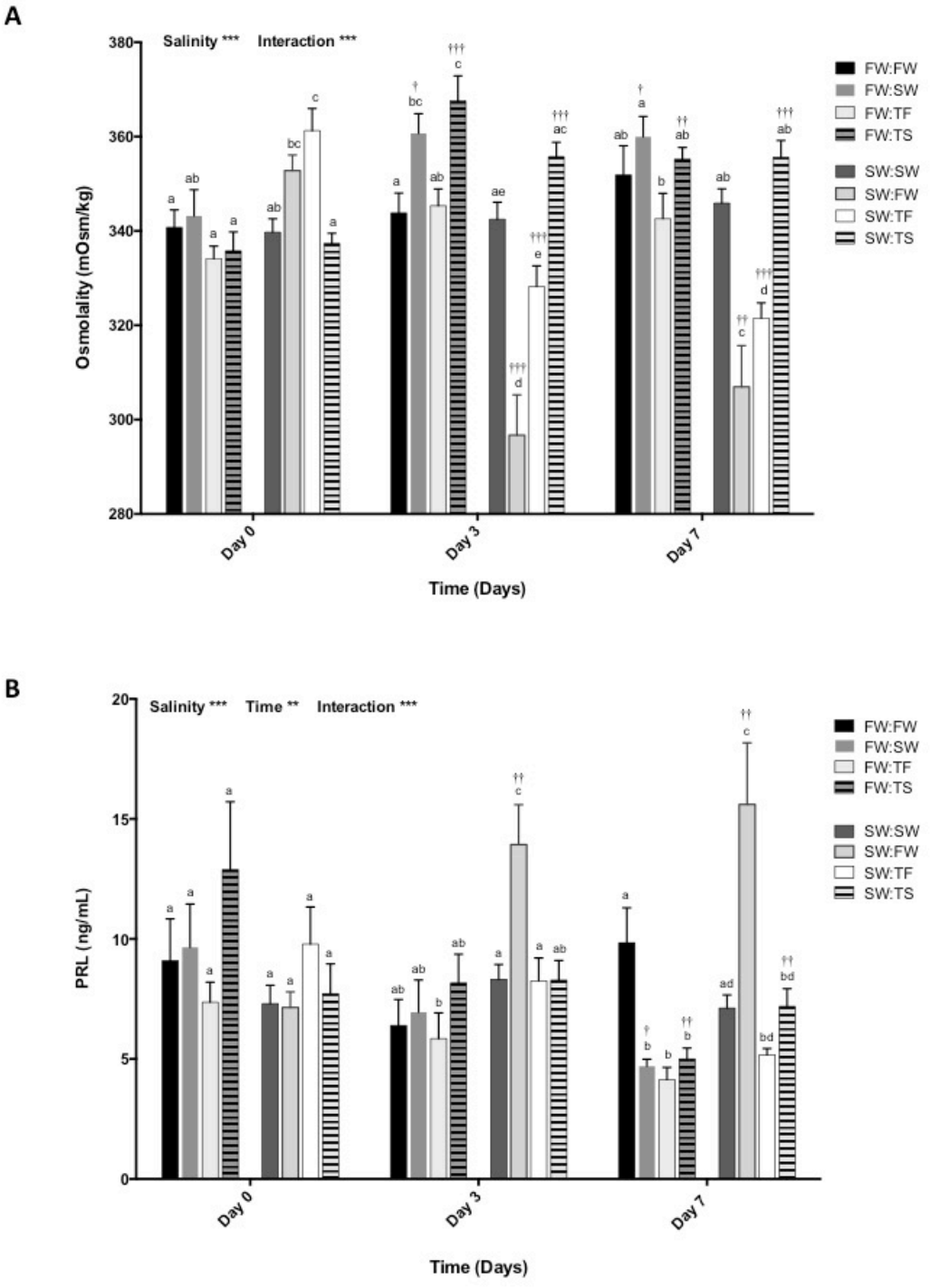

A
Salinity *** Interaction ***
Osmolality (mOsm/kg)
380
360
340
320
300
280
Day 0
Day 3
Day 7
Time (Days)
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS
B
Salinity *** Time ** Interaction ***
PRL (ng/mL)
20
15
10
5
0
Day 0
Day 3
Day 7
Time (Days)
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS

Fig. 4. Branchial gene expression of PRLR1 (A), PRLR2 (B), NCC (C), NKCC1a (D), NKAα1a (E), NKAα1b (F), CFTR (G) and AQP3 (H) in Mozambique tilapia (*Oreochromis mossambicus*) sampled in fresh water (FW), seawater (SW), after transfer from FW to SW and vice versa, and after FW or SW transfers to a tidally-changing salinity (TR), sampled at the end of the FW phase of the tidal cycle (TF), and at the end of the SW phase of the tidal cycle (TS). Bars represent means ± SEM (n= 7-8). Data were analyzed by two-way ANOVA. Where an interaction effect was observed (**P*<0.05, ***P*<0.01, ****P*<0.001), post-hoc comparisons were made between salinity treatments within each time point, and over time within each salinity treatment. Means not sharing the same letter are different ($p < 0.05$; one-way ANOVA with salinity as independent variable, followed by Fisher's LSD test). Daggers (†) indicate difference from Day 0 mean within salinity treatments (**P*<0.05, ***P*<0.01, ****P*<0.001; one-way ANOVA with time as the independent variable, followed by Fisher's LSD test).

Figure 4

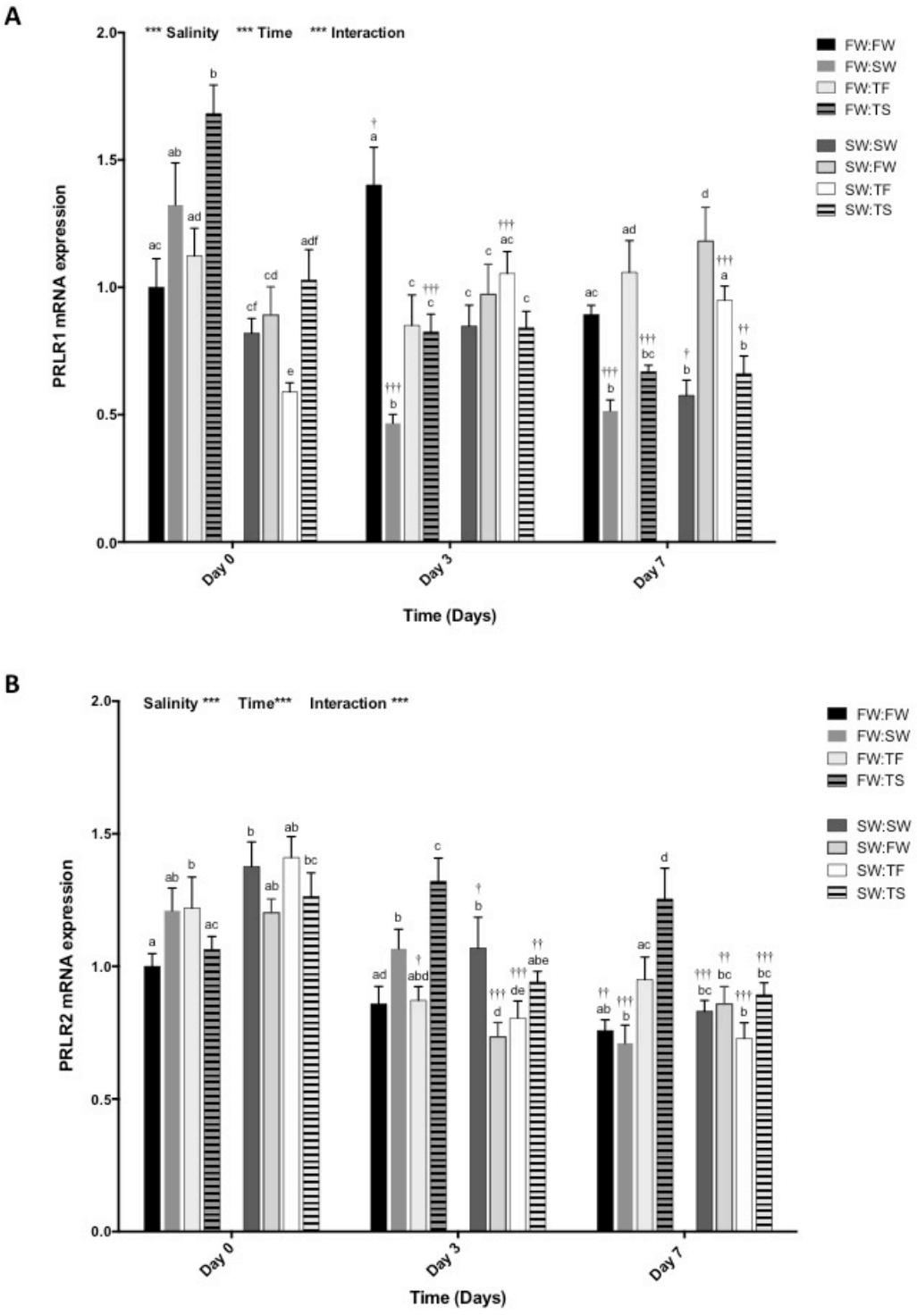

A
*** Salinity *** Time *** Interaction
PRLR1 mRNA expression
Time (Days)
Day 0
Day 3
Day 7
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS
B
Salinity *** Time*** Interaction ***
PRLR2 mRNA expression
Time (Days)
Day 0
Day 3
Day 7
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS

Figure 4

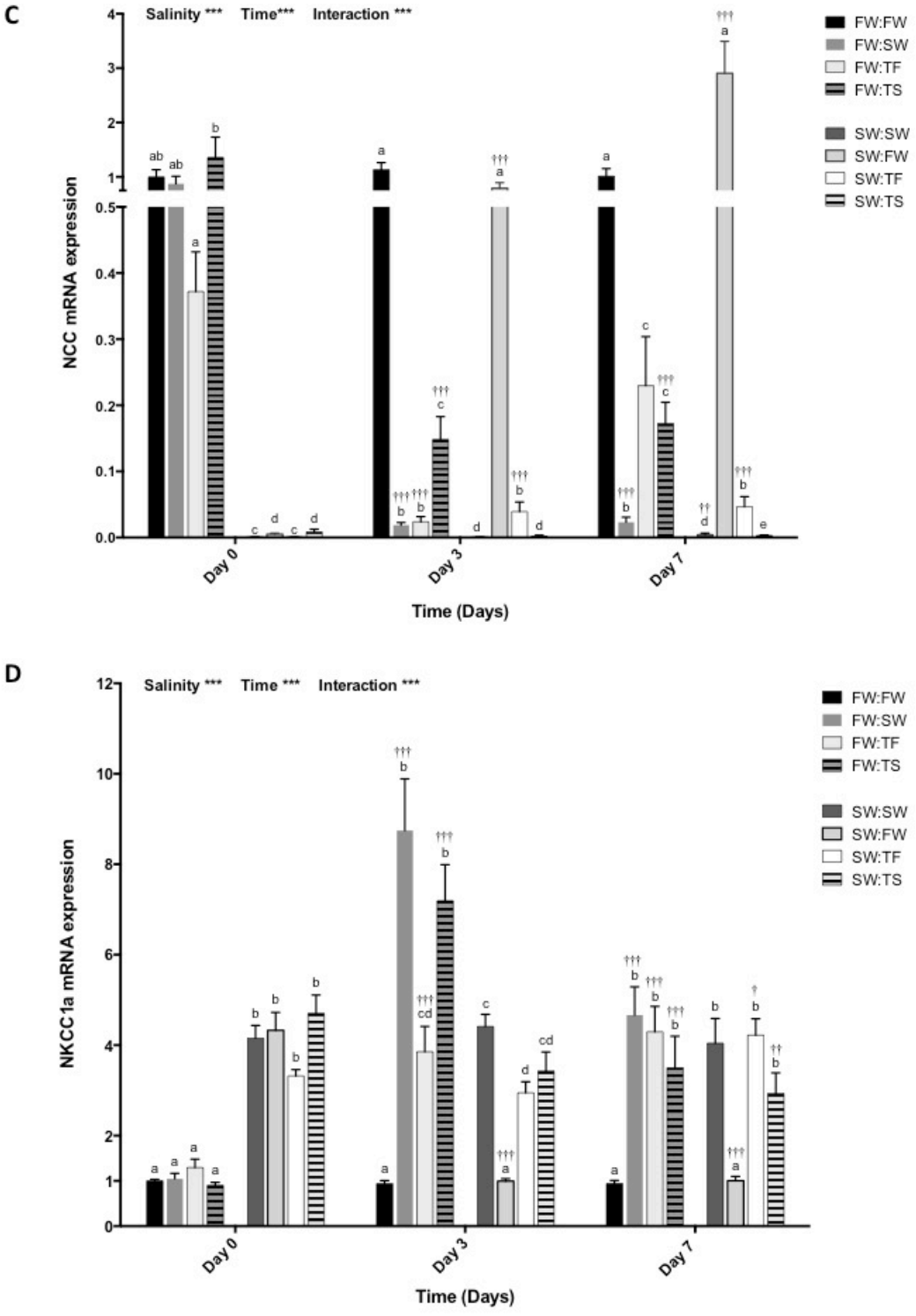

C
Salinity *** Time*** Interaction ***
NCC mRNA expression
Time (Days)
Day 0
Day 3
Day 7
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS
D
Salinity *** Time *** Interaction ***
NKCC1a mRNA expression
Time (Days)
Day 0
Day 3
Day 7
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS

Figure 4

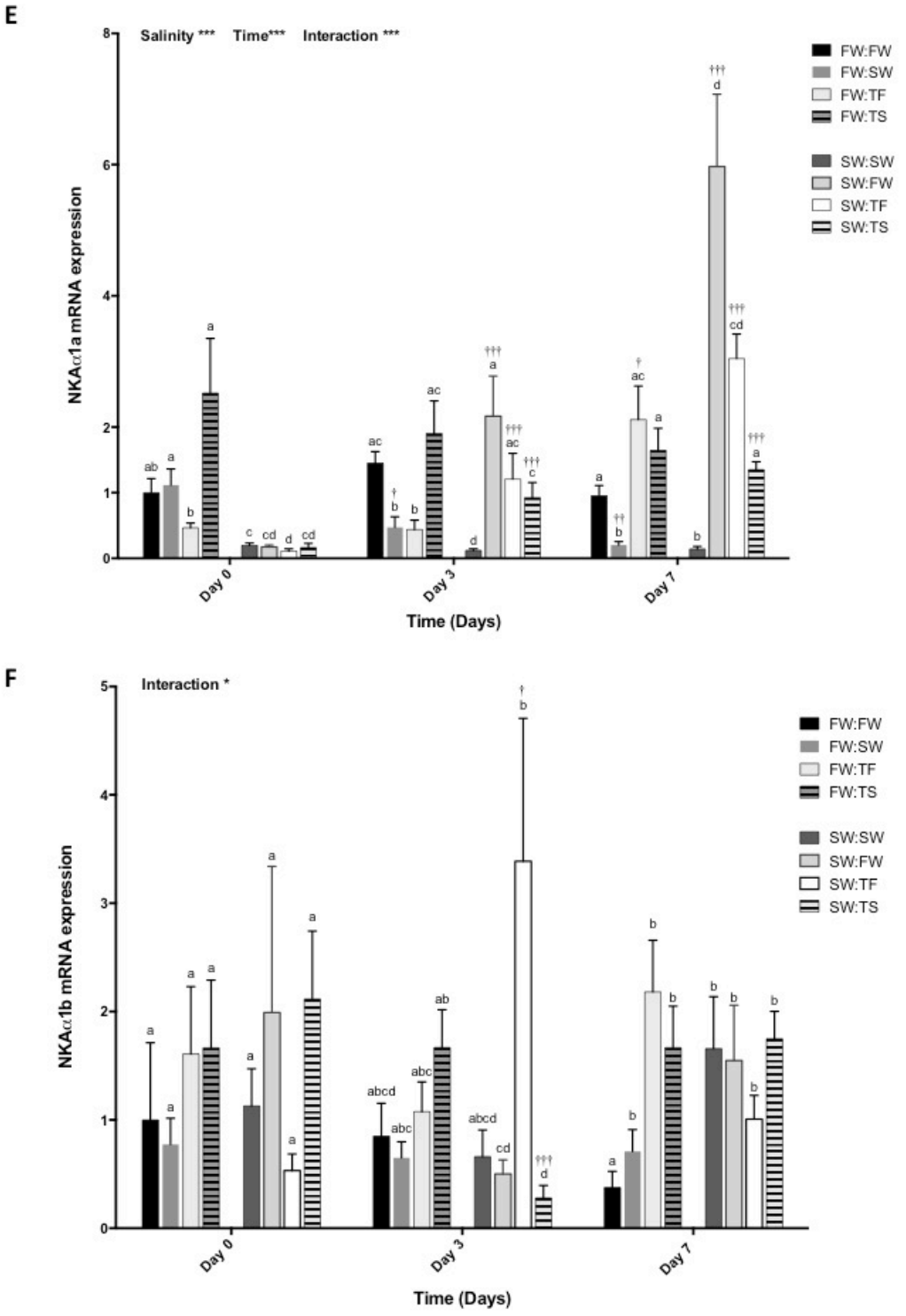
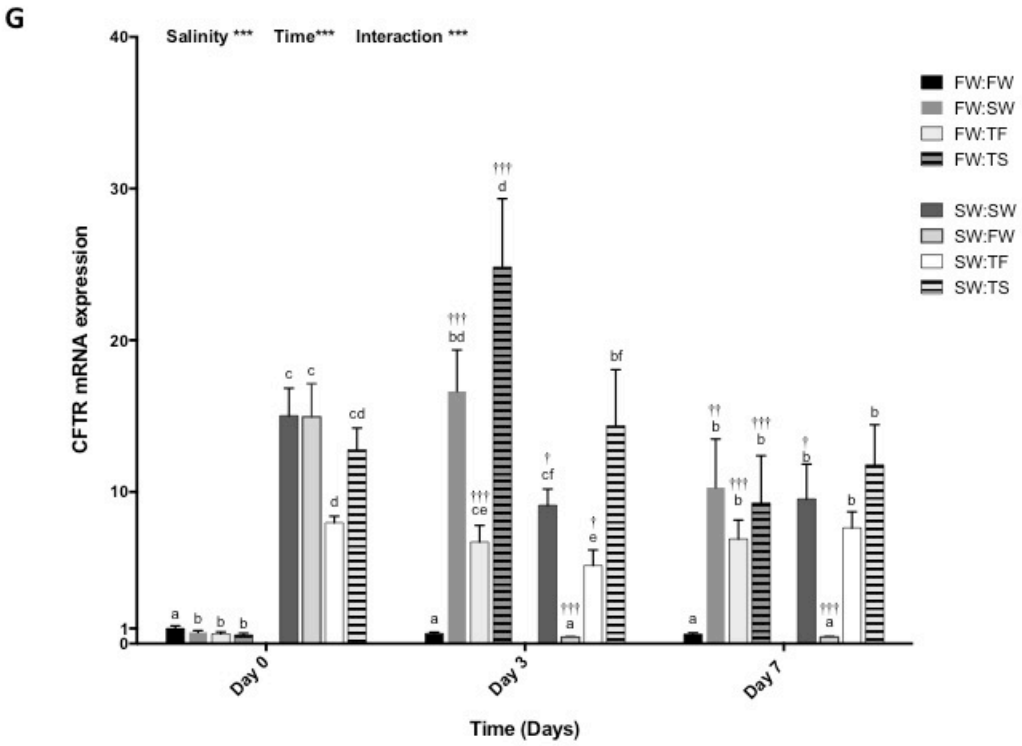
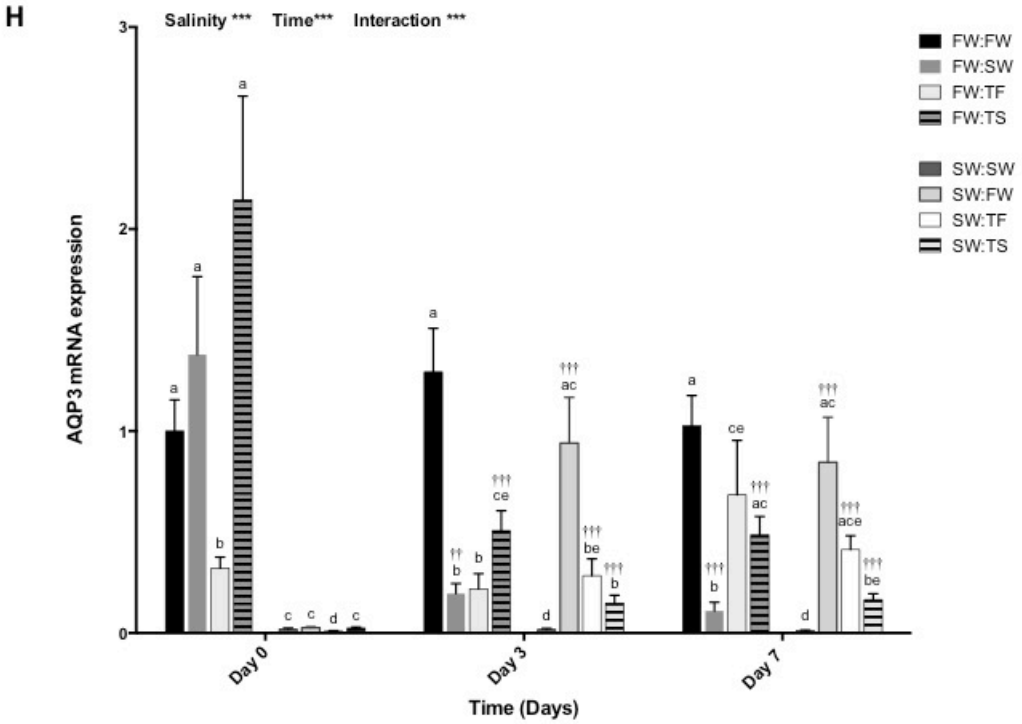

E
Salinity *** Time*** Interaction ***
NKAα1a mRNA expression
Time (Days)
Day 0
Day 3
Day 7
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS
F
Interaction *
NKAα1b mRNA expression
Time (Days)
Day 0
Day 3
Day 7
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS

Figure 4


G
Salinity *** Time*** Interaction ***
CFTR mRNA expression
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS
Day 0
Day 3
Day 7
Time (Days)


H
Salinity *** Time*** Interaction ***
AQP3 mRNA expression
FW:FW
FW:SW
FW:TF
FW:TS
SW:SW
SW:FW
SW:TF
SW:TS
Day 0
Day 3
Day 7
Time (Days)

4. Discussion

The objective of this experiment was to characterize plasma osmolality, PRL and branchial expression of PRLRs, ion transporters and pumps in adult fish reared in TR, and to determine the capacity of adult fish, reared in steady-state FW or SW, to acclimate to TR. This is the first study both to describe an osmoregulatory profile for adult Mozambique tilapia reared for at least two years in a cyclically-changing salinity, similar to the species' native habitat, and to investigate the effects of transfer from FW and SW steady-state rearing conditions to a tidal environment.

The major 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) in TR-acclimated fish, branchial gene expression of effectors of ion transport was generally similar to that of SW fish, with the exception of NKAα1a and AQP3, which were expressed at levels similar to FW fish and intermediate to FW and SW fish, respectively; and (3) within 7 days, the osmoregulatory parameters of fish reared in steady-state FW or SW and transferred to TR were similar to those observed in fish reared in TR since larval stages.

In characterizing a profile of plasma parameters for Mozambique tilapia reared in TR for at least two years, we found that plasma osmolality was higher in SW fish than in FW fish, whether in a steady-state or tidally-changing regimen. This was consistent with previous reports describing a direct relationship between plasma osmolality and external salinity, both in steady-state and tidally-reared Mozambique tilapia (Moorman et al., 2015; Moorman et al., 2014; Seale, et al., 2006a; Seale et al., 2002; Yada et al., 1994). By contrast, plasma PRL was elevated in fish in FW over those in SW, regardless of regimen, which was also in line with previous reports describing an inverse relationship between plasma PRL release and extracellular osmolality (Grau, et al., 1981; Helms, et al., 1991; Seale, et al., 2006b; Seale et al., 2002; Wigham, et al., 1977). In neither set of data, however, was an interaction effect between salinity and regime observed, indicating that there was no difference in plasma osmolality between FW and SW groups within regimens. The similarity in plasma PRL levels in TF and TS fish is consistent with previous reports on TR-acclimated Mozambique tilapia (Moorman et al., 2015; Moorman

et al., 2014). Together, these results indicate that circulating PRL is not as strongly tied to variations in plasma osmolality in fish reared in a tidal regimen, when compared with those reared in steady-state salinities.

Environmental salinity has been shown to modulate the actions of 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., 2016). While branchial PRLR1 expression in TR-acclimated fish was elevated in TS over TF, I observed the opposite pattern in fish that were transferred to TR from either FW or SW steady-state salinities. Elevated expression of branchial PRLR1 in FW relative to SW is consistent with previous reports where fish were sampled in either FW and SW steady-states, transferred from SW to FW, or in FW and SW phases of a tidal regimen (Breves et al., 2011; Fiol et al., 2009; Moorman et al., 2014; Moorman et al., 2015). Furthermore, expression of PRLR1 in gill is stimulated in a dose-dependent manner by PRL (Inokuchi, et al., 2015). However, because plasma PRL in fish sampled in TF was similar to that of fish sampled in TS, it is unlikely that the changes in branchial PRLR1 expression observed are attributable to variations in circulating PRL. Moorman and colleagues (2014) suggested that the differential regulation of branchial PRLR1 expression between the two phases of TR may be attributable to direct transcriptional regulation by environmental salinity at the tissue level (Moorman et al., 2014). At any rate, it is possible that there are nuances in PRLR1 expression in TR-acclimated fish that may be affected by variables other than salinity, such as age, sex or size that may be elucidated with additional studies using this tidal paradigm.

Branchial PRLR2 expression, has also been reported to vary with extracellular osmolality, but unlike PRLR1, its expression increases in hyperosmotic conditions (Fiol et al., 2009; Inokuchi et al., 2015; Seale et al., 2012a). In the present study, branchial PRLR2 expression was higher in SW than in FW in both tidal and steady-state rearing regimens. This finding supports a previously postulated notion that increased PRLR2 expression in hyperosmotic conditions may facilitate acclimation of tilapia to SW (see Inokuchi et al., 2015; Seale et al., 2012a), as binding of PRL to this receptor isoform may not elicit the same response as binding to PRLR1 (see Fiol et al., 2009). Unlike PRLR1,

the dynamic changes in PRLR2 transcription with environmental salinity, regardless of rearing or acclimation regimen, suggest that this isoform is strongly 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 hypoosmotic stimuli and PRL (Breves et al., 2016). The overall similarity in branchial NCC, NKCC1a, and CFTR expression between SW and TR fish was consistent with a previous report on TR-acclimated, 4-month old fish (Moorman et al., 2014), as was branchial AQP3 expression in TF and TS fish, which was intermediate to levels in FW and SW controls. While the branchial expression levels of NKAα1a in TR-acclimated adult fish did not align with this same report, it was consistent with patterns of expression observed in sexually mature, 11-month old fish reared from fry in TR and sampled over a 24-hour period as shown in Chapter III of this thesis. Interestingly, branchial mRNA expression of NKAα1b observed in TR-acclimated fish (Fig. 2F) was opposite to that described by Moorman and colleagues (2014), but was consistent with previous reports of up-regulation in response to increased extracellular osmolality (Breves, et al., 2010a; Hiroi & McCormick, 2007; Inokuchi, et al., 2015; Tipsmark et al., 2011). Overall, these findings suggest that the osmoregulatory profile of the adult fish reared in TR for at least two years is more similar to that of SW fish than of FW fish; this is consistent with observations in four-month old fish reared in TR (Moorman et al., 2014).

Upon conducting the salinity transfer experiments of this study, I found that tilapia reared in both steady state FW and SW could withstand a direct transfer to TR, with 100% survival. This was suggestive of an ability to survive exposure to dynamic salinity changes, regardless of rearing salinity. It is well established that FW-acclimated tilapia cannot survive direct transfer to SW, but are able to survive when first transferred to an intermediate salinity (Breves, et al., 2010b; Moorman et al., 2015; Seale et al., 2012a; Seale et al., 2002; Yada et al., 1994). In light of these observations, it is reasonable to postulate that for FW fish, transition to a cyclically changing salinity is less

severe than to SW. Specifically, FW fish transferred to TR suffered no mortalities despite their initial exposure to full-strength SW within 2 h of the first TS phase post-transfer.

By Day 7 of transfer from steady-state salinities to TR, patterns in plasma parameters and branchial expression of PRLRs and effectors of ion transport 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 described in the TR-acclimated fish sampled in Experiment 1. Hence, the overall trends in the relative expression of osmoregulatory genes in fish transferred from steady-state salinities to TR are similar to those described in fish reared in TR since their larval stages, with the exception of PRLR1. Together, these observations indicate that adult tilapia do not require exposure to salinity changes during their development in order to successfully withstand such changes at later stages in life, as they possess the remarkable capacity to acclimate to a tidal regimen within 7 days, regardless of the steady-state salinity in which they were reared.

In the current study, I was able to measure plasma parameters and branchial gene expression of effectors of ion transport in adult fish to characterize an osmoregulatory profile for TR-acclimated Mozambique tilapia. Using this profile and the findings of previous reports on TR-acclimated fish, I was able to demonstrate the successful acclimation to TR within 7 days by adult fish reared in steady-state FW or SW for at least two years. Therefore, I have provided novel insights to osmoregulation under TR rearing conditions at a life stage not previously examined under this paradigm. It is worthy of mention that in a similar study, fish reared in TR for four months grew faster than fish reared in FW or SW alone (Moorman, et al., 2016). This increase in growth was associated with stimulation of the growth hormone/ insulin-like growth factor (GH/ IGF) system (Moorman, et al., 2016). Future investigation of TR-acclimation on growth and other physiological processes is warranted, as this rearing paradigm could provide an additional means of improving production in tilapia, a widely farmed finfish, which stands to benefit both aquaculture and the world food supply. Additionally, as the TR rearing paradigm approximates the range and dynamism of salinity fluctuations to which the Mozambique tilapia may be exposed in its native habitat, the findings of this study expand what is known about osmoregulation in this euryhaline teleost under these

minimally studied conditions. Use of the TR rearing paradigm, therefore, can foster the elucidation of novel and comprehensive physiological insights, and provide the means to develop optimal rearing conditions for euryhaline fish.

CHAPTER III

Dynamic changes in plasma and gill osmoregulatory parameters over a 24-hour period in Mozambique tilapia reared in a tidally-changing salinities

Abstract

The Mozambique tilapia is a euryhaline teleost species native to estuarine waters characterized by tidal fluctuations in salinity ranging from FW to SW. Previous studies with tilapia have examined osmoregulatory parameters in fish acclimated to FW or SW steady states, or after one-way transfers between the two. Using a previously described tidal regimen (TR) simulating the dynamic salinity changes characterizing the native habitat of the Mozambique tilapia, the current study sought to characterize osmoregulatory parameters in 11-month old fish reared in TR from fry, and sampled every three hours over a 24-hour period. The following parameters were measured: plasma osmolality, PRL, and branchial expression of PRL receptors (PRLR1 and PRLR2), Na^+/Cl^- and $Na^+/K^+/2Cl^-$ co-transporters (NCC and NKCC), Na^+/K^+ ATPase (NKAa1a and NKA a1b), and Cl^- and water channels (CFTR and AQP3). The current study provides insight to how these parameters change throughout multiple points during the FW and SW phases of the tidal cycle (TF and TS, respectively). Throughout the 24-hour period, plasma osmolality fluctuated with TF and TS, while plasma PRL remained steady. Additionally, the branchial gene expression profile of ion transporters during this period suggested that TR fish maintain ionocyte function similar to that of SW-acclimated fish, with some differences. Overall, these results indicate that throughout the diurnal cycle, fish acclimated to TR are able to compensate for broad changes in external salinity while keeping osmoregulatory parameters within a narrow range, and that some nuances in the fluctuations of these parameters may not be as tightly associated with changes in environmental salinity in TR fish as has been observed in FW and SW fish.

1. Introduction

Osmoregulation is critical to life in complex organisms, including most vertebrates. Organisms that osmoregulate must constantly manage the balance of ions and water in the body to maintain osmotic homeostasis (Marshall & Grosell, 2005). Mozambique tilapia and other teleost fishes tightly regulate internal osmotic conditions to maintain plasma osmolality within a narrow range—typically near one-third the osmolality of SW (Stephen D. McCormick, 2001). However, as a euryhaline species indigenous to estuarine environments in Southeast Africa, the Mozambique tilapia in its native habitat is subject to broad fluctuations in salinity, and is capable of maintaining plasma osmolality in the face of exposure to a wide variety of salinities (Marshall & Grosell, 2005; Trewavas, 1983).

In tilapia and other teleost fishes, the gill, kidney and intestine are essential in osmoregulation, with gill as the primary site of Na^+ and Cl^- exchange with the environment (Evans, 2008; McCormick, 2001). In hypoosmotic environments, Mozambique tilapia and other teleosts must counteract solute loss and excessive hydration by producing dilute urine and actively taking up ions across the gill (McCormick, 2001). In hyperosmotic environments, these fishes must counteract fluid loss and the tendency to gain solutes by drinking the surrounding water, and absorbing both water and ions via the gut, and extruding excess ions across the gill and kidney (McCormick, 2001).

Among euryhaline teleost species, the pituitary hormone prolactin (PRL) is essential to osmoregulation in FW (Dharmamba, et al., 1967; Manzon, 2002; Pickford, & Phillips, 1959). In Mozambique tilapia, plasma PRL levels rise in hypoosmotic conditions, and in FW fish, the effect of PRL at the gill is to stimulate ion uptake and to reduce water permeability (Manzon, 2002). These and other downstream effects of PRL are induced by binding PRL receptors 1 and 2 (PRLRs 1 and 2), which have been found in the gill, kidney and gut in Mozambique tilapia (Fiol et al., 2009). *In vitro* studies in tilapia pituitary and gill have demonstrated that PRLR1 and PRLR2 respond differently to osmotic stimuli and PRL. While PRLR1 mRNA expression is stimulated by PRL

(Inokuchi, et al., 2015), PRLR2 expression is upregulated by an increase in environmental salinity (Seale et al. 2012a; Inokuchi, et al., 2015).

Specialized cells called ionocytes facilitate osmoregulatory ion and fluid exchange at the gill. The four types of ionocytes, types I-IV, participate in ion extrusion or ion uptake processes, and as such, can also be categorized into FW- and SW-types (Hiroi et al., 2005; Kaneko et al., 2008). Types I-III are considered FW-type ionocytes, and types I and IV are SW-type (Hiroi et al., 2005). Type I ionocytes are characterized by the presence of basolateral Na^+/K^+ ATPase, which is an ATP-driven pump that maintains an electrochemical gradient across the cell membrane, in turn providing the driving force for both ion uptake and extrusion processes (Hiroi et al., 2008; McCormick, 1995). Two isoforms are expressed in the gill: NKAα1a is abundant in FW-ionocytes, and expression is stimulated in response to decreased extracellular osmolality and increased extracellular PRL (Inokuchi, et al., 2015; Tipsmark et al., 2011), whereas NKAα1b expression is shown to increase when fish are moved from FW to SW (Tipsmark et al., 2011). Type II ionocytes are characterized by the presence of apical Na^+/Cl^- cotransporter (NCC), which is directly regulated by PRL (Breves, et al., 2010d; Inokuchi, et al., 2015). Type IV ionocytes maintain basolateral $Na^+/K^+/2Cl^-$ cotransporter (NKCC1a) and an apical Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR) (Hiroi et al., 2005). AQP3, a water channel in the basolateral membrane, facilitates fluid exchange with the extracellular environment in both FW and SW ionocytes (Connolly, et al., 1998; Watanabe et al., 2005).

While extensive study of Mozambique tilapia has focused on FW- and SW-acclimated fish, as well as those transferred from FW to SW or vice versa, little is known about how these fish respond to rearing under a regimen that simulates the cyclical salinity fluctuations of their native, estuarine environment. In a recent study, Moorman and colleagues reported osmoregulatory parameters in four-month old fish sampled after rearing under a tidal rearing paradigm (TR) the team devised. Specifically, fish were exposed to full salinity changes from FW to SW every six hours to simulate a tidal cycle (Moorman et al., 2015; Moorman et al., 2014). Key findings from Moorman's study were: (1) circulating PRL levels were decoupled from fluctuations in plasma osmolality during the tidal cycle in TR-acclimated fish; (2) gene expression of effectors of ion

uptake in fish acclimated to TR was higher than that of fish acclimated to SW but lower than that of FW fish; and (3) the morphology of ionocytes in TR-acclimated fish largely resembled that of SW-acclimated fish (Moorman et al., 2014). By sampling fish near the end of the FW and SW phases of the tidal cycle (TF and TS, respectively), the results of that study described an osmoregulatory profile for TR-acclimated fish, and provided an approximation of how fish respond to the cyclical increase and decrease in environmental salinity (Moorman et al., 2014). Whether the fluctuations in osmoregulatory parameters observed over a tidal cycle are influenced by a circadian rhythm is unknown. In the current study, my objective was to characterize an extensive osmoregulatory profile for fish acclimated to TR at greater resolution to determine how osmoregulatory parameters fluctuate over a full tidal cycle and are influenced by a diurnal cycle. To accomplish this, I designed an experiment extending over a 24-hour period including multiple iterations of the tidal cycle. I hypothesized that the 24-hour profile would reveal similar TR patterns in plasma parameters and branchial gene expression as observed in Moorman's study, with mid-phase measurements intermediate to end-of-phase measurements. To address this objective, I sampled fish acclimated to FW, SW and TR every 3 h over a 24-hour period, and measured: plasma osmolality, plasma levels of the two PRL isoforms, PRL_{177} and PRL_{188}; and brachial mRNA expression of PRLR1, PRLR2, NCC, NKCC1a, NKAα1a, NKAα1b, CFTR and AQP3.

2. Materials and Methods

2.1 Fish Rearing

Mozambique tilapia (*O. mossambicus*) yolk-sac larvae were collected from broodstocks maintained in FW tanks at the Hawaii Institute of Marine Biology (Kaneohe, HI, USA). Fourteen days post-collection, yolk-sacs were fully absorbed, and the fry were seeded to 700L, outdoor, FW tanks filled to 140L, at a density of 120 fish per tank. Water temperature was maintained at 27± 2 °C in all tanks, and fish were exposed to a natural photoperiod. On the second day post-seeding, tanks were transitioned to brackish water (BW) at 10‰, using SW at 34±1‰ (Kaneohe Bay, Oahu, HI). On the fifth day post-

seeding, BW salinity was increased to 18±2‰. On the eighth day post-seeding, two BW tanks were transitioned to FW, two to SW, and four to tanks alternating FW and SW every 6-hours, simulating a tidally-changing salinity (TR) as recently described (Moorman et al., 2014, 2015). Until transition from BW to FW, SW and TR, fish were fed ground trout chow pellets (Skretting, Tooele, UT, USA) *ad libitum* daily, at which point they began fixed rations of 18% mean body weight divided over two feedings daily (mean body weight 24±1 mg). Rations were decreased by 4% every 21-25 days until they were equivalent to 4% mean body weight and maintained for the remainder of the experiment. The fish were reared in these conditions until sampling. Fish were fasted for the duration of sampling; the final feeding occurred just prior to the first sampling time point.

Four male and four female fish reared under TR were sampled at the end of the FW and SW phases of the tidal cycle, as well as at the mid-point of each phase. For each time point at which TR fish were sampled, corresponding FW and SW control groups were sampled, each also composed of four males and four females. Fish were collected from across all of the replicate tanks for FW, SW and TR at every time point. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

2.2 Treatments and sampling

At the time of sampling, fish were netted and anesthetized with 2-phenoxyethanol (0.3mL/L). After fish were weighed, blood was drawn with a needle and syringe coated with sodium heparin (200 U/ml, Sigma-Aldrich, St. Louis, MO), and fish were euthanized by rapid decapitation. Plasma was separated by centrifugation and stored at -20°C for further analysis. Gill filaments from the second gill arch on the left side of the fish were collected. Gill samples were frozen in liquid nitrogen and stored at -80°C, pending further analysis.

2.3 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), 400 ng of total RNA 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). The mRNA levels of reference and target genes were determined by an absolute quantification standard curve. Elongation factor 1a (EF1a) was used as a reference gene to normalize the mRNA levels of target gene. The PCR mixture (15 uL) contained Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA), 200 nM of each primer, and 2 µl of standard cDNAs or cDNAs prepared from experimental samples. PCR cycling parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The R^2 values and amplification efficiencies for standard curves were as follows, respectively: 0.999 and 85.0-85.3% (EF1a), 0.999 and 84.6-85.5% (PRLR1), 0.999 and 70.1-74.7% (PRLR2), 0.998-0.999 and 90.1-90.3% (NCC), 1 and 85.1-85.6% (NKCC1a), 0.999-1 and 87.9-88.2% (NKA a1a), 0.999-1 and 86.3-87.9% (NKA a1b), 1 and 90.0-90.3% (CFTR), and 0.993-0.995 and 89.5-90.0% (AQP3). All primer pairs have been previously described: NCC (Inokuchi, et al., 2008), NKCC1a (Inokuchi et al., 2008), NKA a1a (Tipsmark et al., 2011), NKA a1b (Tipsmark et al., 2011), EF1a (Breves et al., 2010a), PRLR1 (Pierce et al., 2007), PRLR2 (Breves, et al., 2010c), AQP3 (Watanabe et al., 2005), and CFTR (Moorman et al., 2014).

2.4 Plasma Parameters

Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5100C; Wescor, Logan, UT). Plasma PRL_{177} and PRL_{188} were measured via homologous radioimmunoassay (RIA) as described by Ayson and colleagues (Ayson et al., 1993).

2.5 Statistical Analysis

Statistical analyses were conducted using a two-way analysis of variance (ANOVA) with time and salinity (FW, SW or TR) as independent variables. Significant interaction effects of time and salinity ($P<0.05$) were followed up by Fisher's Least Significant Difference (LSD) test. Data are expressed as means ± S.E.M. Where applicable, individual values were log-transformed 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 Plasma parameters

Throughout the 24-hour sampling period, plasma osmolality in TR fish was elevated in TF over TS, with mid-phase values intermediate to TF and TS (Fig. 5A). A sharp drop in TR plasma osmolality began at hour 18 and continued through the final 6 hours of the 24-hour period. Plasma osmolality in FW and SW fish did not differ for the first 6 hours of the experiment, and began to diverge toward maximum and minimum values, respectively, at hour 12 (21:45). Plasma PRL_{177} levels in FW, SW and TR fish remained below 4 ng/ml for up to 18 hours, at which point a rise to approximately 9 ng/ml in TR fish during the second half of the dark phase coincided with a drop in water salinity associated with the tidal cycle (Fig. 5B). The onset of the rise was delayed by three hours, but resembled a rise observed in FW fish that also began in the second half of the dark hours, spanned 6 hours, and peaked just after the onset of daylight. After the 07:00 peak in PRL_{177} in FW fish, a sharp drop was observed at 09:00. PRL_{177} levels in SW fish remained steady throughout the 24-hour period. Plasma PRL_{188} in FW fish was elevated compared with SW fish for the entire experiment. PRL_{188} levels in TR fish remained steady and similar to those in SW fish for up to 18 hours, after which PRL_{188} rose to approximately 17 ng/ml. The rise in PRL_{188} also coincided with a drop in water salinity associated with the tidal cycle (Fig. 5C). This rise of PRL_{188} in TR fish was also

three hours delayed relative to the onset of a rise in FW fish, which in turn coincided with the FW rise in PRL_{177}. The 07:00 peak in PRL_{188} in FW fish was also followed by a sharp drop by 09:00, as observed in PRL_{177}. PRL_{188} levels in SW fish remained steady throughout the 24-hour period.

3.2 Branchial gene expression of PRL receptors; ion transporters and pumps; and ion and water channels

Branchial gene expression of PRLR1 in FW fish remained consistent and elevated over SW fish, throughout the 24-hour period (Fig. 6A). Expression of PRLR1 in TR fish was similar to that in SW fish for the most part, with occasional excursions to FW-control levels during the TF phase, in both light and dark hours.

By contrast, PRLR2 mRNA expression in SW fish was generally elevated over that in FW fish throughout the sampling period (Fig. 6B). Similar to patterns observed in PRLR1 expression, expression of PRLR2 in TR fish coincided with the expression in SW controls during the TS phases of the tidal cycle, and with the expression in FW controls during the TF phases of the tidal cycle (Fig. 6B).

Throughout the 24-hour sampling period, branchial gene expression of NCC was elevated in FW over SW; SW expression levels were nearly zero. NCC expression in TR fish varied minimally from that of SW fish over 24 h (Fig. 6C). At 03:45, NCC expression in FW controls increased and remained elevated through the final sampling time point at 09:45. This rise in NCC expression coincided with the observed increases in plasma PRL_{177} and PRL_{188} levels in FW controls at the same time (Fig. 5A and 5B). On the other hand, NKCC1a mRNA expression in SW fish ranged from 2-fold higher to nearly 5-fold higher than that in FW fish throughout the 24-hour period (Fig. 6D). NKCC1a expression in TR fish coincided with that in SW fish at all time points, except at 19:00, shortly after the onset of darkness. However, there was no significant time effect on NKCC1a expression (Fig. 6D).

Branchial gene expression of NKAα1a was near zero in SW fish. In FW fish, NKAα1a expression ranged from 0.5 to 1.5 fold higher than in SW fish; expression in TR fish was similar to that of FW fish through most of the 24-hour period. NKAα1a

expression in FW fish reached peak levels compared with SW controls at the first 09:45 sampling time point, and again at the second 09:45 sampling time point. The onset of the rise in NKAα1a expression leading up to the final 09:45 time point occurred during the dark hours at 03:45, similarly timed to the observed onset of increases in plasma PRL_{177} and PRL_{188} levels in FW controls (Fig. 5A and 5B).

There was no effect of salinity on branchial NKAα1b expression (Fig. 6F). An effect of time was observed, with slight reductions in NKAα1b expression in SW and TR fish during the dark hours, between 01:00 and 03:45, and 21:45 and 03:45, respectively (Fig. 6F).

Branchial CFTR gene expression was consistently higher in SW compared with FW; expression in TR fish was similar to that in SW fish at most time points (Fig. 6G).

Branchial AQP3 gene expression, on the other hand, was higher in FW fish compared with SW controls at all time points (Fig. 6H). In TR fish, AQP3 expression was intermediate to that of FW and SW controls. However, there was a peak in AQP3 expression at the final 09:45 time point in TR fish, which was closely timed with the aforementioned rises in plasma PRL_{177} and PRL_{188}. This rise in AQP3 expression in TR fish mirrored that observed in FW fish.

Fig. 5. Plasma osmolality (A), plasma PRL_{177} (B), and plasma PRL_{188} (C) of Mozambique tilapia (*Oreochromis mossambicus*) reared in FW (black dashed), SW (black dotted) and TR (solid black), and sampled over 24 hours (h). Values represent means ± SEM (n= 6-8). Shading denotes dark hours. Grey lines denote mean water salinity measured hourly in fresh water (FW), seawater (SW) and tidally-changing salinity (TR) tanks. Salinity and time effects were analyzed by two-way ANOVA, followed by Fisher's LSD where main or interaction effects were observed, $*P<0.05$, $**P<0.01$, $***P<0.001$). Means not sharing the same letter are different ($p<0.05$); uppercase letters indicate differences across salinities at each sample time, whereas lowercase letters indicate differences over time within each salinity.

Figure 5

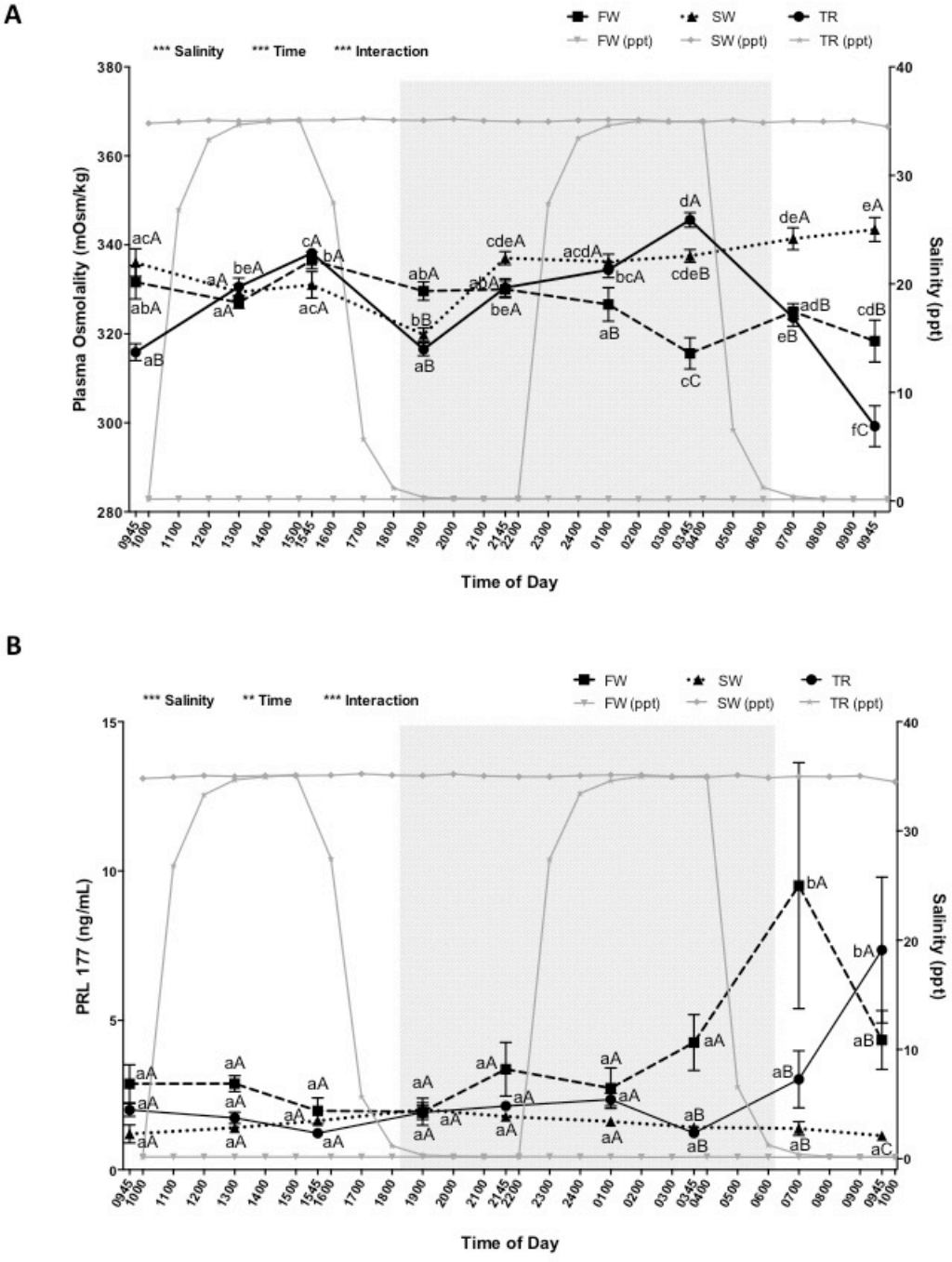

A
FW
SW
TR
FW (ppt)
SW (ppt)
TR (ppt)
*** Salinity
*** Time
*** Interaction
Plasma Osmolality (mOsm/kg)
Salinity (ppt)
Time of Day
B
*** Salinity
** Time
*** Interaction
PRL 177 (ng/mL)
Salinity (ppt)
Time of Day

Figure 5

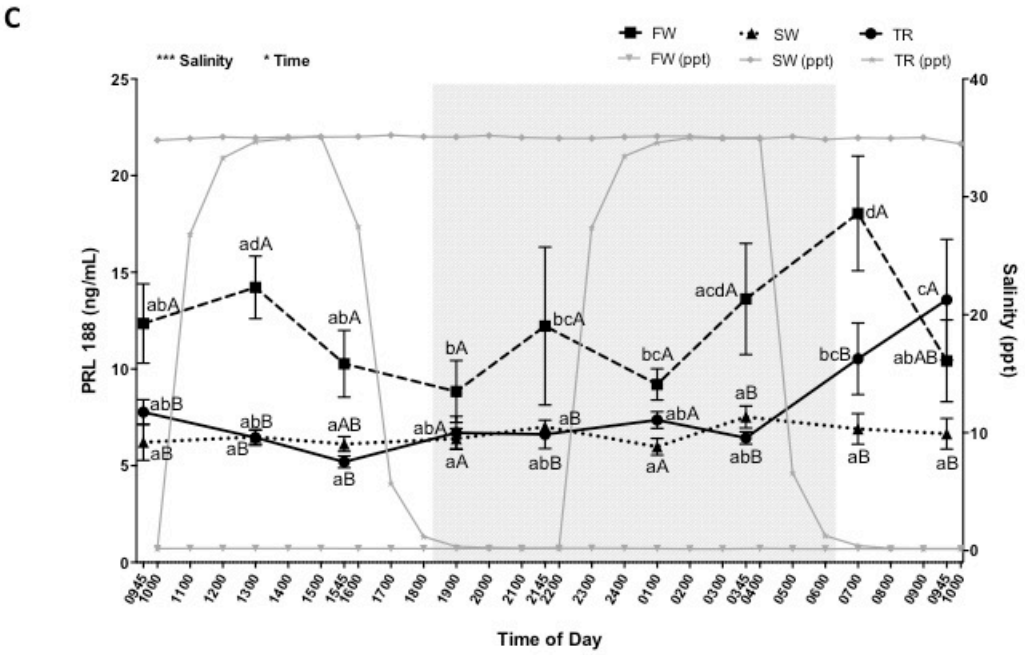

C
*** Salinity
* Time
FW
SW
TR
FW (ppt)
SW (ppt)
TR (ppt)
PRL 188 (ng/mL)
Salinity (ppt)
Time of Day
abA
adA
abA
bA
bcA
bcA
acdA
dA
cA
abAB
bcB
abB
abB
aAB
abA
aB
abA
aB
aB
aB
aB
aA
abB
aA
abB
aB
aB
0945
1000
1100
1200
1300
1400
1500
1545
1600
1700
1800
1900
2000
2100
2145
2200
2300
2400
0100
0200
0300
0345
0400
0500
0600
0700
0800
0900
0945
1000

Fig. 6. Branchial gene expression of PRLR1 (A), PRLR2 (B), NCC (C), NKCC1a (D), NKAα1a (E), NKAα1b (F), CFTR (G) and AQP3 (H) in Mozambique tilapia (*Oreochromis mossambicus*) reared in FW (black dashed), SW (black dotted) and TR (solid black), and sampled over 24 h. Values represent means ± SEM (n= 6-8). Shading denotes dark hours. Grey lines denote mean water salinity measured hourly in fresh water (FW), seawater (SW) and tidally-changing salinity (TR) tanks. Salinity and time effects were analyzed by two-way ANOVA, followed by Fisher's LSD where main or interaction effects were observed, **P*<0.05, ***P*<0.01, ****P*<0.001). Means not sharing the same letter are different ($p<0.05$); uppercase letters indicate differences across salinities at each sample time, whereas lowercase letters indicate differences over time within each salinity.

Figure 6

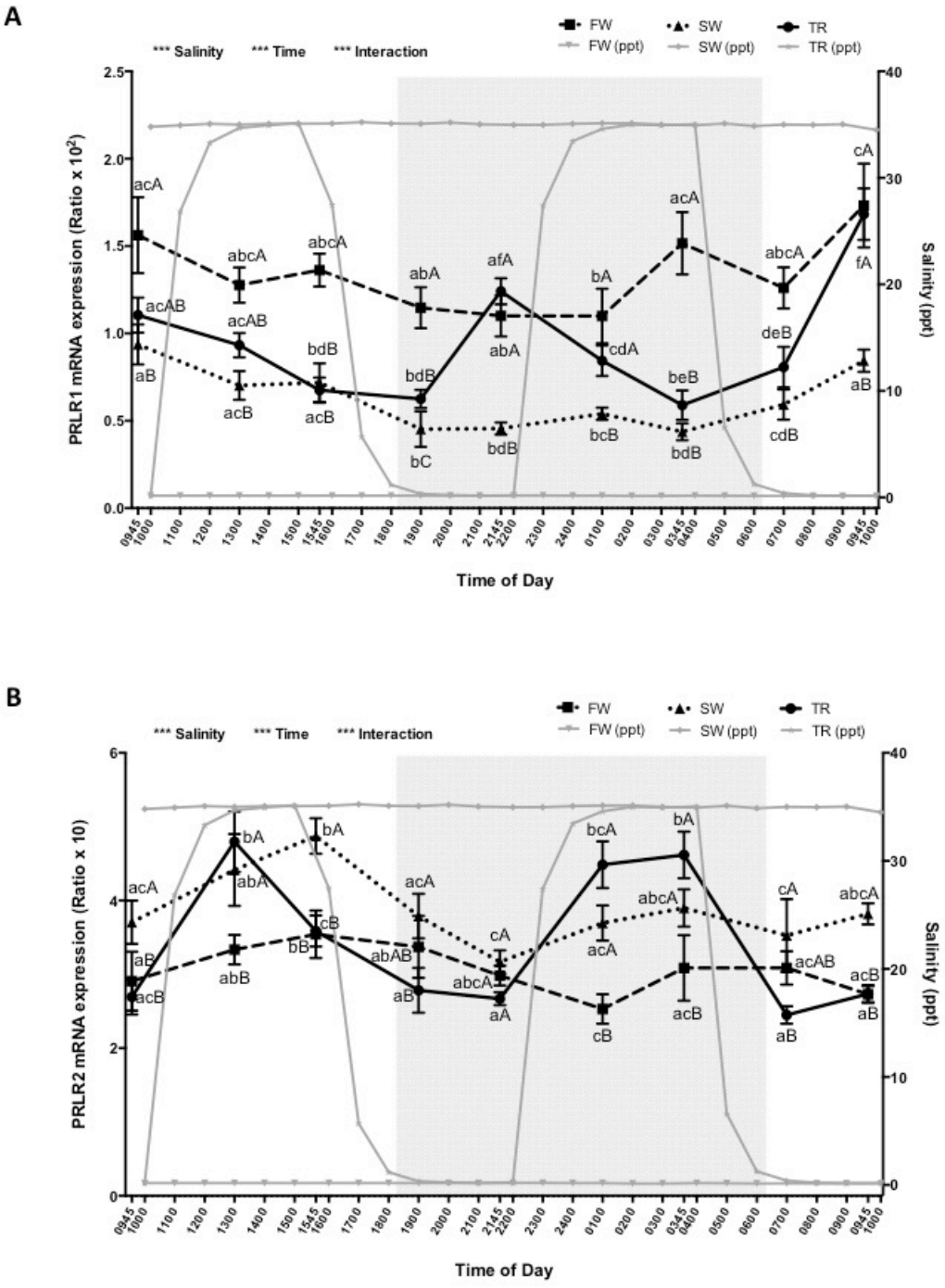

A
*** Salinity
*** Time
*** Interaction
FW
SW
TR
FW (ppt)
SW (ppt)
TR (ppt)
PRLR1 mRNA expression (Ratio x 10²)
Salinity (ppt)
Time of Day
B
*** Salinity
*** Time
*** Interaction
FW
SW
TR
FW (ppt)
SW (ppt)
TR (ppt)
PRLR2 mRNA expression (Ratio x 10)
Salinity (ppt)
Time of Day

Figure 6

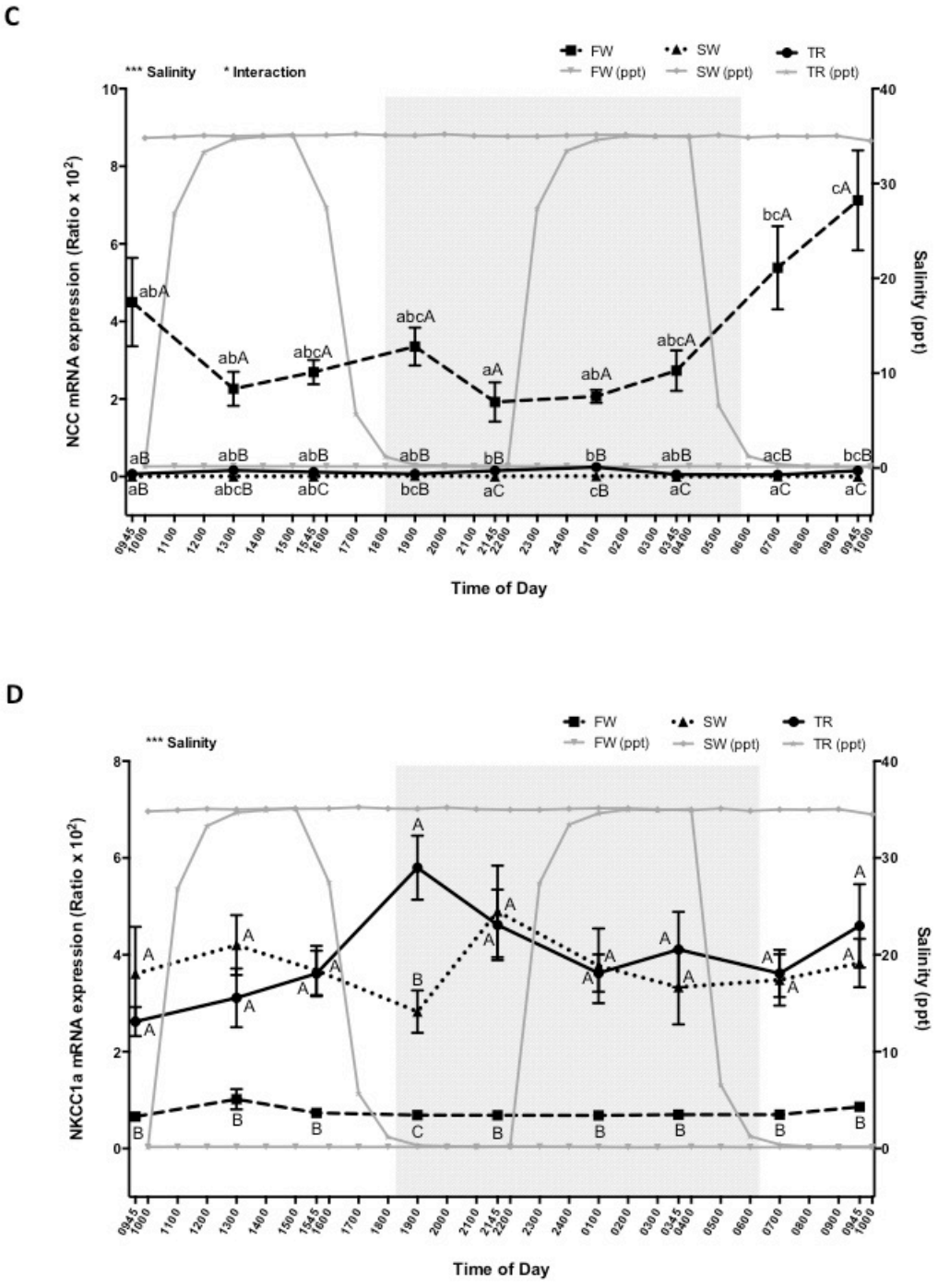

C
*** Salinity
* Interaction
FW
SW
TR
FW (ppt)
SW (ppt)
TR (ppt)
NCC mRNA expression (Ratio x 10²)
Salinity (ppt)
Time of Day
D
*** Salinity
NKCC1a mRNA expression (Ratio x 10²)
Salinity (ppt)
Time of Day

Figure 6

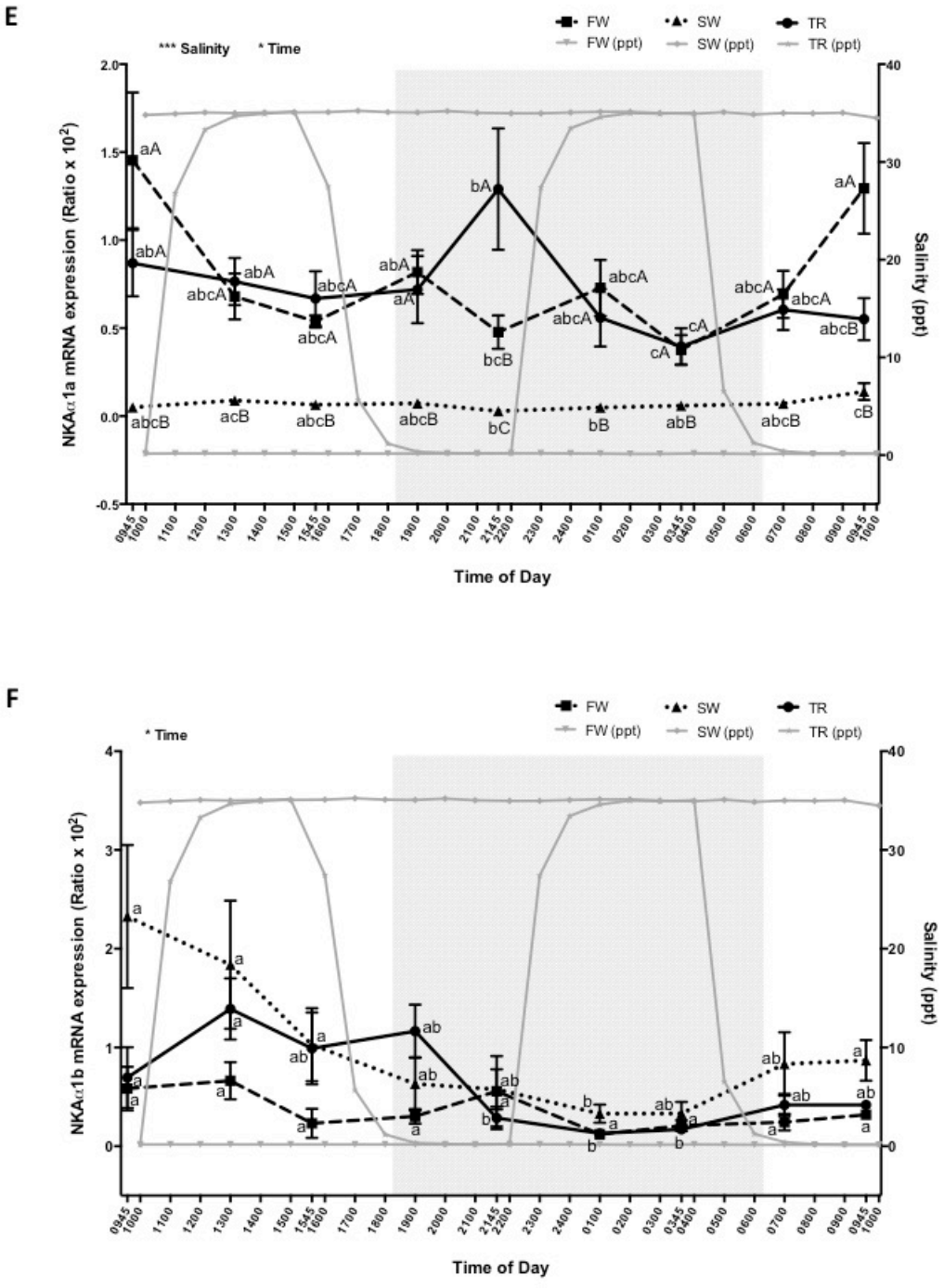
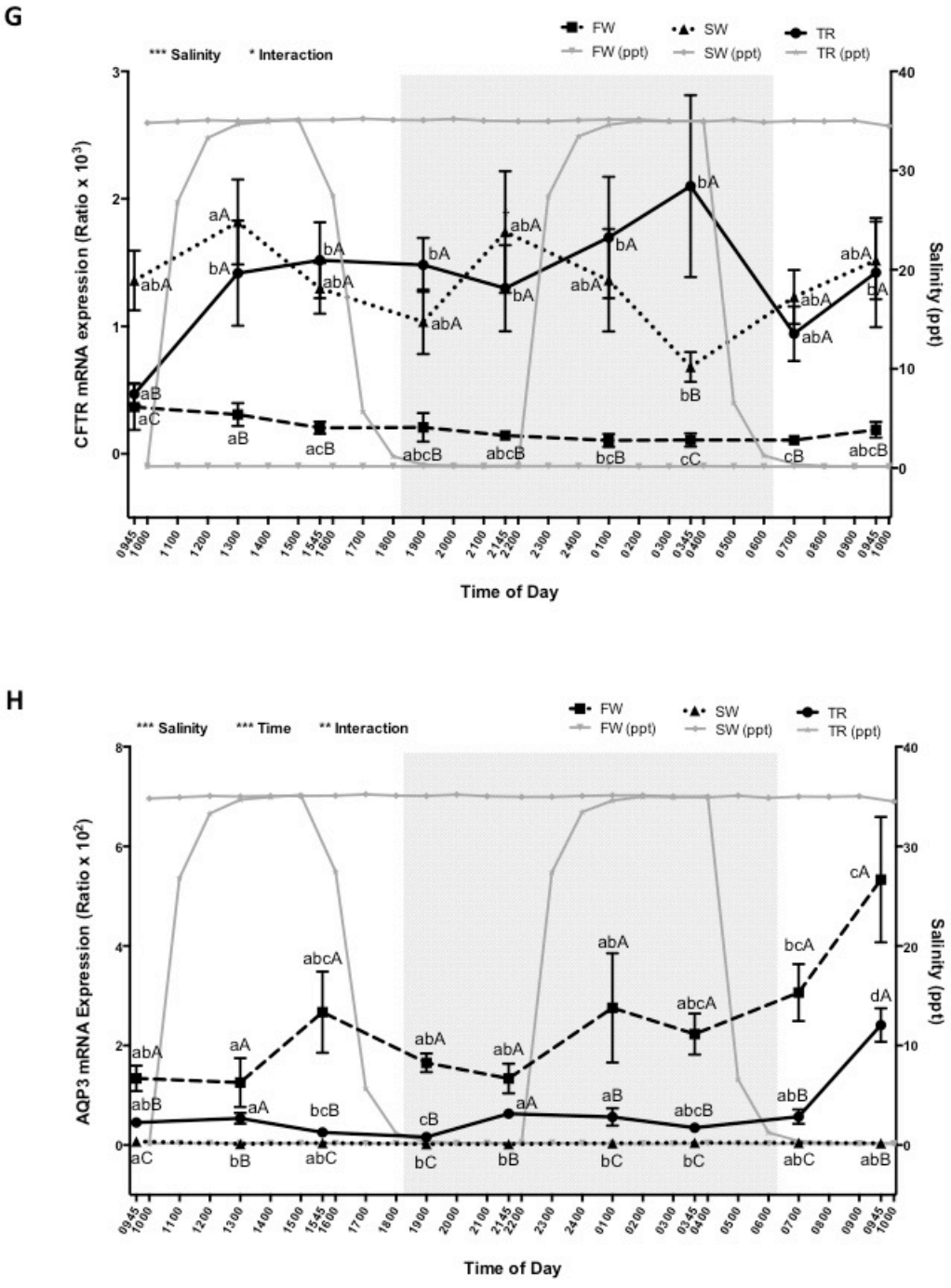

E
FW
SW
TR
FW (ppt)
SW (ppt)
TR (ppt)
*** Salinity
* Time
NKAα1a mRNA expression (Ratio x 10²)
Salinity (ppt)
Time of Day
F
* Time
NKAα1b mRNA expression (Ratio x 10²)
Salinity (ppt)
Time of Day

Figure 6


G
FW
SW
TR
FW (ppt)
SW (ppt)
TR (ppt)
*** Salinity
* Interaction
CFTR mRNA expression (Ratio x 10³)
Salinity (ppt)
Time of Day
H
*** Salinity
*** Time
** Interaction
AQP3 mRNA Expression (Ratio x 10²)
Salinity (ppt)
Time of Day

4. Discussion

The objective of this experiment was to characterize the dynamic changes in plasma osmolality, PRLs, and expression of the branchial effectors of ion transport in a euryhaline teleost, the Mozambique tilapia, in relation to the phases of the tidal cycle, throughout a 24-hour period. This was done by rearing tilapia under a tidal regimen, and then measuring, at the mid- and end-points of the FW and SW phases of the tidal cycle (TF and TS, respectively), both plasma osmolality and circulating PRLs, as well as branchial mRNA expression of PRLR1, PRLR2, NCC, NKCC1a, NKAαla, NKAαlb, CFTR and AQP3. These effectors of ion transport were selected based on their responsiveness to changes in extracellular osmolality and PRL in tilapia. This is the first experiment to examine these parameters in tidally-acclimated fish both at the end of and at intermediate points during the FW and SW phases of the tidal cycle throughout a full diurnal period.

While it has been widely demonstrated that plasma PRL is increased by hypoosmotic stimuli in euryhaline teleosts, studies to date have employed fish reared in steady-state salinities to address this endocrine response. In Mozambique tilapia, PRL release is strongly stimulated by a fall in extracellular osmolality, *in vivo* and *in vitro*; this response is more robust in fish acclimated to FW, as the number of PRL cells and the abundance of stored hormone is greater than those in SW (see Seale et al., 2012b). Recently, however, it was found that in tidally-reared tilapia, plasma PRL was unchanged between FW and SW phases of the tidal cycle, despite the fall and rise, respectively, in plasma osmolality (Moorman et al., 2014). Similar to that study, I found that circulating PRLs were uncoupled from plasma osmolality in TR fish during the initial 18 h of sampling. Moreover, this is the first study to measure PRL_{177} levels in tidally-reared tilapia, therefore enabling a comparison between the two tilapia PRL isoforms under this dynamic rearing paradigm. While plasma levels of PRL_{177} were generally lower than those of PRL_{188}, changes in PRL_{188} levels were generally more robust in response to salinity, both in steady-state or tidally-reared fish. These differences in response are consistent with previous reports (Borski, et al., 1992; Seale et al., 2012a; Yada et al., 1994).

After the first 18 hours of sampling, during the last dark hours and early light hours, plasma osmolality strongly dropped in TR fish. A sharp increase in plasma PRL_{177} and PRL_{188} was observed over the same period. This response is consistent with previous studies in which release of PRL_{177} and PRL_{188} were found to be stimulated by a decrease in external osmolality (Grau, et al., 1981; Helms, et al., 1991; Seale, et al., 2006b; Seale et al., 2002; Wigham, et al., 1977). Interestingly, the rise in plasma levels of both PRLs in FW and TR fish just after the onset of light at 07:00 is similarly timed to a peak in daily PRL levels observed in gulf killifish, *fundulus grandis*, maintained in FW under similar photoperiod and temperature (Spieler, et al., 1978). This suggests that the peak in plasma PRLs of both FW and TR fish observed in the current study could be associated with a diurnal PRL rhythm. Circulating PRLs in SW fish, however, did not change after the onset of light at 07:00. Whether or not associated with a diurnal rhythm, it is worth noting that the peaks in circulating PRLs in TR fish were delayed relative to those in FW fish, a likely reflection of the exposure of TR fish to elevated salinity (TS phase) immediately prior to 07:00. Furthermore, the PRL peak in TR fish also coincides with a large drop in plasma osmolality, a well-established stimulus for PRL release. Thus, the extent to which a diurnal rhythm may modulate PRL release in fish subject to TR is unclear. It is also worth noting that netting and confinement stress elicits a drop in plasma osmolality associated with a rise in plasma PRL_{177} and PRL_{188} in FW-acclimated tilapia within 6 h (Breves et al., 2010c). While the fish in this study were subject to repeated netting every 3 hours, the drop in plasma osmolality before dawn was only observed in TR fish, not FW fish. Moreover, in the current study, plasma PRLs in FW fish decreased after the 07:00 peak, despite being exposed again to netting at the subsequent sampling time point. The findings in the current study suggest, especially in FW-acclimated fish, that the peaks in plasma PRL_{177} and PRL_{188} observed near dawn may be associated, at least in part, with a diurnal PRL rhythm. A targeted, circadian study would be required to fully characterize PRL rhythms in tilapia.

PRLR1 mRNA expression has been shown to be stimulated in a dose-dependent manner by PRL_{177} and PRL_{188} (Inokuchi, et al., 2015), and by transfer of fish from SW to FW (Breves et al., 2011; Fiol et al., 2009). Additionally, a branchial PRLR1 expression in tidally-reared fish fluctuated between FW and SW control levels, with levels in TF fish

elevated over those in TS fish (Moorman et al., 2014). Consistent with these findings, in the current study branchial PRLR1 gene expression was elevated in FW fish over SW fish. PRLR1 expression in TR fish fluctuated between that of FW and SW fish during the 24-hour period, with TF values elevated over TS values. Neither plasma PRL_{177} nor PRL_{188} fluctuated with the tidal cycle, however, suggesting that the actions of PRL in tidally reared fish may be modulated by regulation of its receptors, rather than by changes in plasma PRL levels, as previously postulated (Moorman et al., 2014).

On the other hand, branchial PRLR2 expression has been shown to be osmosensitive, increasing with a rise in extracellular osmolality *in vitro* (Inokuchi, et al., 2015), and *in vivo* (Fiol et al., 2009; Seale et al., 2012a). In the current study PRLR2 expression was higher in the gills of SW controls than FW controls. Additionally, the PRLR2 expression pattern in TR fish was opposite to that of PRLR1, specifically highest and lowest at TS and TF phases, respectively. This pattern was consistent with previously published findings in TR-acclimated tilapia (Moorman et al., 2014). The dynamic changes in expression of both PRLRs in close association with salinity fluctuations of the tidal cycle indicates direct regulation of PRL's effects at the level of the target tissue.

The elevation of NCC expression in FW controls over SW controls throughout the 24-hour period was consistent with previous studies and the accepted role of NCC in ion uptake (Hiroi et al., 2008; Inokuchi et al., 2008; Kaneko et al., 2008; Watanabe et al., 2005) Breves, et al., 2010a; Breves, et al., 2010b; Breves, et al., 2010d). As TR fish have previously been shown to maintain branchial ionocytes of similar morphology to SW-type ionocytes (Moorman et al., 2014), it is not surprising that NCC expression in TR fish was suppressed, similar to that of SW fish. Further, as SW-type ionocytes are partly characterized by the expression of NKCC1a and CFTR (Evans, et al., 2005; Hiroi et al., 2005), it was not unexpected to find mRNA expression for these effectors of ion extrusion in TR fish to be similar to that of SW fish. Consistent with previous reports indicating elevation of NKAα1a expression in response to decreased extracellular osmolality and PRL (Breves, et al., 2010a; Inokuchi, et al., 2015; Tipsmark et al., 2011), I found NKAα1a expression to be higher in FW fish than in SW fish. Despite overall elevated expression of effectors of ion extrusion, and low expression of NCC, TR fish maintained branchial NKAα1a expression close to that of FW controls. While this does

not align with previously reported TF and TS expression of NKAα1a at levels intermediate to FW and SW controls (Moorman et al., 2014), the stability in expression across TR phases and the elevation over SW levels is similar. The sustained elevation in expression of NKAα1a, concurrently with elevated mRNA expression of key effectors of ion extrusion, may be indicative of the need to sustain an electrochemical gradient to drive ion uptake and extrusion between FW and SW phases of the tidal cycle. Furthermore, as TR expression of NKAα1a remained elevated despite low levels of PRL in circulation, and was neither associated with the pronounced rise in circulating levels of both PRLs during the second half of the dark hours, nor to the similarly timed decrease in plasma osmolality, it appears that the effects of PRLs and external salinity on NKAα1a expression may be muted under a cyclically changing salinity regimen, in comparison with the effects seen in steady-state salinities or one-way salinity transfers.

Contrary to prior reports indicating elevation of branchial NKAα1b expression in response to an increase in external osmolality (Tipsmark et al., 2011), there was no difference in NKAα1b expression across FW, SW or TR groups. This was also inconsistent with previous findings where NKAα1b expression in TF and TS fish were intermediate to that in FW and SW controls (Moorman et al., 2014). There was, however, a gradual reduction in NKAα1b expression in SW fish over time. Together, these data suggest that the α1a isoform is the prevalent NKA in TR fish.

Branchial AQP3 expression in FW fish was elevated at all time points over SW controls, consistent with prior reports on European eel, Japanese eel, Japanese medaka, Atlantic killifish, Atlantic salmon and Mozambique tilapia, describing elevated mRNA expression of AQP3 in FW compared to SW (Cutler & Cramb, 2002; Jung, et al., 2012; Lignot, et al., 2002; Madsen, et al., 2014; Moorman et al., 2015; Tipsmark, et al., 2010; Tse, et al., 2006). Also consistent with previously reported data on TR-acclimated fish, branchial AQP3 expression levels were intermediate to FW and SW controls, though closer to that of SW fish (Moorman et al., 2014). Also, expression rose in FW and TR fish with similar timing to the rise in plasma PRLs beginning at 01:00 and 03:45, respectively. This is also consistent with the recently reported PRL-induced upregulation of branchial AQP3 expression in tilapia (Breves, et al., 2016).

Overall, I found that patterns in osmoregulatory parameters in TR-acclimated fish were largely maintained throughout the tidal cycle, not only confirming those from previous studies comparing single time points, but providing for the first time a detailed profile of changes over a 24-hour period. Specifically, I showed that throughout a 24-hour period, tilapia reared under a tidal regimen were largely able to withstand wide fluctuations in external salinity while maintaining plasma osmolality and circulating levels of PRL_{177} and PRL_{188} within a narrow range. These data support the notion that, rather than cyclically adjusting circulating PRLs with each change in salinity, TR-acclimated fish regulate ion transport at the level of the target tissue. Despite relatively steady PRL levels in TR fish, the changes in transcription of both PRLRs in tandem with the phases of the tidal cycle suggest that the potency of PRL's effects may vary according to salinity in dynamically changing conditions. In addition, the branchial expression of ion channels, pumps and transporters in TR-acclimated fish may be directly osmosensitive, as shown to be the case in gill filaments from FW-acclimated tilapia (Inokuchi et al., 2016). Together, these findings suggest that in dynamically changing salinities, tilapia are regulating ion transport locally, at the level of target tissues, rather than systemically (i.e. through the pituitary). I have also shown that the branchial gene expression profile of PRLRs and effectors of ion transport in TR fish throughout a 24-hour period most closely resembled that of SW-acclimated fish. Additionally, I found that in the osmoregulatory parameters measured throughout the entire diurnal cycle, there were nuances in their changes that were not as closely tied with environmental salinity. These nuances would otherwise remain undetected with the TR approach of previous studies, which was limited to a single sample of tidal phases (Moorman et al, 2014, 2015). Hence, this 24-hour profile of osmoregulatory parameters in TR-acclimated fish (1) expands upon the current knowledge available for the tidal rearing (TR) paradigm, and (2) provides insight into the interactions between cycles entrained by salinity and daylight. Future studies employing this paradigm will unveil novel aspects of dynamic osmoregulation in euryhaline teleosts.

CHAPTER IV
FINAL REMARKS

In this thesis, I reported two studies I conducted that were aimed at characterizing the osmoregulatory effects of acclimation to a tidal rearing environment (TR) in Mozambique tilapia, using a recently described paradigm (Moorman et al., 2015; Moorman et al., 2014). I collected data to address two main objectives. The first was to characterize plasma osmolality, plasma PRL and branchial expression of ion transporters and pumps in adult fish reared under TR for a minimum of two years, and to determine the capacity of FW or SW-reared fish of similar age, to acclimate to TR. The second objective was to provide detailed resolution of the dynamic changes in plasma osmolality, plasma PRLs, and expression of the branchial effectors of ion transport in tilapia acclimated to a tidal cycle throughout a 24-hour period. Aggregating the findings of both studies, two key themes associated with acclimation to a tidal environment are apparent. These are (1) TR-acclimated fish are able to compensate for broad changes in external salinity while keeping osmoregulatory parameters within a narrow range; and (2) the branchial expression profile of ion transporters in TR fish suggests maintenance of ionocytes that are similar in function to, but not the same as, those of SW fish.

In the first study (Chapter II), I hypothesized that adult fish reared in FW and SW would be able to fully acclimate to TR within seven days of transfer, by exhibiting osmoregulatory parameters similar to those of fish that had been reared in TR from fry. My findings largely supported this hypothesis. Briefly, I found that within 7 days, both in fish acclimated to TR and in those transferred to TR, plasma osmolality varied directly with external salinity. When fish were transferred from steady-state SW to FW, and from FW to SW, PRL levels increased and decreased within 7 days, respectively; PRL levels, however, did not change with salinity in TR fish. Branchial expression of PRLR2 increased with salinity, and remained similar to SW control levels in both TR-acclimated fish and fish transferred to TR. Also similar to levels observed in SW control fish were branchial expression of NCC, NKCC1a, NKAα1b, NKAα1b and CFTR, both in fish that were acclimated to TR and in those transferred to TR, whereas in these same fish expression of AQP3 mRNA in gill was intermediate to FW and SW controls. Together, these findings indicate that fish reared in FW and SW steady-states were capable of fully

acclimating to TR within 7 days. This capacity for rapidly acclimating to different salinities can be further exploited in the development of a TR rearing paradigm for aquaculture production, as growth rates in tilapia have been largely associated with salinity. In fact, a recent study has shown that fish reared in TR grew faster than fish reared in either FW or SW (Moorman et al., 2016). Along these lines, further investigation of the relationship between TR acclimation and growth, aimed at identifying whether enhanced growth can be induced in later life stages in tilapia reared in steady state salinities through exposure to the tidal environment, may benefit aquaculture of this widely farmed species.

In the second study (Chapter III), I hypothesized that the 24-hour profile in osmoregulatory parameters would reveal similar end-of-phase patterns in plasma parameters and branchial gene expression previously reported in fish reared in TR (Moorman et al., 2014), with mid-phase measurements intermediate to end-of-phase measurements, and possible differences in these parameters in fish sampled during the same phases but in light versus dark hours. Aside from being the first study to measure osmoregulatory end points in TR fish over multiple iterations of the tidal cycle spanning both the light and dark phases of the diurnal period, this study was also the first to characterize plasma PRL_{177} in TR-acclimated fish. Briefly, I found that as in a previous study (Moorman et al., 2014) and in Chapter II of this thesis, plasma osmolality and plasma PRL_{188} were largely uncoupled throughout the tidal cycle. PRL_{177} in plasma followed a similar pattern to PRL_{188}, but at reduced circulating levels, as expected based on previous reports (Borski, et al., 1992; Seale et al., 2012a; Yada et al., 1994). It was also notable that while plasma levels of PRLs remained steady, PRLR1 mRNA expression in gill increased with a fall in plasma osmolality and external salinity, which seems to support the notion that the effects of PRL in tidally reared fish may be modulated by regulation of PRL receptors, rather than changes in plasma PRL levels (see Moorman et al., 2014). Further supporting this idea, and in line with a previous report both showing increased PRLR2 expression in SW and a role for PRLR2 in increasing osmotolerance (Fiol et al., 2009), PRLR2 mRNA expression in gill increased with a rise in plasma osmolality and external salinity throughout the tidal cycle. As observed in Chapter II, in TR-acclimated fish, branchial expression of NCC, NKCC1a, and CFTR

was similar to that of SW control fish. While the elevated branchial NKAα1a expression in TR-acclimated fish over FW and SW controls in the current study is dissimilar to Moorman's findings (Moorman et al., 2014), the stability in expression across TR phases and the elevation over SW levels is similar. The lack of difference between branchial mRNA expression of NKAα1b in TR fish compared to FW and SW fish in this study contrasted with previously reported expression levels in TR-acclimated fish that were intermediate to FW and SW controls (Moorman et al., 2014). The reason for the lack of similarity in this parameter across the two studies is unclear, and is further complicated by the fact that my findings were also contrary to prior reports indicating elevation of branchial expression in response to an increase in external osmolality (Tipsmark et al., 2011). Branchial expression of AQP3 in TR fish in the current study, however, did align with results reported previously (Moorman et al., 2014), in which it was found that branchial expression of AQP3 was intermediate to expression in FW and SW controls, and closer to that of SW fish. In sum, the findings of this study largely support my hypothesis, as many of the patterns observed to occur over much of the diurnal period in tidal fish resembled those described previously in TR fish. However, while the nuances observed over 24 h throughout the iterations of the tidal cycle seem to be less closely tied with salinity than other patterns observed, it is not possible to conclude whether they are associated specifically with the light and dark phases of the diurnal period.

An important consideration of these studies is whether the observed changes in gene expression are reflective of protein abundance and distribution in the cell membrane. While in this study I have not measured protein levels, a recent study has provided evidence that protein levels of effectors of ion transport in gill are indeed related to changes observed in gene expression (Moorman et al., 2014). Immunohistochemistry results from this previous study showed that in FW, immunoreactivity for the NCC protein was highest whereas in SW, immunoreactivity for NKCC was prevalent, and CFTR was only observed in SW; these patterns were reflective of the gene expression levels observed. Further, the group found that ionocytes of tidal fish exhibited ionocytes that were intermediate to those of FW and SW fish, which was also generally similar to the patterns in gene expression (Moorman, et al., 2014). In my study, however, I did acquire detailed insight to the patterns of these changes in gene expression across the two

phases of the tidal cycle. Interestingly, while some genes appear to be expressed at intermediate levels across both the FW and SW phases of the tidal cycle and do not seem to fluctuate much with salinity, others are strongly responsive to salinity changes within the tidal cycle. For example, branchial expression of PRLR1, PRLR2, NKCC, NKAα1a did show fluctuation, whereas branchial expression of NCC, AQP3, CFTR and NKAα1b did not change as much with salinity across phases of tidal cycle, remaining closer to levels in their steady state counterparts. Overall, these patterns provide important insight to environmental regulation of genes.

In summary, both of the studies described in this thesis have provided novel insights to osmoregulation in Mozambique tilapia, by characterizing osmoregulatory parameters associated with acclimation to a recently-developed tidal rearing paradigm. The importance of this new paradigm is underscored by the fact that the native distribution of Mozambique tilapia encompasses estuarine regions of southeastern Africa (Trewavas, 1983). For populations of tilapia that inhabit estuarine areas, tidal fluctuations between FW and SW are not uncommon. In light of this distribution, it is surprising that few studies have addressed the physiology of this fish in rearing conditions that closely approximate their native habitat; most prior studies in Mozambique tilapia have focused on fish reared in FW or SW, or after exposure to one-way salinity transfer between the two. Hence, my studies contribute directly to physiologically defining a salinity regimen that most closely resembles the natural history of the Mozambique tilapia. As this fish is important both as a model organism for understanding acclimation to extreme environments and as a widely farmed finfish, my studies and any future studies employing this rearing paradigm will be instrumental in not only advancing our understanding of tilapiine physiology, but also in development of new rearing strategies aimed at enhancing the sustainability and yield of aquaculture production of this and other euryhaline species.

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