THE EFFECTS OF REARING MOZAMBIQUE TILAPIA IN A TIDALLY-CHANGING SALINITY ON OSMOREGULATION AND GROWTH

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ABSTRACT

The native distribution of Mozambique tilapia, *Oreochromis mossambicus*, is characterized by estuarine areas subject to salinity variations between fresh water (FW) and seawater (SW) with tidal frequency. This dissertation characterized the effects of rearing tilapia in a tidally-changing salinity environment on osmoregulation and growth.

Osmoregulation in the face of changing environmental salinity is largely mediated through the neuroendocrine system and involves the activation of ion uptake and extrusion mechanisms in osmoregulatory tissues. The immunohistochemistry results of this study revealed that tilapia reared in tidally-changing salinities maintain SW-type ionocytes in both the FW and SW phases of the tidal cycle. The study also indicated that tilapia reared in tidally-changing salinities can compensate for large changes in external salinity while maintaining PRL expression and FW ion transporters, pumps, and channels within a narrow range in both phases of the tidal cycle that are closer to the levels observed in SW-acclimated tilapia.

A second study characterized the differences in osmoregulatory capacity among Mozambique tilapia reared in FW, SW, or under a tidally-changing salinity by exposing them to abrupt changes in salinity. The results suggested that Mozambique tilapia reared in a tidally-changing salinity, a condition that more closely represents their natural history, gain an adaptive advantage compared with fish reared in FW when facing a hyperosmotic challenge.

Lastly, a third study focused on the effect of rearing tilapia in a tidally-changing salinity on growth and the growth hormone (GH)/ insulin-like growth factor (IGF) axis. This study indicated that rearing tilapia in a tidally-changing salinity does not cause endocrine parameters associated with growth to fluctuate between levels seen in FW or SW fish. The study also showed that tilapia reared in a tidally-changing salinity had enhanced growth rates and at some time points had significantly higher feed conversion rates than FW or SW fish.

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SYMBOLS AND ABBREVIATIONS

AQP3 = Aquaporin 3

BW = Brackish water

CFTR = Cystic fibrosis transmembrane conductance regulator

EF1α = Elongation Factor 1α

FCR = Feed conversion ratio

FW = Fresh water

GH = Growth hormone

GHR2 = Growth hormone receptor 2

IGF-I = Insulin-like growth factor-I

IGF-II = Insulin-like growth factor-II

IHC = Immunohistochemistry

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 = quantitative real-time PCR

RIA = Radioimmunoassay

RPD =Rostral pars distalis

SW = Seawater

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

The maintenance of salt and water balance is fundamental to life in complex organisms. Physical and chemical conditions must be maintained within a narrow range to maintain the proper structure and function of macromolecules. Aquatic organisms are faced with maintaining homeostasis in the presence of a strong and sometimes fluctuating osmotic gradient depending on their external environment. Two main strategies have been described through which aquatic organisms are able to maintain the stable intracellular environment that is necessary for proper cell function. Osmoconformers maintain cellular homeostasis by regulating the composition of ions as well as other osmolytes that are essential for maintaining optimal enzyme function even in extreme environments (Yancey, 2001). Osmoregulators, by contrast, maintain a nearly constant intracellular osmotic environment for the proper functioning of cellular biochemical reactions. In changing environments, osmoregulators may evolve enzyme systems that are optimized for specific physical/chemical conditions, rather than having to operate sub-optimally over a range of environmental osmotic concentrations. For this reason, many organisms invest considerable energy in controlling precisely the composition of both intracellular and extracellular fluids. In fish, for example, osmoregulation can consume a high proportion of available energy, ranging from 25 to 50% of total metabolic output (Bushnell and Brill, 1992; Boeuf and Payan, 2001).

Teleosts represent a highly successful group of vertebrates that have extensively colonized nearly all aquatic-habitats. Fewer than 5% of teleost species are truly euryhaline, meaning that they can maintain internal osmotic homeostasis in environmental salinities ranging from fresh water (FW) to seawater (SW). They provide excellent models for investigating various strategies employed to maintain salt and water balance in vertebrates. This dissertation focuses on a euryhaline cichlid, the Mozambique tilapia (*Oreochromis mossambicus*), which is native to estuaries and near-shore rivers from the lower Zambezi River to the southeast coast of South Africa (Trewevas, 1983). A detailed understanding of tilapiine species is important for both their importance as experimental models for studies related to osmoregulatory physiology and their importance as a world aquaculture resource.

Teleost Osmoregulation

The majority of vertebrates maintain an internal environment that is around one third the osmotic strength of SW. Teleosts maintain a similar internal osmotic strength regardless of whether they are in FW, SW, an intermediate of the two, or a fluctuating environment. There are two basic challenges that fish encounter based on the environment in which they are living. Teleost fishes inhabiting a freshwater (FW) environment are at constant risk of excessive hydration and electrolyte loss across body surfaces. To counteract this tendency, FW-acclimated fish actively take up ions across the gill and gut and eliminate excess water by producing dilute urine (Evans et al., 2005). By contrast, osmoregulation for teleosts in seawater (SW) is facilitated through the active extrusion of monovalent ions by the gill and the conservation of water through re-absorption by the gastrointestinal tract (Marshall, 2006). In euryhaline species, the gill, kidney, and gut are important osmoregulatory organs. Such organs possess the functional plasticity to efficiently direct ion and water transport processes to support life in both hypo-osmotic and hyper-osmotic environments.

Ionocytes in tilapia

Ionocytes in the gills are integral to maintaining ionic balance in fish and a change in salinity causes ionocytes to undergo rapid changes in functional morphology (Kaneko, 2008). Based on the distribution of ion transporters, four types of ionocytes have been identified in tilapia; FW-acclimated tilapia possess type I, II, and III, while SW-acclimated tilapia have type I and IV (Hiroi et al., 2005; Kaneko, 2008). Type II ionocytes are characterized by the presence of NCC in the apical membrane (Hiroi et al., 2008). An apical Na^+/H^+ exchanger 3 (NHE3), which plays a role in both osmoregulation and acid/base regulation, is found in type III ionocytes (Watanabe et al., 2008). All four types of ionocytes include Na^+/K^+ ATPase (NKA), which actively pumps ions across the basolateral membrane to establish an electrochemical gradient (Richards et al., 2003). Type IV ionocytes, which are present in SW tilapia, form multicellular complexes with

accessory cells and are responsible for ion extrusion; they are defined by the apical Cl^- channel, cystic fibrosis transmembrane conductance regulator (CFTR), and basolateral NKA and $Na^+/K^+/2Cl^-$ cotransporter 1a (NKCC1a) (Hiroi et al., 2005).

The Importance of Tilapia in Aquaculture

According to the United Nations Food and Agriculture Organization data for 2012, tilapias are geographically the most wide spread species for aquaculture production in the world with close to 140 countries and territories recorded for farming of tilapias. Aquaculture produced 60 million tons of food fish with an estimated value of US$119 billion (FAO, 2012). Of the fish produced, tilapia is ranked third after carp and salmon and in 2008 its production approached 2.8 million tons. Tilapiine species are well suited for aquaculture for many reasons. They thrive and spawn in captivity, exhibit high growth rates on low-protein diets, are amenable to handling and confinement stress, are resilient to a wide range of environmental conditions, including temperature and salinity, and show low susceptibility to diseases (Trewevas, 1983). Due to the increasing importance of tilapia in aquaculture it is important to understand how growth can be optimized to keep up with the ever increasing demand.

Regulation of Growth in Tilapia

Growth in tilapia is regulated through the coordinated actions of the GH/IGF-I axis which regulates cellular differentiation and proliferation. The GH/IGF-I axis can be affected by nutritional state, environmental factors, and other physiological factors. GH is a pituitary hormone that is central to regulating tissue growth (Bern, 1983). GH has been shown to both act directly on target tissue and by stimulating the synthesis and release of IGF-I. IGF-I can act as both an autocrine and paracrine hormone and plays a role in a variety of growth promoting actions in most tissues (Butler and Le Roith, 2001; Le Roith, 2003; Fan et al., 2009). The GH/IGF-I axis acts in all major physiological processes, including growth, development, osmoregulation, metabolism, immune function,

reproduction, and behavior (cf. Duan, 1998; Reinecke et al., 2005; McCormick and Bradshaw, 2006; Sakamoto and McCormick, 2006).

GH and IGFs act by binding to membrane receptors (Kajimura et al., 2004b; Pierce et al., 2007; Duan et al., 2010). Two GH receptors, GHR1 and GHR2, have been identified and are upregulated in tilapia hepatocytes by GH (Kajimura et al., 2004b; Pierce et al., 2007; Pierce et al., 2012). Further studies determined that GHR2 is the receptor for GH while GHR1 is a putative receptor for somatolactin, a teleost protein whose function has not been fully determined (Pierce et al., 2012).

Goals and Objectives

The two main objectives of this dissertation were to understand the mechanisms of osmoregulation and growth in Mozambique tilapia reared in a tidally-changing salinity regimen. I studied the expression of key ion transporters, pumps, and channels in the gills along with PRL and its receptors to address the osmoregulatory mechanisms. I also focused on gene expression and hormone levels of key elements of the GH/IGF axis to address the effect that rearing fish in a tidally-changing environment has on growth. The second chapter describes the effects of different rearing salinities on PRL expression and plasma levels, as well as on the expression of key ion transporters in branchial ionocytes. The third chapter compares the osmoregulatory physiology of fish reared in different salinity regimes after they were transferred to either FW or SW for 6 hours, 1 day, 2 days, or 7 days. The fourth chapter focuses on growth, feed conversion ratio (FCR), and endocrine parameters associated with growth in fish reared in FW, SW, and in tidally-changing salinities. Lastly, chapter V provides general conclusions, implications and future perspectives on the work described in this dissertation. Chapter II has been published in General and Comparative Endocrinology (Moorman et al., 2014), chapter III has been submitted for publication, and chapter IV is being prepared for publication.

CHAPTER II

The osmoregulatory effects of rearing Mozambique tilapia in a tidally-changing salinity

Abstract

In nature, Mozambique tilapia, *Oreochromis mossambicus*, inhabit estuarine waters subject to salinities that vary with tidal frequency between fresh water (FW) and seawater (SW). The neuroendocrine system plays a central role in regulating salt and water balance in the face of changing environmental salinity controlling appropriate ion uptake and extrusion mechanisms in osmoregulatory tissues. I compared plasma osmolality, plasma prolactin (PRL), pituitary PRL mRNA, and mRNA of branchial ion pumps, transporters, channels, and PRL receptors in tilapia reared in FW, SW, brackish water (BW) and in tidally-changing salinity which varied between FW (TF) and SW (TS) every six hours. Plasma PRL was higher in FW tilapia than in SW, BW, TF, and TS tilapia. Unlike tilapia reared in FW or SW, fish in tidally varying salinities showed no correlation between plasma osmolality and PRL. In FW fish, gene expression of PRL receptor 1 (PRLR1), Na^+/Cl^- cotransporter (NCC), aquaporin 3 (AQP3) and two isoforms of Na^+/K^+-ATPase (NKA α1a and NKA α1b) was higher than that of SW, BW or tidally-changing salinity fish. Gene expression of the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1a), and the cystic fibrosis transmembrane conductance regulator (CFTR) were higher in fish in SW, BW or a tidally-changing salinity than in FW fish. Immunocytochemistry revealed that ionocytes of fish in tidally-changing salinities resemble ionocytes of SW fish. This study indicated that tilapia reared in a tidally-changing salinity can compensate for large changes in external osmolality while maintaining osmoregulatory parameters within a narrow range closer to that observed in SW-acclimated fish.

1. Introduction

Osmoregulation is a fundamental prerequisite for life in complex organisms. Teleost fishes inhabiting a freshwater (FW) environment are at constant risk of excessive hydration and electrolyte loss across body surfaces. To counteract this tendency, FW-acclimated fish actively take up ions across the gill and gut and eliminate excess water by producing dilute urine (Evans et al., 2005). By contrast, osmoregulation for teleosts in seawater (SW) is facilitated through the active extrusion of monovalent ions by the gill and the conservation of water through re-absorption by the gastrointestinal tract (Marshall, 2006). In euryhaline species, the gill, kidney, and gut are important osmoregulatory organs. Such organs possess a functional plasticity that allows the efficient regulation of ion and water transport processes that support optimal physiology in both hypo-osmotic and hyper-osmotic environments.

The Mozambique tilapia (*Oreochromis mossambicus*) is a euryhaline teleost with a worldwide tropical distribution in estuaries and coastal rivers from the lower Zambezi River to the southeast coast of South Africa generally not more than a mile from the tidal ebb and flow (Trewevas, 1983). They tolerate external salinities ranging from FW to double-strength SW (Stickney, 1986; Fiess et al., 2007). Like other teleosts, Mozambique tilapia maintain plasma osmolality within a narrow physiological range, equivalent to about one-third the osmolality of SW. Previous work on osmoregulation in tilapia has focused on fish reared under steady-state salinity conditions (e.g. FW and SW) or following the one-way transfer of tilapia from FW to SW or vice-versa (Heijden et al., 1997; Seale et al., 2002; Seale et al., 2006b; Inokuchi et al., 2008; Inokuchi et al., 2009; Breves et al., 2010c; Breves et al., 2010d; Breves et al., 2010b; Breves et al., 2011; Velan et al., 2011; Seale et al., 2012b). While important for identifying factors involved in the adaptation to changes in salinity, rearing Mozambique tilapia under steady-state salinity conditions and subjecting them to one-way transfers, does not fully reflect how fish osmoregulate in salinities that vary with the tide. Until now, there has been no report comparing the osmoregulatory performance of tilapia acclimated to FW, SW, or brackish water (BW) with that of tilapia reared in a tidally-changing salinities.

The neuroendocrine system plays a central role in the homeostatic regulation of salt and water balance in vertebrates (cf. McCormick and Bradshaw, 2006). Studies have shown that prolactin (PRL) is essential for FW osmoregulation in euryhaline teleosts, acting on target tissues such as the gill, kidney, and gut to promote ion uptake and water extruding processes (cf. McCormick, 2001; Manzon, 2002; Seale et al., 2006b). Consistent with its osmoregulatory activity, PRL release in the Mozambique tilapia has been found to increase in response to physiologically relevant reductions in extracellular osmolality both *in vivo* and *in vitro* (Nagahama et al., 1975; Wigham et al., 1977). Two PRL receptors (PRLRs) have been identified in tilapia, PRLR1 and PRLR2 (Fiol et al., 2009). Branchial PRLR1 is up-regulated when tilapia are transferred from SW to FW and down-regulated after transfer from FW to SW (Breves et al., 2011). The function of PRLR2, however, is unclear inasmuch as it is up-regulated in SW fish when PRL levels are relatively low (Fiol et al., 2009; Breves et al., 2011; Seale et al., 2012b). In Mozambique tilapia acclimated to FW, prolactin has also been shown to up-regulate the Na^+/Cl^- cotransporter (NCC), a mediator of ion uptake localized in ionocytes (Breves et al., 2010b).

Ionocytes in the gills are integral to maintaining ionic balance in fish and a change in salinity causes ionocytes to undergo rapid changes in functional morphology (Kaneko, 2008). Based on the distribution of ion transporters, four types of ionocytes have been identified in tilapia; FW-acclimated tilapia possess type I, II, and III, while SW-acclimated tilapia have type I and IV (Hiroi et al., 2005; Kaneko, 2008). Type II ionocytes are characterized by the presence of NCC in the apical membrane (Hiroi et al., 2008). An apical Na^+/H^+ exchanger 3 (NHE3), which plays a role in both osmoregulation and acid/base regulation, is found in type III ionocytes (Watanabe et al., 2008). All four types of ionocytes include Na^+/K^+ ATPase (NKA), which actively pumps ions across the basolateral membrane to establish an electrochemical gradient (Richards et al., 2003). Type IV ionocytes, which are present in SW tilapia, form multicellular complexes with accessory cells and are responsible for ion extrusion; they are defined by the apical Cl^- channel, cystic fibrosis transmembrane conductance regulator (CFTR), and basolateral NKA and $Na^+/K^+/2Cl^-$ cotransporter 1a (NKCC1a) (Hiroi et al., 2005). Recently, two isoforms of NKA, NKA α1a and NKA α1b, have been found to be differentially

expressed in tilapia ionocytes as a function of acclimation salinity (Tipsmark et al., 2011). While gene expression of NKA α1a was augmented in the gills of tilapia transferred from SW to FW, NKA α1b, which had been previously established in salmonids as the SW isoform of NKA, was prevalent in the gills of tilapia transferred from FW to SW (McCormick et al., 2009).

A previous study of the euryhaline killifish, *Fundulus heteroclitus*, found that the branchial transepithelial potential in fish subjected to tidally-changing salinities was similar to that of SW-acclimated fish. The branchial transepithelial potential is a measurement of the charge produced by the movement of ions into or out of the cell. In both SW and under changing salinities gills of fish showed positive values which suggest that this euryhaline fish adaptively favors ion extrusion (Wood and Grosell, 2009). To determine whether euryhaline tilapia reared under tidally-changing salinities exhibit osmoregulatory mechanisms that are similar to those found in fish acclimated to FW, BW or SW, I examined pituitary and branchial endpoints of endocrine and ion transport. Specifically, I characterized plasma PRL levels; pituitary expression of PRL and PRLRs; branchial expression of ion pumps, channels, transporters, and PRLRs; and morphological localization of transporters, pumps, and channels in ionocytes of tilapia reared under a tidally-changing environment, as compared with tilapia reared in FW, SW, or BW.

2. Materials and Methods

2.1 Fish Rearing

Mozambique tilapia (*Oreochromis mossambicus*) yolk-sac fry were collected from broodstock tanks maintained in FW at the Hawaii Institute of Marine Biology (Kaneohe, HI). The fry were kept in 75 L glass aquaria supplied with circulating FW until yolk-sac absorption was complete. Fry were then combined into one 75 L aquarium containing FW. Two days after yolk-sac absorption they were distributed into five 75 L glass aquaria supplied with FW (3 L/min) and stocked at a density of 100 fry per tank (mean weight, 12±1 mg, was not significantly different between the two replicate

experiments). Water temperature was maintained at 25±1°C in all tanks. The fish were exposed to a 12L:12D cycle. Fish were fed crushed Silver Cup Flake food (Silver Cup, Harrietta, MI) *ad libitum* daily. After two days in FW, four of the five tanks were transitioned from FW to BW over the course of 3 hours (10 ‰), composed of sea water (SW; 35 ‰ Kaneohe Bay, HI) diluted with FW. After an additional two days, one of the BW tanks was transitioned to SW, two other tanks were maintained under a tidally-changing salinity regime, and the fourth BW tank was kept in a BW condition. As a result, the salinity in five tanks was adjusted to FW, SW, BW, and two were maintained in a tidally-changing salinity.

Salinity in the tanks subjected to the tidally-changing salinity alternated between FW and SW every 6 h yielding a complete salinity transfer within 1.5 h. FW and SW flowing to the tanks with a tidally-changing salinity were controlled by two magnetic drive utility pumps (Pondmaster, Islandia, NY) that were connected to two electric timer outlets (Sylvania, Danvers, MA). The FW and SW pumps were set to switch every 6 h and were set 6 h out of phase. The fish were maintained in these conditions until sampling. Forty fish from each tank were sampled after four months. Fish reared in a tidally-changing salinity were sampled at the end of the FW and SW phases of the cycle. The experiment was replicated by employing different tanks, whose treatments were set up in a different order. 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-phenoxyethenol (0.3 ml/L). Blood was collected with a needle and syringe coated with sodium heparin (200 U/ml, Sigma-Aldrich, St. Louis, MO). Plasma was separated by centrifugation and stored at −80°C until later analyses. Fish were rapidly decapitated and the pituitary was removed. Filaments from the second gill arch on the left side of the fish were harvested. Pituitary and gill samples were frozen in liquid nitrogen and stored at −80°C prior to RNA extraction. An additional gill arch was collected and fixed in 4% paraformaldehyde

(PFA) in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C, then stored in 70% ethanol for immunohistochemistry.

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 High Capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA), 500 ng of total RNA was reverse transcribed into cDNA. The 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 the standard curve generated from serial dilutions of cDNA transcribed from FW gill mRNA. Elongation factor 1α (EF1α) 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. Primers for CFTR (forward: 5′-CATGCTCTTCACCGTGTTCT-3′, reverse: 5′-GCCACAATAATGCCAATCTG-3′) were designed using Primer3 (Untergasser et al., 2012). The amplification efficiency of CFTR primer pair was 105%, and the amplification of single product was confirmed by melt curve. All other primer pairs have been previously described: NCC (Inokuchi et al., 2008), NKCC1a (Inokuchi et al., 2008), NKA α1a (Tipsmark et al., 2011), NKA α1b (Tipsmark et al., 2011), EF1α (Breves et al., 2010a), PRLR1 (Pierce et al., 2007), PRLR2 (Breves et al., 2010b), AQP3 (Watanabe et al., 2005), NHE3 (Inokuchi et al., 2008), and PRL_{188} (Magdeldin et al., 2007).

2.4 Plasma Parameters

Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5100C; Wescor, Logan, UT). Two forms of PRL; PRL_{177} and PRL_{188}, are produced in the

rostral pars distalis (RPD) of the tilapia pituitary. While PRL_{177} and PRL_{188} release from cultured RPDs or dispersed PRL cells have shown similar trends in response to changes in osmolality, the response of PRL_{188} is more robust (Seale et al., 2012b). For this reason only plasma PRL_{188}, referred to as PRL in this study, was measured. Determination of plasma PRL was carried out by homologous radioimmunoassay (RIA) as described by Ayson and colleagues (Ayson et al., 1993).

2.5 Whole-mount immunohistochemisty

For detection of NKA-immunoreactive ionocytes by immunohistochemisry, I used a rabbit polyclonal antiserum raised against a synthetic peptide corresponding to part of the highly conserved region of the NKA α-subunit (NAK121) (Uchida et al., 2000). To detect NKCC1a and NCC, I employed a mouse monoclonal T4 antibody (T4; developed by Christian Lytle and Bliss Forbush III, and obtained from the Developmental Studies Hybridoma Bank, IA). While T4 was directed against 310 amino acids at the C terminus of human colonic NKCC, the antibody has been shown to react with NCC in the apical region of FW tilapia ionocytes (Hiroi et al., 2005; Hwang and Lee, 2007; Hiroi et al., 2008; Inokuchi et al., 2008). Although the T4 antibody binds to both NCC and NKCC1a, it is possible to distinguish between both proteins based on the localization of the signal in ionocytes; while a basolateral T4 signal is associated with NKCC1a, an apical signal reflects the presence NCC (Hiroi et al. 2005; Inokuchi et al. 2008). Branchial CFTR was detected using anti-human CFTR antibody raised in mice (R&D systems, Minneapolis, MN), which has been previously employed in tilapia research (Hiroi et al., 2008).

Preparation of filaments for IHC followed previously described methods (Breves et al., 2010b). Briefly, fixed gill filaments were rehydrated in 0.01 mol/L phosphate-buffered saline containing 0.2% Triton X-100 (PBST) for 1 h, and incubated overnight at room temperature with the mixture of anti-NKA, T4 antibody and anti-CFTR diluted 1:1000, 1:1000 and 1:500, respectively, with PBST containing 10% normal goat serum, 0.1% bovine serum albumin, 0.02% keyhole limpet hemocyanin, and 0.01% sodium azide (NB-PBS). The samples were incubated overnight at room temperature with a mixture of goat anti-rabbit IgG labeled with Alexa Fluor 488, goat anti-mouse IgG2a

labeled with Alexa Fluor 555, and goat anti-mouse IgG1 labeled with Alexa Fluor 647 (Molecular Probes/ Life Technologies, Carlsbad, CA), all diluted 1:500 with NB-PBS. Samples were observed with a confocal laser scanning microscope LSM 710 (Zeiss, Jena, Germany) with excitation at 488, 561, and 633 nm, and emission collected with bandpass filters of 493-551, 563-640, and 638-755 nm.

2.6 Statistical Analysis

Statistical analyses were conducted using a one-way analysis of variance (ANOVA) with salinity regime as the independent variable. Significant main effects of salinity regime ($P<0.05$) were followed up by the Bonferroni multiple comparison test. Data are expressed as means ± S.E.M. Where applicable, individual values were log-transformed to meet assumptions of normality and equal variance. Each replicate experiment was analyzed individually and after determining that the results were consistent between the two experiments the data were combined. Statistical calculations were performed using a statistical software program, Prism 5.0 (GraphPad, La Jolla, CA).

3. Results

3.1 Plasma parameters

Plasma osmolality in tilapia reared in tidally-changing salinities varied according to the salinity of the cycle. Fish that were sampled in the SW phase of the tidal cycle (TS) had plasma osmolalities ranging between 340 and 345 mOsmolal, similar to those found in fish fully adapted to constant SW conditions. By contrast, plasma osmolality observed after a one-way transfer between FW to SW rises above 400 mOsmolal by 6 h (Wang et al., 2009; Breves et al., 2010c; Seale et al., 2012b). On the other hand, fish sampled in the FW phase of the tidal cycle (TF) had plasma osmolalities ranging between 318 and 325 mOsmolal, values that are similar to those in fish held in FW and BW (Fig. 1A). Plasma PRL levels did not differ between fish sampled in the two phases of the tidal cycle and were similar to those of fish kept in SW and BW. In contrast, PRL levels of FW fish were

higher than in any other group and were 2-fold greater than those sampled in TF fish (Fig. 1B).

3.2 Pituitary gene expression of PRL and its receptors

Pituitary PRL mRNA expression was 10-fold greater in FW-acclimated tilapia compared with that in SW fish. Fish in TF, TS, and BW had 2-fold greater PRL mRNA expression than fish in SW (Fig. 2A). Pituitary PRLR1 gene expression did not differ among FW, SW, BW, and TF fish; TS fish, however, had significantly higher mRNA expression of PRLR1 (Fig. 2B). Pituitary PRLR2 mRNA expression was higher in SW fish than in FW fish; similarly, the expression of PRLR2 mRNA was enhanced in TS fish relative to TF fish, but to a greater extent than between FW and SW fish. PRLR2 mRNA in BW-acclimated fish was intermediate between SW and TS fish (Fig. 2C).

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

Branchial PRLR1 mRNA expression was significantly higher in FW-acclimated fish than in SW and TS fish, and was intermediate to them in BW and TF fish. The difference in branchial PRLR1 mRNA expression between FW and SW fish was 2-fold greater than the difference between its expression in TF and TS fish (Fig. 3A). Conversely, branchial PRLR2 expression in tilapia reared in a tidally-changing salinity showed a pattern opposite to that of PRLR1 in that TS fish had higher expression of PRLR2 than did TF fish. Furthermore, the difference in PRLR2 expression between TF and TS fish was greater than the difference in its expression between FW and SW-acclimated fish (Fig. 3B). I observed that branchial NCC mRNA expression was 500-fold higher in FW fish relative to that in SW fish, which is consistent with tilapia's osmoregulatory requirements in FW (Breves et al., 2010b). By comparison, NCC mRNA in fish reared in a tidally-changing salinity differed only 3–fold between TF and TS; in both of these groups, NCC mRNA was maintained significantly higher than in SW fish (Fig. 3C). Branchial NKCC1a expression was lowest in FW fish, intermediate in SW,

BW, and TF fish and highest in TS fish (Fig. 3D). NHE3 mRNA expression was 2-fold greater in tilapia acclimated to FW than those acclimated to SW; there were no differences in NHE3 expression among TF, TS, and BW fish which had intermediate expression levels (Fig. 3E). The pattern of CFTR mRNA expression was quite similar to that of NKCC1a (Fig. 3F). The expression of NKA α1a was 10-fold greater in FW fish relative to that observed in SW-acclimated fish, whereas no difference was observed among TF, TS, and BW fish which had intermediate expression of their mRNA respectively (Fig. 3G). Branchial NKA α1b mRNA expression was lowest in SW-acclimated fish and highest in FW-acclimated fish. Under a tidally-changing salinity condition, NKA α1b mRNA expression was higher in TF fish than TS fish, but both were intermediate to mRNA expression in FW and SW fish (Fig. 3H). Branchial mRNA expression of AQP3 was 50-fold greater in FW fish than that in SW fish. Fish reared in BW and a tidally-changing salinity showed higher expression of AQP3 mRNA than SW fish, while the expression in BW and TF fish was maintained lower than in TS fish (Fig. 3I)

3.4 Immunohistochemistry

Na^+/K^+-ATPase immunoreactive cells were prevalent in branchial ionocytes of fish reared under all salinity regimes (Fig. 4A, E, I, M, and Q). Within the NKA immunoreactive cells, NCC immunoreactivity was present in the FW fish, but was observed only in a few cells in the TF fish, and was not apparent in those of SW, BW, and TS fish (Fig. 4B, F, J, N, and R). NKCC1a immunoreactive cells were present in SW, BW, TF, and TS fish, but not in FW fish (Fig. 4B, F, J, N, and R). CFTR immunoreactive cells were present in SW, BW, and TS fish, but only in a few cells in the TF fish, and were not present in the FW fish (Fig. 4C, G, K, O, and S). Merged images of ionocytes in each salinity regimen show the co-localization of NKA and NCC in cells of FW fish, NKA, NKCC1a and CFTR in cells from SW, BW, and TS fish and NKA and NKCC1a in cells from TF fish (Fig. 4D, H, L, P, and T).

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), brackish water (BW), 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 = 45-60). Means not sharing the same letter are significantly different (one-way ANOVA, Bonferroni multiple comparison test, $P < 0.05$).

Figure 1

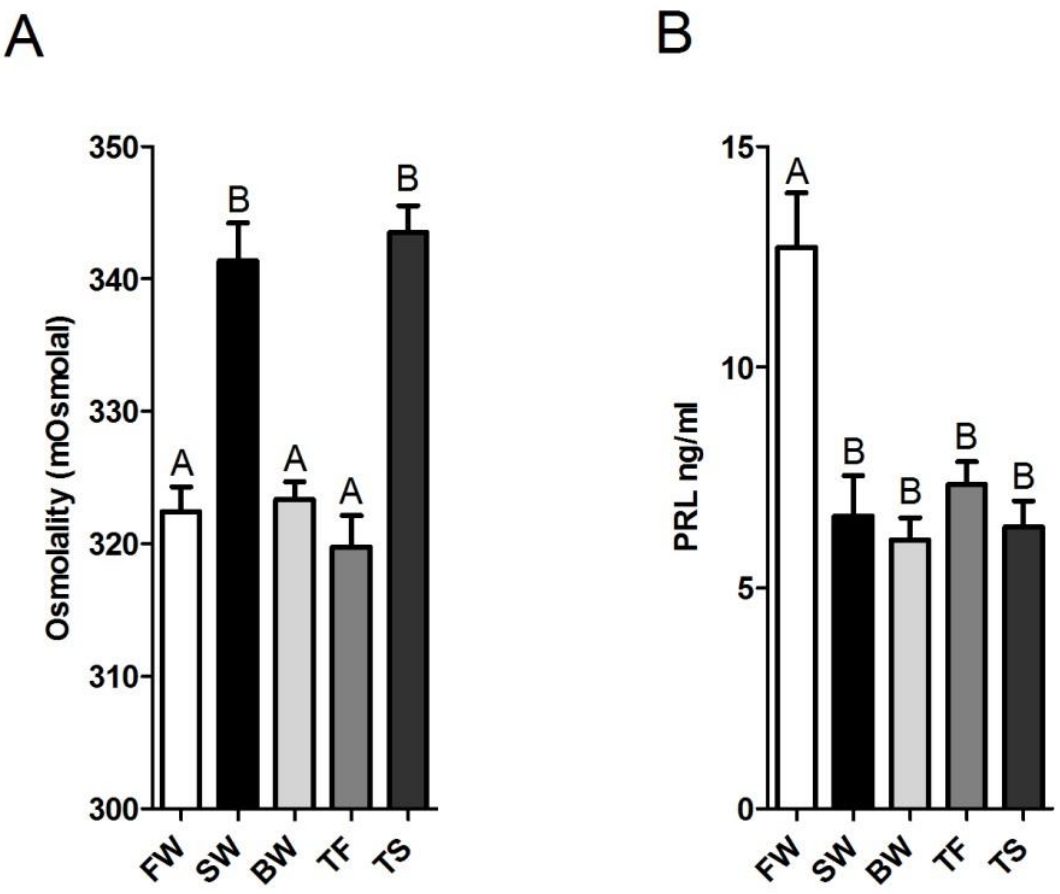

A
B
Osmolality (mOsmolal)
350
340
330
320
310
300
A
B
A
A
B
FW
SW
BW
TF
TS
PRL ng/ml
15
10
5
0
A
B
B
B
B
FW
SW
BW
TF
TS

Fig. 2. Effects of rearing condition on pituitary gene expression of prolactin (A), prolactin receptor 1(PRLR1) (B), and prolactin receptor 2 (PRLR2) (C) in Mozambique tilapia (*Oreochromis mossambicus*) sampled in fresh water (FW), seawater (SW), brackish water (BW), 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 = 35-40). Means not sharing the same letter are significantly different (one-way ANOVA, followed by Bonferroni multiple comparison test, $P < 0.05$).

Figure 2

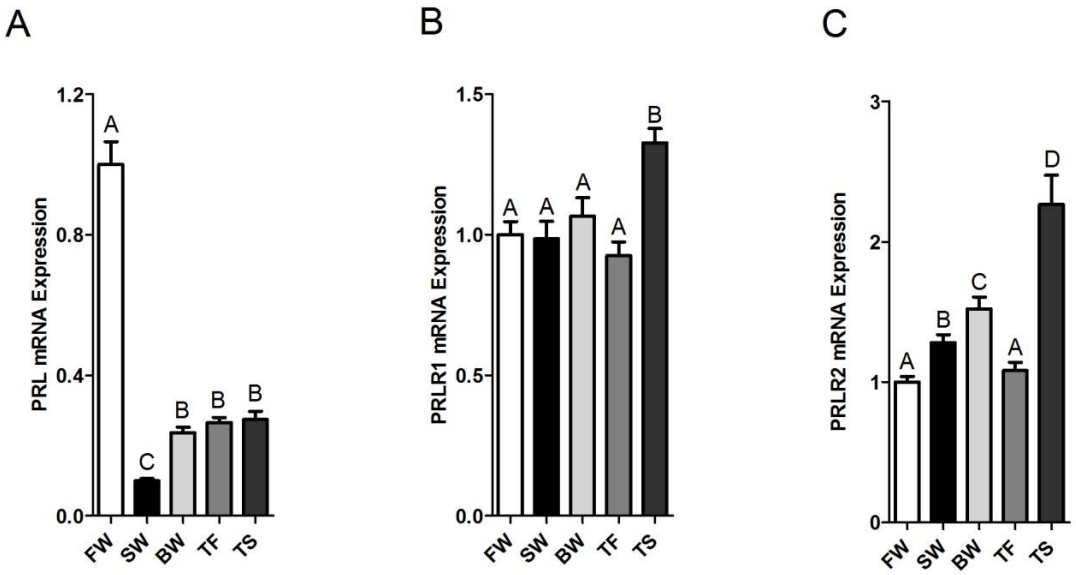

A
B
C
PRL mRNA Expression
PRLR1 mRNA Expression
PRLR2 mRNA Expression
FW
SW
BW
TF
TS

Fig. 3. Effects of rearing condition on branchial gene expression of PRLR1 (A), PRLR2 (B), NCC (C), NKCC1a (D), NHE3 (E), CFTR (F), NKA α1a (G), NKA α1b (H), AQP3 (I) in Mozambique tilapia (*Oreochromis mossambicus*) sampled in fresh water (FW), seawater (SW), brackish water (BW), 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 = 60-70). Means not sharing the same letter are significantly different (one-way ANOVA, followed by Bonferroni multiple comparison test, $P < 0.05$).

Figure 3

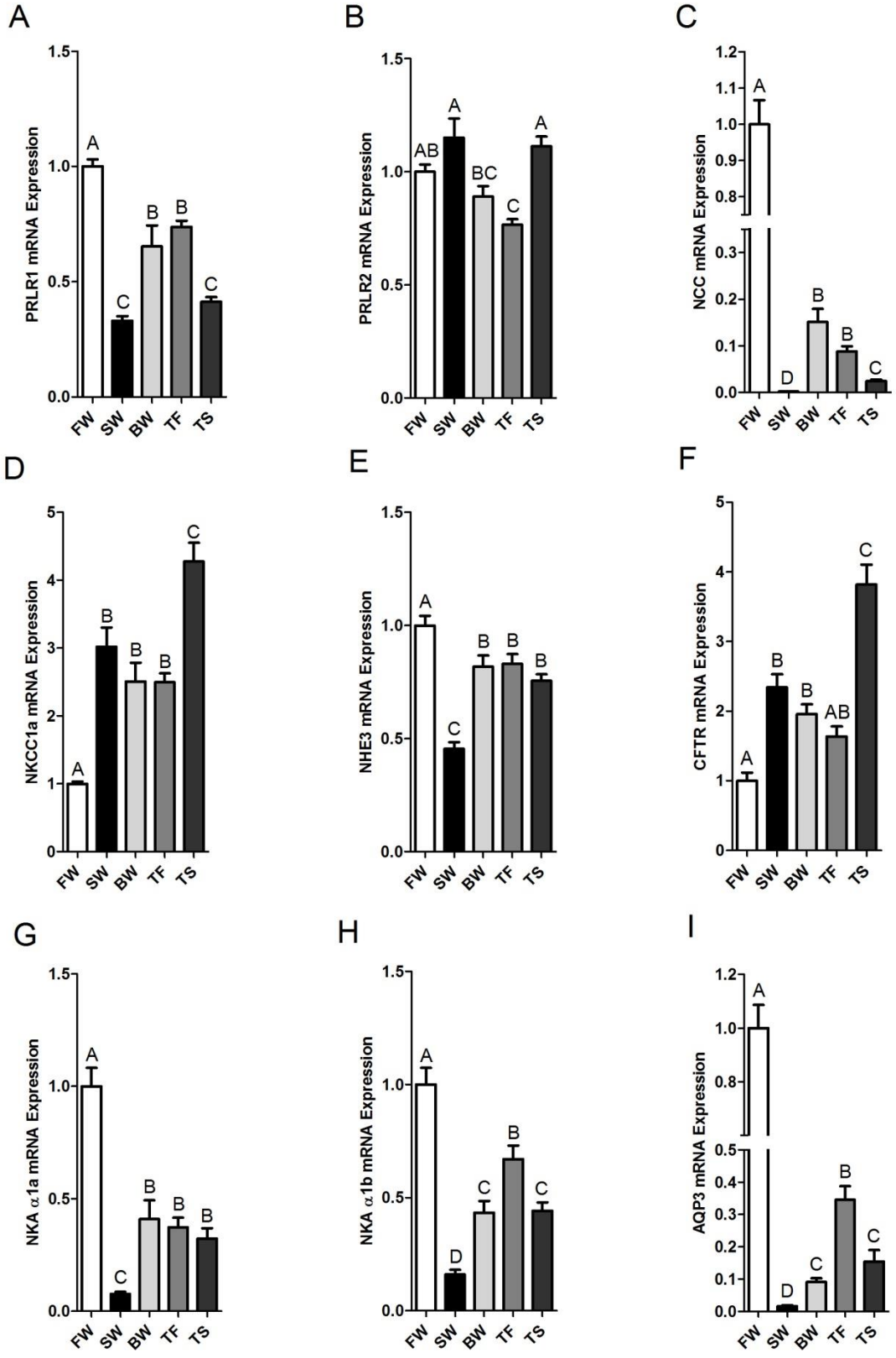
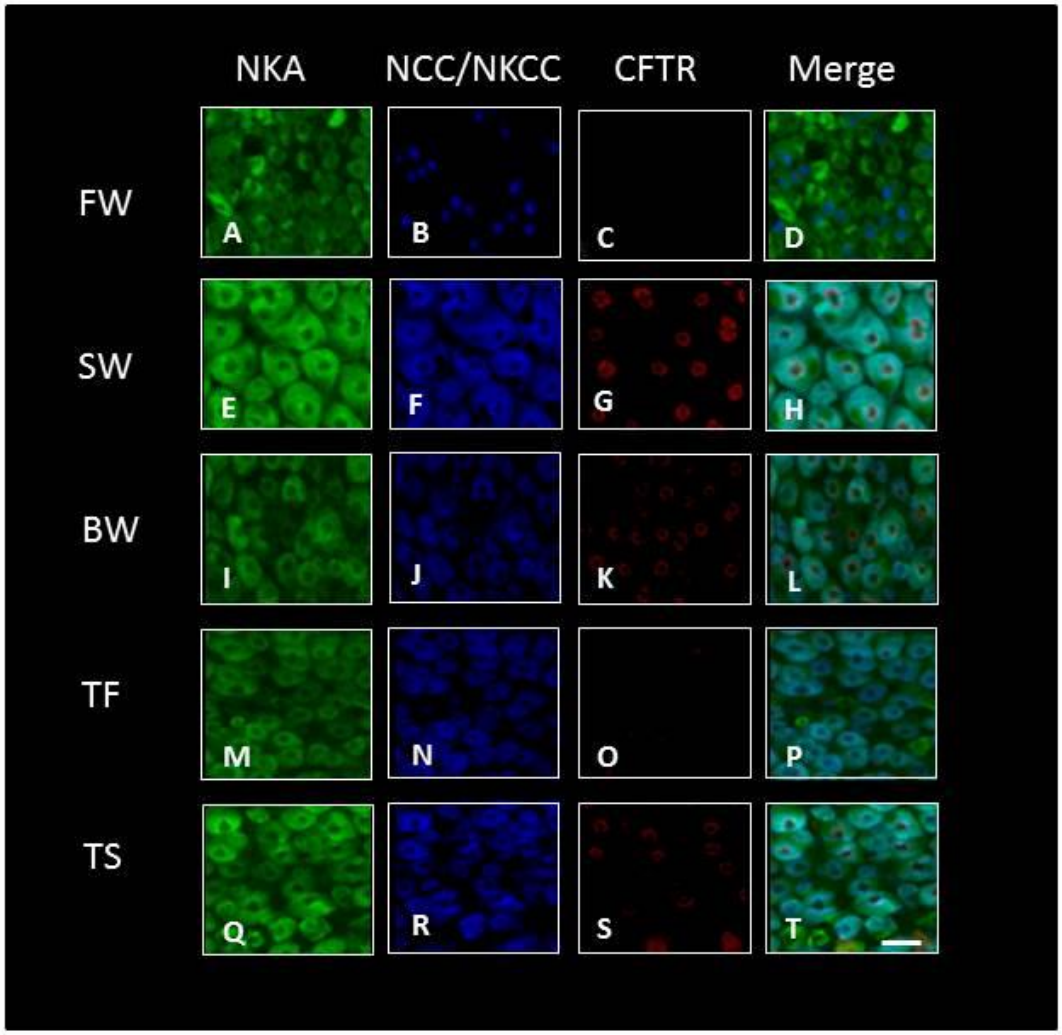

A
PRLR1 mRNA Expression
1.5
1.0
0.5
0.0
A
C
B
B
C
FW SW BW TF TS
B
PRLR2 mRNA Expression
1.5
1.0
0.5
0.0
AB
A
BC
C
A
FW SW BW TF TS
C
NCC mRNA Expression
1.2
1.1
1.0
0.9
0.8
0.3
0.2
0.1
0.0
A
D
B
B
C
FW SW BW TF TS
D
NKCC1a mRNA Expression
5
4
3
2
1
0
A
B
B
B
C
FW SW BW TF TS
E
NHE3 mRNA Expression
1.5
1.0
0.5
0.0
A
C
B
B
B
FW SW BW TF TS
F
CFTR mRNA Expression
5
4
3
2
1
0
A
B
B
AB
C
FW SW BW TF TS
G
NKA α1a mRNA Expression
1.5
1.0
0.5
0.0
A
C
B
B
B
FW SW BW TF TS
H
NKA α1b mRNA Expression
1.5
1.0
0.5
0.0
A
D
C
B
C
FW SW BW TF TS
I
AQP3 mRNA Expression
1.2
1.0
0.8
0.5
0.4
0.3
0.2
0.1
0.0
A
D
C
B
C
FW SW BW TF TS

Fig. 4. Triple immunofluorescence staining with anti-Na^+/K^+-ATPase (green), anti-NCC/NKCC1a (blue), and anti- CFTR (red) in gill filaments of Mozambique tilapia (*Oreochromis mossambicus*) sampled in fresh water (FW;A-D), seawater (SW; E-H), brackish water (BW; I-L), at the end of the FW phase of the tidal cycle (TF; M-P), and at the end of the SW phase of the tidal cycle (TS; Q-T). D,H,L,P,T Merged images. Scale bar, 20 μm.

Figure 4:



4. Discussion

The purpose of this study was to characterize differences in the activity of various elements of the osmoregulatory machinery of Mozambique tilapia raised in a tidal environment compared with activity in fish raised in either FW, SW or BW. I reared euryhaline tilapia in a tidally-changing environment to assess endocrine and osmoregulatory output in an experimental paradigm that models their natural environment. Our major findings were: 1) plasma PRL levels do not vary in concert with changes in plasma osmolality in fish reared in a tidal regimen; 2) tidal fish maintain gene expression of proteins involved in ion uptake at higher levels than steady-state SW fish, but much lower than those of FW fish, whereas the transcripts of ion extrusion-promoting molecules were found to be at levels which were similar to those of SW fish; and 3) in both phases of the tidal cycle, ionocytes in the gill of tidally-reared fish most closely resemble the ionocytes of SW fish. To our knowledge, this is the first time that these fish have been studied under experimental conditions that model their typical native environment, and therefore, more accurately reflect their physiology under the attendant conditions of changing salinity.

Previous experiments with Mozambique tilapia have demonstrated that upon transfer to SW, tilapia either reared in FW or held in FW for prolonged periods, exhibit temporary increases in plasma osmolality to levels well beyond those observed in SW-acclimated fish. Acute transfer of FW fish to SW results in osmolalities above 400 mOsmolal within 6 h, followed by a return to levels seen in SW-acclimated fish after 7 days (Wang et al., 2009; Breves et al., 2010c; Seale et al., 2012b). When SW-reared fish are transferred to FW, plasma osmolality falls to 300 mOsmolal or lower (Breves et al., 2010d; Breves et al., 2011; Seale et al., 2012b). Inasmuch as tidal changes in salinities impart osmotic challenges of the same level as those that occur in a one-way SW to FW transfer, or vice-versa, it was hypothesized that the plasma osmolality of fish reared in a tidally-changing salinity would vary as seen in previous salinity transfer experiments. Instead, our results showed that the difference in plasma osmolality in TF and TS fish was not greater than the small difference in osmolality between steady-state FW and SW fish. Specifically, I did not observe the dramatically large increase in plasma osmolality

that occurs when FW-reared fish are transferred to SW. This suggests that tidal fish are physiologically prepared to adapt to salinity changes in a manner that steady-state FW or SW fish subjected to one-way changes in salinity are not.

PRL has been shown to be vital for FW osmoregulation in euryhaline teleosts examined (cf. Manzon, 2002). Consistent with its role in maintaining ion balance, PRL reduces water permeability and increases ion uptake, at least in part, by upregulating NCC mRNA expression in the gills (Breves et al., 2010b). It has been shown that the release of PRL from the pituitary is directly regulated by decreases in extracellular osmolality that accompany FW acclimation (Wigham et al., 1977; Grau et al., 1981; Helms et al., 1991; Borski et al., 1992; Seale et al., 2002; Seale et al., 2006b; Seale et al., 2012b; Seale et al., 2012a). In addition, circulating levels of PRL are higher in FW tilapia than SW tilapia (Yada et al., 1994; Seale et al., 2002; Seale et al., 2006b). When tilapia are moved from FW to SW, plasma PRL levels decrease; conversely, tilapia moved from SW to FW exhibit rapid increases in plasma PRL (Yada et al., 1994; Seale et al., 2002; Seale et al., 2006b; Seale et al., 2012b). Our measurements of the steady-state plasma PRL levels and pituitary PRL gene expression in FW and SW are consistent with those of previous studies, showing an inverse relationship between PRL and plasma osmolality. By contrast, plasma PRL levels in both TF and TS fish did not change with plasma osmolality or with environmental salinity and were similar to the range observed in SW fish. Pituitary mRNA expression of PRL in TF, TS and BW fish, on the other hand, was 2.5-fold greater than in SW fish and pituitary mRNA expression of PRL in FW fish was 10-fold greater than in SW fish. These results suggest that, *in vivo*, high levels of PRL in circulation may only be triggered by abrupt declines in plasma osmolality that extend below the 320-340 mOsmolal range. Due to the more intermediate nature of being reared in BW or in a tidally-changing salinity, I hypothesize that the maintenance of osmotic homeostasis does not require fish in these environments to elevate PRL levels to the degree that is required by fish reared in FW. Nevertheless, the moderately elevated level of PRL mRNA expression in fish reared in tidally-changing salinities and in BW may provide a pre-adaptive advantage for these fish that is not required by fish living solely in SW.

I also observed an effect of rearing salinity on PRLR1 and PRLR2 expression in both the pituitary and gills. It is worth noting that the response of PRLR1 was not the same between these tissues. As with previous studies, PRLR1 mRNA was more highly expressed in the gills of FW tilapia than in SW fish (Fiol et al., 2009). This pattern of salinity-dependent gene expression was also observed in TF and TS fish, indicating that in cycling salinity conditions the actions of PRL may be augmented by enhanced receptor availability rather than by greater abundance in circulation. Consistent with previous results, pituitary gene expression of PRLR1 did not differ between FW- and SW-acclimated tilapia (Seale et al., 2012b). In contrast with the pattern of branchial expression seen in fish reared in a tidally-changing salinity, pituitary PRLR1 expression was higher in TS than in TF, indicating that PRL's effects may be differentially regulated by environmental salinity at the tissue level. The reason for this difference is unclear but it suggests that in TS inhibitory autocrine regulation of pituitary PRL synthesis might prevent circulating levels of PRL from rising.

Unlike PRLR1 mRNA, PRLR2 mRNA expression has been shown to increase in response to an increase in external salinity in Mozambique tilapia (Fiol et al., 2009; Seale et al., 2012a). This response has been suggested to confer an increased ability to adapt to a hyper-osmotic environment (Fiol et al., 2009). Previously, two variants were found for PRLR2; the functional long variant (55 kDa) and the short variant (35 kDa). Whereas the long variant is coupled to different signaling pathways from PRLR1, the short variant lacks extracellular domain and is not able to trigger any downstream responses. In a study by Fiol and co-workers western blot analysis revealed that only 35 kDa protein abundance was upregulated in the SW tilapia gill (Fiol et al., 2009). The short variant of PRLR2 may dimerize with the long variant to sequester the long variant and inhibit PRL binding (Fiol et al., 2009). The transient increases in branchial PRLR2 mRNA expression during the SW phase of the tidal cycle may contribute to the low reactivity of PRL due to its inability to bind to PRLR2 and elicit a downstream response.

To characterize differences in ionoregulatory mechanisms in the gills of Mozambique tilapia reared in different salinities, I examined gene expression of key ion transporting proteins previously characterized in this species. Consistent with their role in ion uptake and with the results of previous reports, I observed higher mRNA expression

of NCC, NHE3, and NKA α1a in FW tilapia than in SW tilapia (Watanabe et al., 2005; Hiroi et al., 2008; Inokuchi et al., 2008; Kaneko, 2008). Because of its role in water transport, the expression of AQP3 protein in basolateral membrane of ionocytes was also measured (Watanabe et al., 2005). Consistent with a previous study, I found that branchial NKA α1a mRNA expression was higher in FW-acclimated tilapia when compared with that in SW-acclimated tilapia (Tipsmark et al, 2011). The results for NKA α1b, however, were inconsistent with a study by Tipsmark and co-workers which reported higher NKA α1b mRNA expression in SW-fish when compared with FW-fish. The reason for this discrepancy may be due to differences in methodology employed between the studies. While in the current experiment both FW and SW fish were sampled concurrently and the NKA α1b qRT-PCR assay was run together, the conclusions of Tipsmark and co-workers were based on the results of two separate one-way transfer experiments. Those experiments showed NKA α1b mRNA expression increasing with a transfer from FW to SW and decreasing with a transfer from SW to FW. A study on black-chinned tilapia, *Sarotherodon melanotheron*, from West Africa also found branchial NKA mRNA expression to be higher in FW-acclimated fish compared with SW fish, although they did not distinguish between different NKA α1 isoforms (Tine et al., 2011). The authors of that study suggest that the salinity-dependent difference in branchial NKA mRNA expression may be related to the marine ancestral origin of black-chinned tilapia, which may have adapted mechanisms for minimal energetic requirements in SW. Likewise, our present results for NKA in Mozambique tilapia are consistent with the notion of a SW-adapted ancestral lineage.

Expression of PRLR1, NCC, NKA α1a, NKA α1b, and AQP3 mRNA in fish reared in a tidally-changing salinity was intermediate to those observed in fish reared solely in FW or SW. NCC mRNA expression in FW-acclimated tilapia was much higher than that in SW-acclimated tilapia (Breves et al., 2010c; Breves et al., 2010d; Breves et al., 2010b). The magnitude of the difference in branchial NCC mRNA expression between FW and SW fish is 15-fold greater than the difference in NCC mRNA expression between TF and TS fish. This suggests that tilapia can function in cycling salinities with relatively low NCC expression, but if kept in FW for an extended period,

will become increasingly reliant on NCC for proper maintenance of salt and water balance; these results closely parallel our observations of circulating PRL levels.

Aquaporin 3 showed the same pattern of gene expression as NCC, indicating that both ion uptake and water permeability, functions known to be affected by PRL, change proportionally with one another under the rearing salinity regimes tested (Manzon 2002). I also found that the gene expression of NKCC1a and CFTR, which are involved in ion extrusion, was higher in SW versus FW fish, consistent with previous studies using Mozambique tilapia (Hiroi et al., 2005; Breves et al., 2010d; Breves et al., 2010b; Breves et al., 2011). Most striking, however, is the observation that fish reared in a tidally-changing salinity change gene expression of NKCC1a and CFTR between the two phases of the tidal cycle. The higher expression of NKCC1a and CFTR in TS fish compared with that of TF fish, suggest that these genes are immediately necessary for survival when fish are in SW, at least over a 6 h interval. Our immunocytochemical results show that NKCC1a immunoreactivity was similar between TF and TS fish, at an intensity that was intermediary between FW and SW fish. By contrast, CFTR signals were stronger in TS than in TF fish. The change in CFTR immunoreactivity between both phases of the tidal cycle suggests that branchial CFTR is immediately important for SW adaptation and immediately detrimental if fish are maintained in FW.

Gene expression of NKA and a voltage-dependent anion channel (VDAC), a non-selective Cl^- channel in the outer mitochondrial membrane, was shown to be higher in black-chinned tilapia living in an environment characterized by salinity changes (Tine et al., 2011). It was suggested that these fish maintain higher mRNA levels in a changing environment so that they are prepared to quickly respond and adapt to shifts in salinity. The dynamic changes in gene transcripts encoding for proteins involved in branchial osmoregulation observed in the current experiment provide evidence that Mozambique tilapia experimentally subjected to a tidal regimen are also better prepared for salinity changes relative to fish kept in steady-state conditions.

The results of both gene expression measurements and immunohistochemistry indicate that fish reared in a tidally-changing salinity maintain SW-type ionocytes in both phases of the tidal cycle. This is consistent with the similarity in trans-epithelial potential between killifish, *Fundulus heteroclitus*, raised in SW and a tidal environment (Wood

and Grosell, 2009). Both TF and TS fish maintain ionocytes similar to that of SW fish and are adapted for ion extrusion, except for the dramatic decrease of CFTR in TF fish. A possible explanation for low CFTR immunoreactivity in TF is that the membrane trafficking of the CFTR protein is salinity dependent (Marshall and Singer, 2002). The low abundance of CFTR may explain, at least in part, why TF fish are able to endure low salinities even though they have very few FW-type ionocytes.

In summary, the rearing paradigm for Mozambique tilapia employed in this study experimentally simulated the conditions found in their common native habitat and provided a model to study the osmoregulatory physiology of euryhaline fish under naturally occurring dynamic salinity conditions. Mozambique tilapia reared under tidally-changing salinities were able to compensate for large changes in external osmolality while maintaining osmoregulatory parameters within a narrow range. Their endocrine and osmoregulatory responses, together with branchial ionocyte functional morphology, under changing salinities most closely resembled those of SW-acclimated fish. These findings are consistent with the notion that, over evolutionary history, the ancestral lineage of this species may have gradually added FW-tolerance to a constitutive background of SW adaptability. Future studies employing this experimental paradigm are likely to further reveal the mechanisms of physiological adaptation employed by euryhaline teleosts under their native environmental conditions.

CHAPTER III

The Effects of Acute Salinity Challenges on Osmoregulation in Mozambique Tilapia Reared in a Tidally-Changing Salinity

Abstract

This study characterizes the differences in osmoregulatory capacity among Mozambique tilapia, *Oreochromis mossambicus*, reared in fresh water (FW), seawater (SW), or under tidally-driven changes in salinity. This was addressed through the use of an abrupt exposure to a change in salinity. I measured changes in: 1) plasma osmolality and prolactin (PRL) levels; 2) pituitary expression of prolactin (PRL) and its receptors, PRLR1 and PRLR2; 3) branchial expression of PRLR1, PRLR2, Na^+/Cl^- cotransporter (NCC), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), α1a and α1b isoforms of Na^+/K^+-ATPase (NKA), cystic fibrosis transmembrane conductance regulator (CFTR), aquaporin 3 (AQP3) and Na^+/H^+ exchanger 3 (NHE3). Mozambique tilapia reared in a tidal environment successfully adapted to SW while fish reared in FW did not survive a transfer to SW beyond the six hour sampling. With the exception of CFTR, the change in the expression of ion pumps, transporters, and channels was more gradual in fish transferred from tidally-changing salinities to SW than in fish transferred from FW to SW. Upon transfer to SW, the increase in CFTR expression was more robust in tidal fish than in FW fish. Tidal and SW fish successfully adapted when transferred to FW. These results suggest that Mozambique tilapia reared in a tidally-changing salinity, a condition that more closely represents their natural history, gain an adaptive advantage compared with fish reared in FW when facing a hyperosmotic challenge.

1. Introduction

The rigorous control of osmotic homeostasis is essential to life for most vertebrates. Teleost fish in fresh water (FW) face the risk of excessive hydration and electrolyte loss across body surfaces. To counteract this tendency, FW-acclimated fish actively take up ions across the gill and gut and eliminate excess water by producing dilute urine (Evans et al., 2005). By contrast, teleost fishes in seawater (SW) must adapt to a dehydrating environment. Osmoregulation in SW is facilitated through the active extrusion of monovalent ions by the gill and the acquisition of water through re-absorption by the gastrointestinal tract (Marshall, 2006). Mozambique tilapia (*Oreochromis mossambicus*) tolerate external salinities ranging from FW to double-strength SW (Stickney, 1986; Uchida et al., 2000; Fiess et al., 2007). Their natural distribution includes estuaries and the lower reaches of rivers, not generally more than a mile from the ebb and flow of the tide, from the Zambezi River to the southeast coast of South Africa (Trewevas, 1983). As with other teleosts, Mozambique tilapia maintain their plasma osmolality within a narrow physiological range, 305-330 mOsmolal in FW and 335-360 mOsmolal in SW (Seale et al., 2006a).

The hypophyseal hormone, prolactin (PRL), plays an essential role in FW osmoregulation in euryhaline teleosts (Pickford and Phillips, 1959; Dharmamba et al., 1967; Bern, 1983; cf. Manzon, 2002). Consistent with its activity in FW, PRL release in Mozambique tilapia increases in response to physiologically relevant reductions in extracellular osmolality both *in vivo* and *in vitro* (Grau et al., 1981; Yada et al., 1994; Seale et al., 2002; Seale et al., 2006b; Seale et al., 2012c). Conversely, Mozambique tilapia transferred from FW to BW (22 ‰) or SW, showed a reduction in plasma levels of PRL within 6 h (Yada et al., 1994; Seale et al., 2012b). Prolactin is thought to act principally by stimulating ion uptake and reducing water intake across osmoregulatory epithelia, following binding to its receptors (PRLRs). The receptors, PRLR1 and PRLR2 are disparately regulated by extracellular osmolality (Breves et al., 2011; Fiol et al., 2009; Seale et al., 2012b). Branchial PRLR1 expression is induced when tilapia are transferred from SW to FW, in concert with the rise in PRL release from the pituitary. By contrast, PRLR2 expression is induced following transfer to SW. While the function of PRLR2 is

unclear, its downstream signaling pathway has been shown to be distinct from that of PRLR1 (Fiol et al, 2009).

The gill is a target for the actions of PRL and is regarded the primary site of net ion transport by the activities of highly specialized ionocytes (Kaneko, 2008; Hiroi and McCormick, 2012). Driven by the osmoregulatory requirements associated with euryhalinity, different ionocyte cell types undergo rapid differentiation when faced with a change in salinity. Cell numbers of both new and pre-existing ionocytes change when FW fish are moved to SW and vice versa, indicating that existing ionocytes are regularly replaced with newly-differentiated cells under constant or changing ambient salinity**.** In tilapia, a Na^+/Cl^- cotransporter (NCC) and a Na^+/H^+ exchanger (NHE3) are specifically expressed in the apical membrane of FW-type ionocytes while a Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR), and a $Na^+/K^+/2Cl^-$ cotransporter (NKCC) are expressed in the apical and basolateral membranes, respectively, of SW-type ionocytes (Hiroi et al., 2005; Inokuchi et al., 2008; Watanabe et al., 2008). The expression of NCC is directly regulated by PRL (Breves et al., 2010b). The electrochemical gradient that drives transmembrane ion transport is provided by the basolaterally-located ion pump, Na^+/K^+ ATPase (NKA) (Richards et al., 2003). In tilapia, salmonids, and killifish, two isoforms of NKA, α1a and α1b, are differentially expressed in gill, according to the salinity of the habitat (Richards et al., 2003; Madsen et al., 2009; McCormick et al., 2009; Tipsmark et al., 2011; Berdan and Fuller, 2012). In tilapia, NKAα1a expression predominates in FW-acclimated fish relative to SW- fish, while α1b expression has been shown to be greater or unchanged in SW-acclimated fish when compared with FW- fish (Tipsmark et al., 2011). Water transport in ionocytes of both FW- and SW- acclimated tilapia is mediated through basolaterally-located aquaporin 3 (AQP3) (Watanabe, 2005). Together with PRL expression and release, an examination of the expression patterns of genes that encode effectors of ion and water transport can provide a means for investigating how osmoregulatory pathways are governed in a particular environmental salinity rearing regime.

Extensive work has provided an understanding of how tilapia in FW and SW adapt to acute osmotic challenges (Dharmamba et al., 1967; Dharmamba et al., 1973; Ayson et al., 1993; Yada et al., 1994; Heijden et al., 1997; Sakamoto et al., 1997;

Shepherd et al., 1999; Seale et al., 2002; Seale et al., 2006b; Inokuchi et al., 2008; Inokuchi et al., 2009; Breves et al., 2010c; Breves et al., 2010d; Breves et al., 2010b; Breves et al., 2011; Velan et al., 2011; Seale et al., 2012b). While the effects of salinity change on PRL cells and branchial mediators of ion transport are well documented in tilapia subjected to one-way-transfers between two salinities, less is known about fish subjected to cyclic variations in salinity. Recently, I reared Mozambique tilapia in tidally-changing conditions to model some of the natural estuarine environments in which this species is found (Moorman et al., 2014). In spite of the changes in external salinity every 6 h, Mozambique tilapia reared under a tidal regimen maintained a constant plasma level of PRL whether in SW or FW (Moorman et al., 2014). In addition, I found that branchial expression of mediators of ion transport in fish reared in a tidally-changing salinity were intermediate to those of fish reared in FW or SW. To examine this osmoregulatory paradigm in greater depth, I hypothesized that, relative to fish reared in steady-state FW or SW, rearing fish in tidally-varying salinities would more readily facilitate subsequent osmotic adaptation to SW or FW respectively. In the current study, I tested this hypothesis by comparing the osmoregulatory responses among fish transferred from one steady-state salinity to another (i.e. FW to SW and vice-versa), with those in fish that were reared in tidally-changing salinities and then subsequently maintained in either FW or SW.

To examine the effects of rearing condition on the adaptability of Mozambique tilapia to acute salinity challenges, I measure a variety of osmoregulatory endpoints in the pituitary and gill. Specifically, I measured: 1) plasma osmolality and PRL levels; 2) pituitary expression of PRL, PRLR1 and PRLR2; and 3) branchial expression of PRLR1, PLR2, NCC, NKCC1a, NKA α1a, NKA α1b, CFTR, AQP3 and NHE3 in fish reared in FW, SW or tidally-changing salinities faced with acute salinity challenges.

2. Materials and Methods

2.1 Fish Rearing

Mozambique tilapia (*Oreochromis mossambicus*) yolk-sac fry were collected from broodstock tanks maintained in FW at the Hawaii Institute of Marine Biology (Kaneohe, HI, USA). The fry were kept in 75 l glass aquaria supplied with circulating FW until yolk-sac absorption was complete. Fry were then combined into one 75 l aquarium, containing FW. Two days after yolk-sac absorption they were distributed into eight 75 l glass aquaria supplied with FW (3 L/min) and stocked at a density of 100 fry per tank (mean weight, 12±1 mg, was not significantly different between the two replicate experiments). Water temperature was maintained at 25±1°C in all tanks. The fish were exposed to a 12L:12D cycle. Fish were fed crushed Silver Cup Flake food (Silver Cup, Harrietta, MI, USA) and fed *ad libitum* daily. After two days in FW, six of the eight tanks were transitioned from FW to BW (10 ‰) over the course of 3 h, composed of sea water (SW; 35 ‰ Kaneohe Bay, HI, USA) diluted with FW. After an additional two days, two of the BW tanks were transitioned to SW and the other four other tanks were maintained under a tidally-changing salinity. As a result, the salinity in eight tanks was adjusted as follows: two FW, two SW, and four in a tidally-changing salinity.

The rearing of tilapia in tidally-changing salinities has been recently described (Moorman et al., 2014). Briefly, tanks subjected to the tidally-changing salinity alternated between FW and SW every 6 h yielding a complete salinity transfer within 1.5 h. The fish were maintained in these conditions for four months. After four months the salinity of one of the FW tanks and one of the tidal tanks was switched to SW, and that of one SW tank and one tidal tank was switched to FW (Fig. 5). It took 1.5 h for a complete salinity transfer. Ten fish were sampled from each tank at 6 h, 24 h, 48 h, and 7 d after the transfer. TF fish and TS fish refer to fish sampled 30 minutes prior to the end of the FW and SW phases of the tidal cycle respectively. 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-phenoxyethenol (0.3 ml/L). Blood was collected with a needle and syringe coated with sodium heparin (200 U/ml, Sigma-Aldrich, St. Louis, MO, USA). Plasma was separated by centrifugation and stored at -80°C until later analyses. Fish were rapidly decapitated and the pituitary was removed. Filaments from the second gill arch on the left side of the fish were harvested. Pituitary and gill samples were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

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, USA) and then quantified with a Nano-Drop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), 500 ng of total RNA was reverse transcribed into cDNA. The quantitative real-time PCRs (qRT-PCR) were set up as previously described (Pierce et al., 2007). The mRNA levels of reference and target genes were determined by the relative quantification method as specified by StepOne Software v 2.0 (Life Technologies). Standard curves were generated from 5-fold serial dilutions of cDNA transcribed from FW pituitary mRNA for pituitary samples and FW gill mRNA for gill samples. The R^2 values and amplification efficiencies for standard curves were as follows, respectively: 0.993 and 98.2% (EF1α), 0.998 and 95.9% (PRL_{188}), 0.999 and 93.2% (PRLR1), 0.992 and 83.1% (PRLR2), 0.992 and 98.1% (NCC), 0.992 and 89.8% (NKCC1a), 0.997 and 103% (NKA α1a), 0.997 and 98.1% (NKA α1b), 0.984 and 105% (CFTR), 0.968 and 106% (AQP3), and 0.999 and 96.3% (NHE3). All primer pairs employed in this study have been previously described: NCC, NHE3, and NKCC1a (Inokuchi et al., 2008); NKA α1a and NKA α1b (Tipsmark et al., 2011); EF1α (Breves et al., 2010a); PRLR1 (Pierce et al., 2007); PRLR2 (Breves et al., 2010b); AQP3

(Watanabe et al., 2005); CFTR (Moorman et al., 2014); and PRL_{188} (Magdeldin et al., 2007). The PCR mixture (15 uL) contained 7.5 µl of 2X Power SYBR Green PCR Master Mix (Life Technologies), 200 nM of each primer, and 2 µl of cDNA. The following cycling parameters were employed: 2 min at 50°C, 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min using the StepOnePlus real-time PCR system (Life Technologies). The measured values of target genes were normalized to those of EF1α, which did not vary significantly across treatments (One-way ANOVA, $p > 0.05$). Data are expressed as fold-change from FW values.

2.4 Plasma Parameters

Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5100C, Logan, UT, USA). Two forms of PRL, PRL_{177} and PRL_{188} are produced in the *rostral pars distalis* (RPD) of the tilapia pituitary. While PRL_{177} and PRL_{188} release from cultured RPDs or dispersed PRL cells have shown a similar pattern of response following changes in osmolality, the response in PRL_{188} is more robust (Seale et al., 2012b). For this reason, only plasma PRL_{188}, referred to as PRL in this study, was measured. Determination of plasma PRL was carried out by homologous radioimmunoassay (RIA) as described by Ayson and colleagues (Ayson et al., 1993).

2.5 Statistical Analysis

Statistical analyses were conducted by two-way analysis of variance (ANOVA) with salinity rearing regime and time as the independent variables. Significant main effects of salinity rearing regime and time ($P<0.05$) were followed up by the Fisher LSD test. Data are expressed as means ± S.E.M. When necessary, individual values were log-transformed to meet assumptions of normality and equal variance. Each replicate experiment was analyzed individually and after determining that the results were consistent between the two experiments the data were combined. Statistical calculations were performed using a statistical software program, Prism 6.0 (GraphPad, La Jolla, CA, USA).

3. Results

3.1 Plasma parameters

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were observed on plasma osmolality. Plasma osmolality increased to 550 mOsmolal within 6 h when FW fish were transferred to SW and the fish did not survive to the 24 h sampling time. By contrast, the plasma osmolality of fish at the end of the FW phase of the tidal cycle (TF) transferred to SW increased only to 350 mOsmolal after 6 h and then decreased to steady-state SW levels within 24 h. When transferred to FW, the osmolality of fish reared in SW like that of tidally-reared fish at the end of the SW phase of the tidal cycle (TS) declined to 300 mOsmolal within 48 h and then returned to steady-state FW levels within 7 d (Fig. 6A). Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.01$) were observed on plasma PRL. Plasma PRL in FW fish transferred to SW declined from 15 ng/ml to 2 ng/ml within 6 h. Plasma PRL levels of TF fish transferred to SW, on the other hand, was not significantly different at any of the time points between 6 h and 7 d. When transferred to FW, plasma PRL in both SW- and TS-reared fish increased sharply by 6 h, but by 48 h, though still well above pre-transfer levels, had declined to levels that were similar to those of FW-reared fish (Fig. 6B).

3.2 Pituitary gene expression of PRL and its receptors

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were observed on pituitary PRL expression. Pituitary PRL expression in FW fish was 4-fold greater than that in TF and TS fish and was 10-fold greater than SW fish. PRL expression declined by 1/3 within 6 h after FW fish were transferred to SW. Within 48 h, PRL expression in TF fish transferred to SW fell to levels that were similar to its expression in SW fish. When SW and TS fish were transferred to FW, PRL increased and peaked at 48 h (Fig. 7A). Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were also observed on pituitary PRLR1 expression. PRLR1 expression

fell within 6 h when FW fish were transferred to SW. PRLR1 expression rose by 6 h when TF fish were transferred to SW and then declined by 7 d to about 1/2 of that observed in steady-state FW and SW. The transfer of SW and TS fish to FW produce a rise in PRLR1 expression which peaked at 48 h. PRLR1 expression in SW fish transferred to FW increased and then returned to steady-state FW and SW levels after 7 d. On the other hand, PRLR1 expression in TS fish transferred to FW fell to 1/2 steady-state FW and SW levels after 7 d (Fig. 7B).

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were also observed on pituitary PRLR2 expression. The transfer FW fish to SW produced an increase in gill PRLR2 expression of 15-fold within 6 h. PRLR2 expression increased 5-fold at 6 h when TF fish were transferred to SW and then expression levels declined to levels that were not significantly different from those seen in steady-state FW and SW fish by 48 h. The transfer of SW fish to FW brought about a rise in PRLR2 expression that peaked at 24 h before returning to baseline FW and SW levels at 48 h. The transfer of TS fish to FW was without effect on PRLR2 expression (Fig. 7C).

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

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.01$) were observed on branchial PRLR1 expression. The transfer of FW fish to SW reduced branchial PRLR1 expression by 25% at 6 h. The transfer of TF fish to SW reduced PRLR1 expression to levels that were similar to those of steady-state SW fish at 6 h. The transfer of both SW and TS fish to FW produced an increase in PRLR1 expression with a peak at 6 h (Fig. 8A).

Significant effects of salinity rearing regime ($P<0.01$) and time ($P<0.0001$) were observed on branchial PRLR2 expression. The transfer of FW fish to SW produced a 4-fold increase in PRLR2 expression at 6 h. The transfer of TF fish to SW produced a 1.5-fold rise in PRLR2 expression that peaked at 24 h before falling to initial TF levels. There was no significant difference in PRLR2 expression when SW fish were transferred

to FW. The transfer of TS fish to FW produced an increase in PRLR2 expression at 24 h and 48 h with a return to initial TS levels after 7 d (Fig. 8B).

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were observed in branchial NCC expression. The transfer of FW fish to SW brought about a decline in NCC expression within 6 h to levels observed in tilapia reared in steady-state SW. The transfer of TF fish to SW produced a decline in NCC expression which nevertheless remained above levels in fish reared in SW until 48 h after transfer. NCC expression of SW and TS fish increased in response to a transfer to FW and peaked at 48 h (Fig. 9A).

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were observed on branchial NKCC expression. The transfer of FW fish to SW produced a 3-fold increase in NKCC expression at 6 h. The transfer of TF fish to SW produced a 1.5-fold increase in NKCC expression that peaked at 24 h. The transfer of both SW and TS fish to FW brought about a fall in the expression of NKCC within 48 h which reached levels that were similar to those in fish reared in FW (Fig. 9B).

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were observed on branchial NKA α1a expression. The transfer of FW fish to SW produced an 80% decline in NKA α1a expression at 6 h. NKA α1a expression in TF fish was 50% of what was seen in FW fish before the start of the transfer and by 48 h the expression in TF fish decreased to $1/10^{th}$ of its initial expression levels. The transfer of both SW and TS fish to FW produced a 10-fold increase in NKA α1a expression by 24 h which continued to increase through to 7 d after transfer. The peak expression of NKA α1a was greater in SW fish than TS fish when both were transferred to FW (Fig. 9C).

Significant effects of salinity rearing regime ($P<0.0001$) were observed on branchial NKA α1b expression. The transfer of FW fish to SW brought about a fall in the expression of NKA α1b within 6 h. The transfer of TF fish to SW brought about significant reduction in NKA α1b expression, but only at 24 h after transfer. The transfer of SW fish to FW produced a 4-fold increase in NKA α1b expression at 24 h and at 7 d with an intervening decline to pre-transfer levels at 48 h. The transfer of TS fish to FW produced a 2.5-fold increase in NKA α1b at 6 h, a rise that was maintained through 7 d (Fig. 9D).

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were observed on branchial CFTR expression. The transfer of FW fish to SW yielded a 2-fold increase in CFTR expression by 6 h. Expression of CFTR in TF fish transferred to SW increased 3-fold by 6 h and then declined to pre-transfer levels by 24 h. The transfer of SW fish to FW brought about a fall in CFTR expression by 6 h. CFTR expression of TS fish transferred to FW fell significantly by 6 h, falling further by 24 h to levels that were similar to those of fish reared in FW (Fig. 10A).

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.01$) were observed on branchial AQP3 expression. The transfer of FW fish to SW had no significant effect of AQP3 expression at 6 h. By contrast, the transfer of TF fish to SW produced a 90% decline in AQP3 expression by 48 h. The transfer of SW fish to FW produced an increase in AQP3 expression within 6 h which continued to rise to the end of the study at 7 d. The magnitude of the change in AQP3 expression was greater in TS fish after FW transfer was greater than that observed in SW fish transferred to FW; TS fish transferred to FW had greatest AQP3 expression 48 h after transfer (Fig 10B).

Significant effects of salinity rearing regime ($P<0.0001$) were observed on branchial NHE3 expression. When FW fish were transferred to SW, NHE3 expression declined by 1/2 in 6 h. The transfer of TF fish to SW was without significant effect on NHE3 expression at 6 h or 24 h, but produced a significant decrease by 48 h. The transfer of SW fish to FW had no significant effect on NHE3 expression at either 6 h or 24 h after transfer, but produced a 2.5-fold rise by 48 h. The transfer of TS fish to FW produced an increase in NHE3 expression in within 6 h, a rise that continued to increase to a peak at 24 h before falling to levels observed in FW-reared fish (Fig. 10C).

Figure 5: Illustration of the tank setup for the experiment showing the initial salinity of the tank and the transferred condition. Fish were reared in fresh water (FW), seawater (SW), or a tidal environment (Tidal) which alternated between FW and SW every 6 h. The sampling conditions were FW, SW, a tidal condition with the fish sampled at the end of the fresh water phase of the cycle (TF) or sea water phase of the cycle (TS).

Figure 5

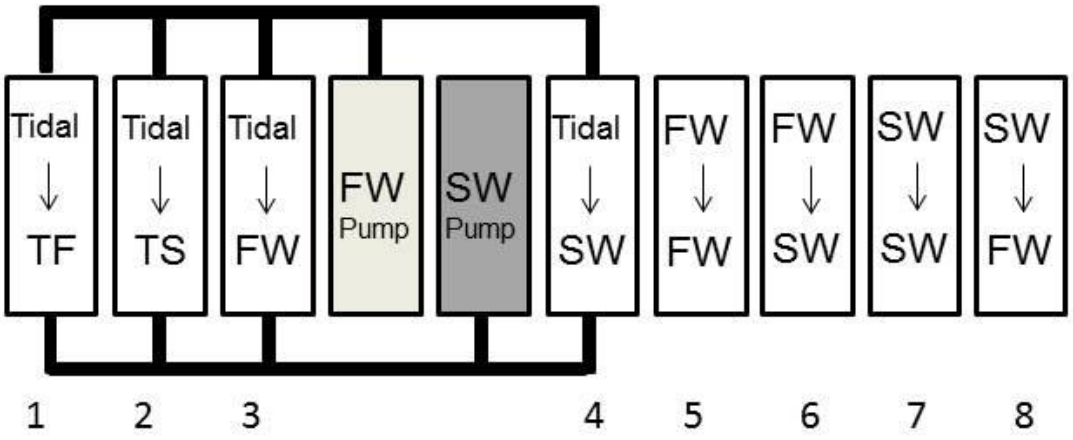
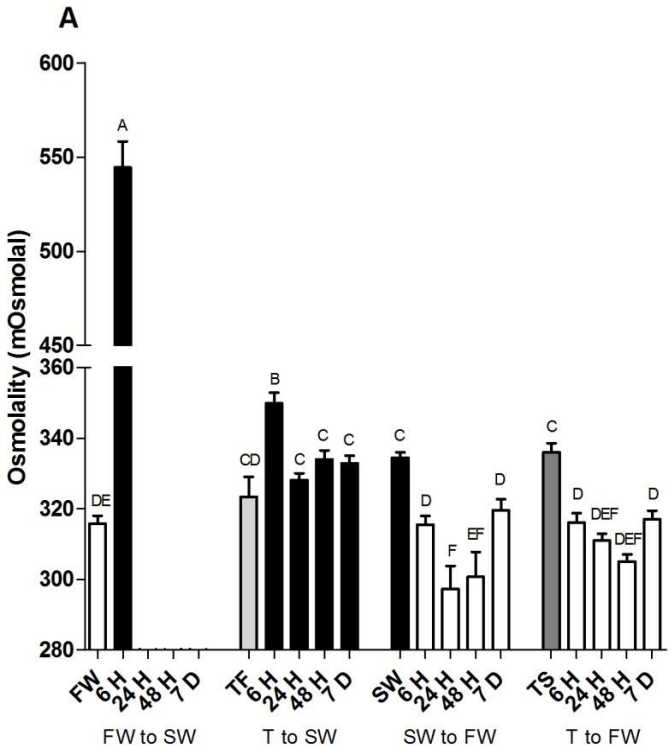

Tidal
TF
Tidal
TS
Tidal
FW
FW
Pump
SW
Pump
Tidal
SW
FW
FW
FW
SW
SW
SW
SW
FW
1
2
3
4
5
6
7
8

Figure 6: Effects of acute salinity challenges on plasma osmolality (A) and plasma prolactin (B) in Mozambique tilapia (*Oreochromis mossambicus*) transferred from fresh water (FW) to seawater (SW), a tidally-changing salinity to SW, SW to FW, and a tidally-changing salinity to FW. Fish were sampled 6 h, 24 h, 48 h, and 7 d after the salinity transfers. Values are expressed as means ± S.E.M. (n = 10-20). Means not sharing the same letter are significantly different (two-way ANOVA, Fisher LSD test, $P < 0.05$).

Figure 6


A
Osmolality (mOsmolal)
600
550
500
450
360
340
320
300
280
A
DE
B
CD
C
C
C
C
D
F
EF
D
C
D
DEF
DEF
D
FW
6 H
24 H
48 H
7 D
TF
SW
TS
FW to SW
T to SW
SW to FW
T to FW

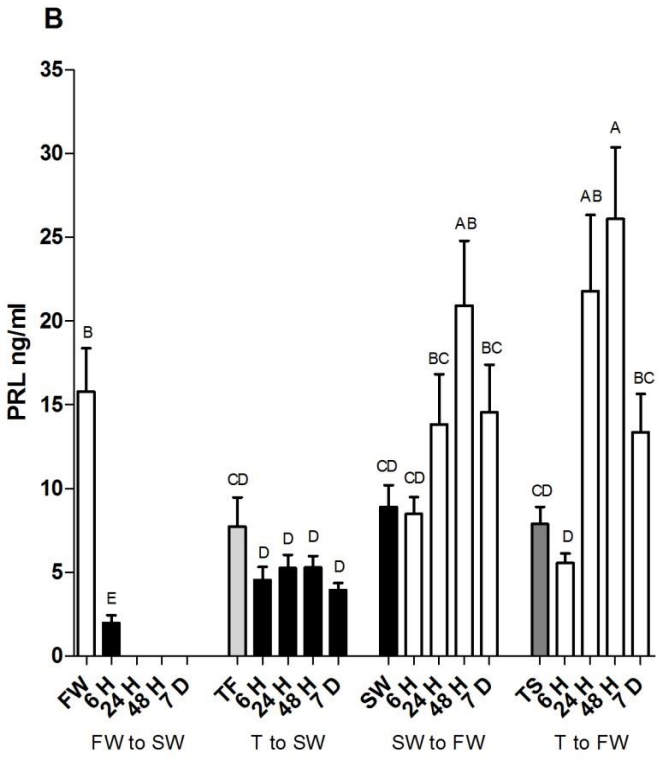

B
PRL ng/ml
35
30
25
20
15
10
5
0
B
E
CD
D
D
D
CD
CD
BC
AB
BC
CD
D
AB
A
BC
FW
6 H
24 H
48 H
7 D
TF
SW
TS
FW to SW
T to SW
SW to FW
T to FW

Figure 7: Effects of acute salinity challenges on pituitary PRL mRNA expression (A), PRLR1 mRNA expression (B), and PRLR2 mRNA expression (C) in Mozambique tilapia (*Oreochromis mossambicus*) transferred from fresh water (FW) to seawater (SW), a tidally-changing salinity to SW, SW to FW, and a tidally-changing salinity to FW. Fish were sampled 6 h, 24 h, 48 h, and 7 d after the salinity transfers. Values are expressed as means ± S.E.M. (n = 10-20). Means not sharing the same letter are significantly different (two-way ANOVA, Fisher LSD test, $P < 0.05$).

Figure 7

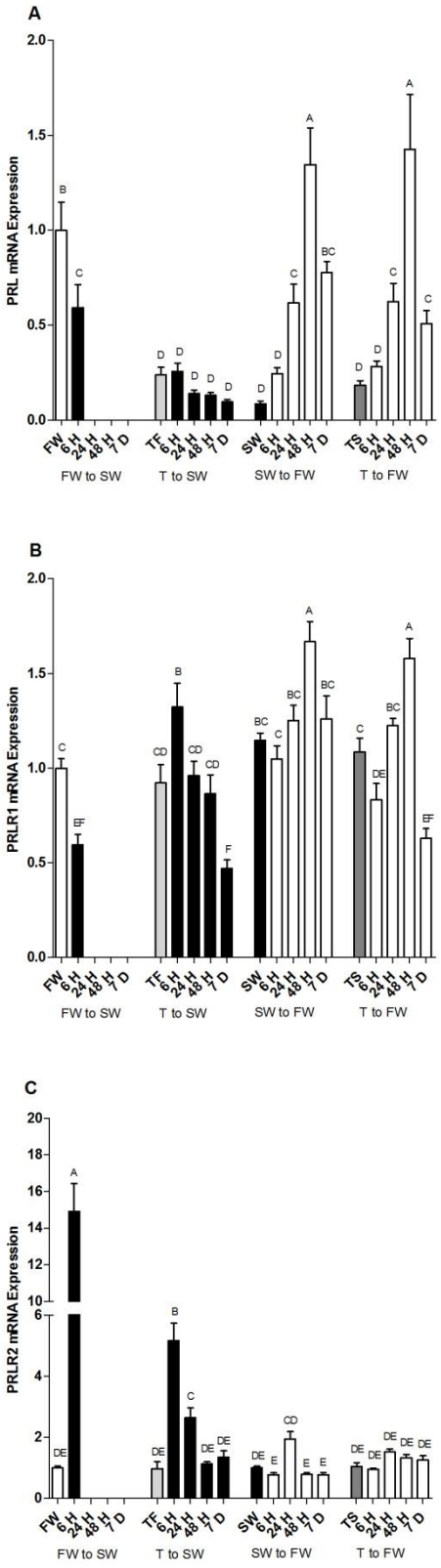

A
PRL mRNA Expression
2.0
1.5
1.0
0.5
0.0
FW
6 H
24 H
48 H
7 D
TF
SW
TS
FW to SW
T to SW
SW to FW
T to FW
B
PRLR1 mRNA Expression
C
PRLR2 mRNA Expression
20
18
16
14
12
10
6
4
2
0

Figure 8: Effects of acute salinity challenges on branchial PRLR1 mRNA expression (A) and PRLR2 mRNA expression (B) in Mozambique tilapia (*Oreochromis mossambicus*) transferred from fresh water (FW) to seawater (SW), a tidally-changing salinity to SW, SW to FW, and a tidally-changing salinity to FW. Fish were sampled 6 h, 24 h, 48 h, and 7 d after the salinity transfers. Values are expressed as means ± S.E.M. (n = 10-20). Means not sharing the same letter are significantly different (two-way ANOVA, Fisher LSD test, $P < 0.05$).

Figure 8

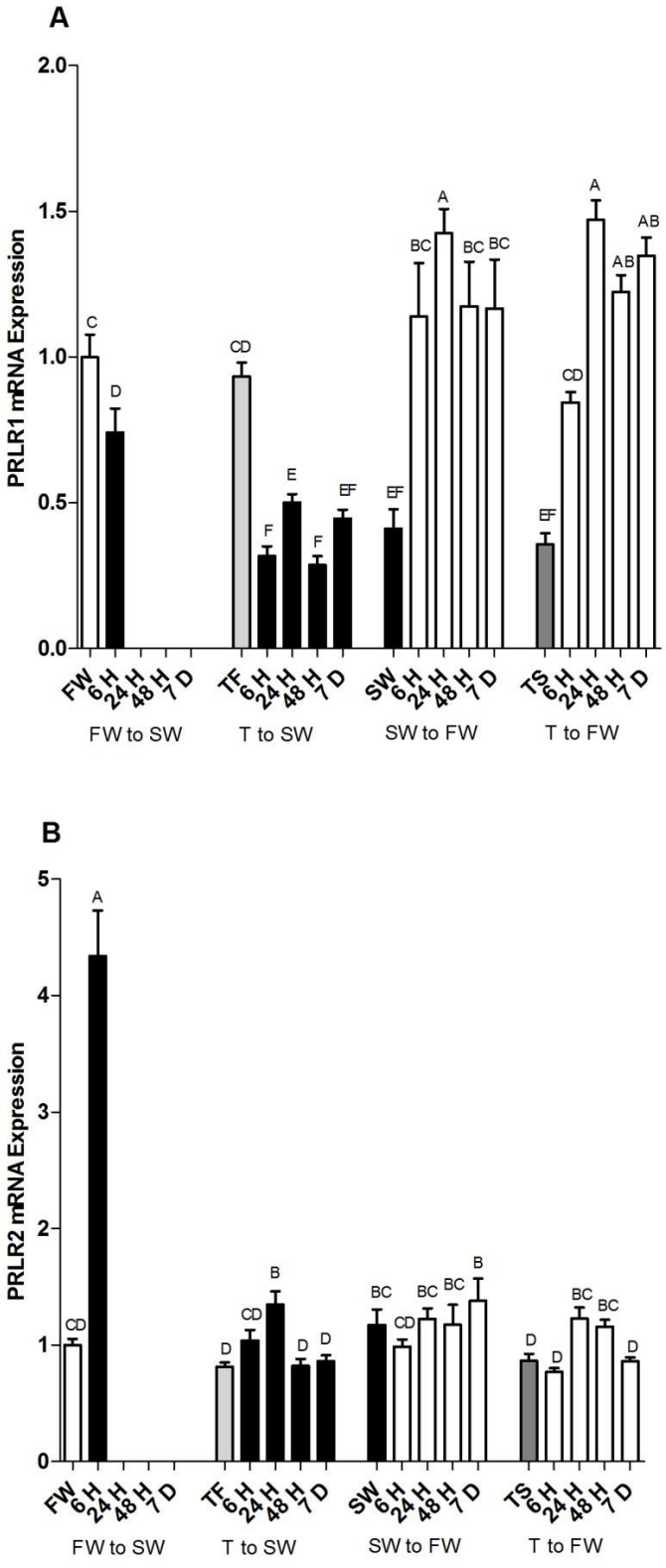

A
PRLR1 mRNA Expression
2.0
1.5
1.0
0.5
0.0
C
D
CD
F
E
F
EF
EF
BC
A
BC
BC
EF
CD
A
AB
AB
FW
6 H
24 H
48 H
7 D
TF
SW
TS
FW to SW
T to SW
SW to FW
T to FW
B
PRLR2 mRNA Expression
5
4
3
2
1
0
A
CD
D
CD
B
D
D
BC
CD
BC
BC
B
D
D
BC
BC
D

Figure 9: Effects of acute salinity challenges on branchial NCC mRNA expression (A), NKCC1a mRNA expression (B), NKA α1a mRNA expression (C), and NKA α1b mRNA expression (D) in Mozambique tilapia (*Oreochromis mossambicus*) transferred from fresh water (FW) to seawater (SW), a tidally-changing salinity to SW, SW to FW, and a tidally-changing salinity to FW. Fish were sampled 6 h, 24 h, 48 h, and 7 d after the salinity transfers. Values are expressed as means ± S.E.M. (n = 10-20). Means not sharing the same letter are significantly different (two-way ANOVA, Fisher LSD test, $P < 0.05$).

Figure 9

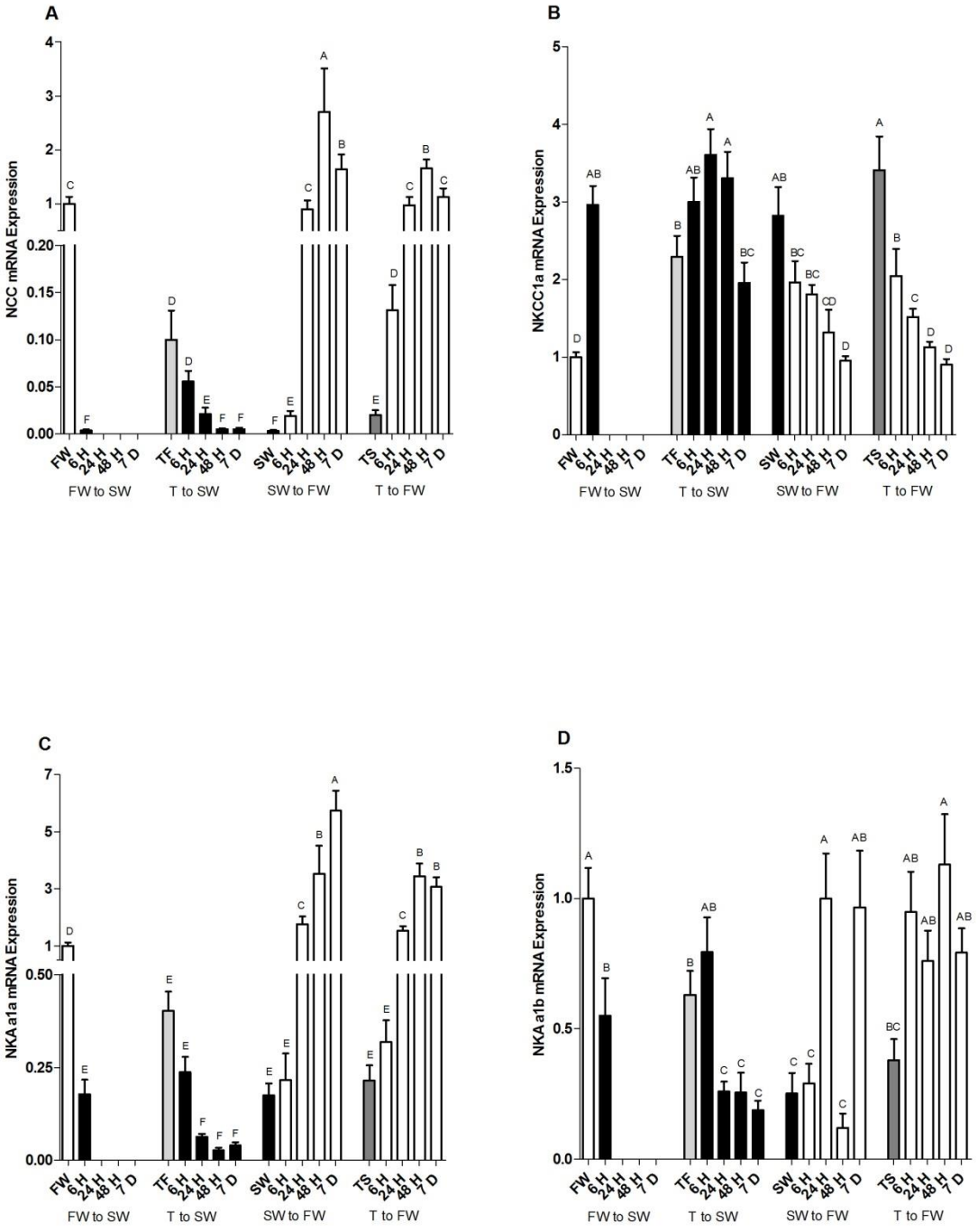
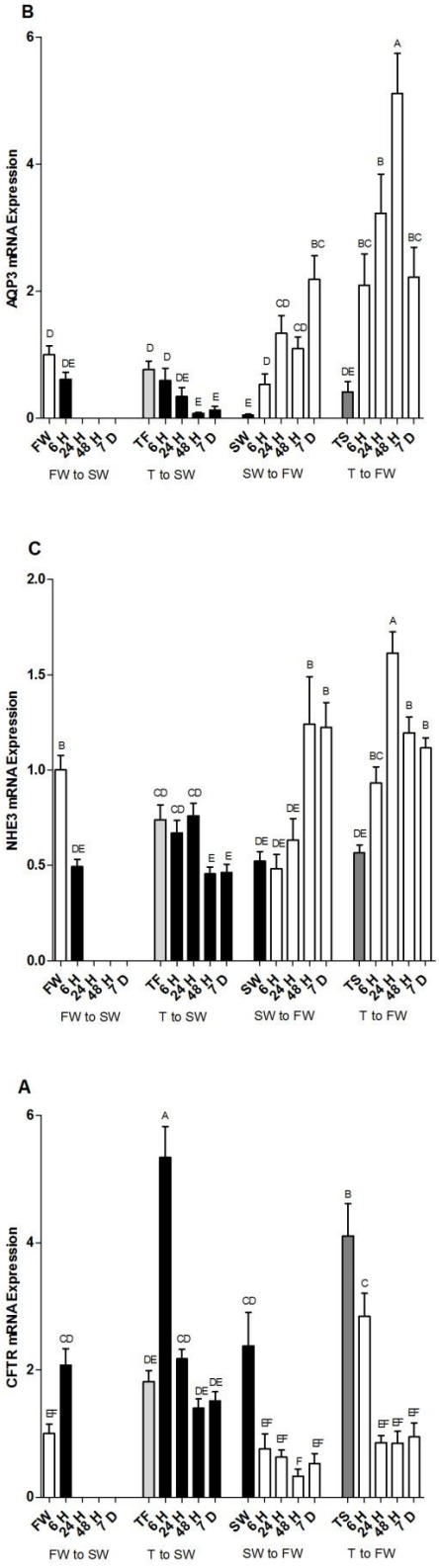

A
NCC mRNA Expression
B
NKCC1a mRNA Expression
C
NKA a1a mRNA Expression
D
NKA a1b mRNA Expression
FW
TF
SW
TS
6 H
24 H
48 H
7 D
FW to SW
T to SW
SW to FW
T to FW

Figure 10: Effects of acute salinity challenges on branchial CFTR mRNA expression (A), AQP3 mRNA expression (B), and NHE3 mRNA expression (C) in Mozambique tilapia (*Oreochromis mossambicus*) transferred from fresh water (FW) to seawater (SW), a tidally-changing salinity to SW, SW to FW, and a tidally-changing salinity to FW. Fish were sampled 6 h, 24 h, 48 h, and 7 d after the salinity transfers. Values are expressed as means ± S.E.M. (n = 10-20). Means not sharing the same letter are significantly different (two-way ANOVA, Fisher LSD test, $P < 0.05$).

Figure 10


B
AQP3 mRNA Expression
FW to SW
T to SW
SW to FW
T to FW
C
NHE3 mRNA Expression
A
CFTR mRNA Expression

4. Discussion

The principal objective of the present study was to compare endocrine and osmoregulatory responses of Mozambique tilapia reared in either FW, SW, or in salinity that varied with tidal frequency between FW and SW. This was done by measuring the effects of changes in salinity on PRL, its receptors as well as on osmoregulatory elements of the gill. This was done in conjunction with the assessment of the ability of fish reared under different salinity regimes to adapt to a sustained change in salinity. The main findings of this study were that: 1) rearing fish in a tidally-changing salinity, which more closely represents their natural history, significantly improves the ability to survive the transfer to SW compared with that of FW-reared tilapia; 2) tilapia reared either SW or in a tidal salinity regime easily adapt when transferred to FW; 3) When transferred to FW, SW-reared tilapia, like those reared in a tidally-changing salinity, show similar patterns of change in ion transporter mRNA; and 3) unlike the other ion transporters measured, CFTR mRNA expression changed more robustly in fish transferred from a tidally-changing salinity to SW than in FW fish transferred to SW.

In both the current experiment and previous experiments with Mozambique tilapia, it has been shown that osmolality dramatically increases when fish are transferred from FW to SW. Similarly, the extent of the osmolality increase and survival rate are dependent on the rate at which they are transferred. Tilapia are able to survive when transferred to an intermediate salinity before being transferred to SW, but are unable to survive a direct transfer (Yada et al., 1994; Seale et al., 2002; Breves et al., 2010d; Seale et al., 2012b). It has been shown through many studies that the survival threshold is between 450 mOsmolal and 550 mOsmolal (Yada et al., 1994; Seale et al., 2002; Kajimura et al., 2004a; Wang et al., 2009; Breves et al., 2010c; Breves et al., 2010d; Seale et al., 2012b). Tilapia reared in a tidally-changing salinity and then exposed to SW for seven days moved to SW with no mortality, suggesting that they were completely acclimated to SW. I have observed previously that plasma osmolality of tilapia reared in a tidally-changing salinity fluctuated between 320 mOsmolal and 345 mOsmolal in the FW and SW phases of the cycle respectively (Moorman et al., 2014). Previous experiments have shown that 320 mOsmolal and 345 mOsmolal are consistent with the

plasma osmolality of Mozambique tilapia held for prolonged periods in FW and SW respectively (Seale et al., 2002; Magdeldin et al., 2007; Breves et al., 2010b).

PRL is sine qua non for maintaining osmoregulatory homeostasis by euryhaline teleosts in FW (Pickford and Phillips, 1959; Dharmamba et al., 1967). Prolactin acts by reducing water permeability and increasing ion uptake, at least in part, by upregulating NCC mRNA expression in the gills (Breves et al., 2010b). Pituitary PRL release is regulated directly by decreases in extracellular osmolality that accompany FW acclimation (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1981; Helms et al., 1991; Borski et al., 1992; Shepherd et al., 1999; Seale et al., 2002; Seale et al., 2006b; Seale et al., 2012b; Seale et al., 2012a). Likewise, circulating plasma PRL levels in FW tilapia are higher than in SW tilapia (Yada et al., 1994; Seale et al., 2002; Seale et al., 2006b). Moving tilapia from FW to SW leads to a fall in plasma PRL levels; conversely, moving tilapia from SW to FW produces a rapid increase in plasma PRL (Yada et al., 1994; Seale et al., 2002; Seale et al., 2006b; Seale et al., 2012b). In our recent study, I found that Mozambique tilapia, reared in a tidally-changing salinities, do not exhibit an increase in plasma PRL. This suggests that the PRL-sensitive osmoregulatory elements are already in action and do not require a "burst" of PRL to be activated. Thus, when exposed to the 6 h FW phase of the "tidal" cycle, TS tilapia are able to regulate osmolality at 320 mOsmolal, exactly as do fish maintained in FW (Moorman et al., 2014). In the current study, I found that PRL significantly increased only after TS fish had been held in FW for 24 h, a pattern that was paralleled in PRL gene expression in the pituitary. This suggests that there is a threshold between 6 and 24 h post-FW exposure for which an elevation in plasma PRL becomes necessary to maintain osmotic balance.

I also examined the impact of rearing salinity, i.e. FW, SW or tidally varying, on the responses of gill and pituitary PRLR1 and PRLR2 expression to a change in salinity. It is to be noted that in this study the response of PRLR1 and PRLR2 expression differs between the gill and pituitary. In the current study, branchial PRLR1 expression was higher in FW tilapia than in those in SW. This is consistent with previous work on PRLR1 gene expression and PRLs role in promoting ion uptake (Fiol et al., 2009; Moorman et al., 2014). The expression of PRLR1 mRNA fell more rapidly in the gill than in the pituitary when fish were transferred from a tidally-changing salinity to SW.

The cause of the difference in PRLR1 expression between the two tissues is not clear, but may be related to tissue-specific regulatory patterns, or differences in the rates at which each tissue is exposed to the changes in environmental salinity. The similarity in response to the transfer to FW for fish reared in SW and those reared in a tidally-changing salinity suggests that neither rearing history confers an adaptive advantage over the other during FW acclimation.

While PRLR1 expression responded more robustly to salinity changes in the gill than in the pituitary, the reverse was true for PRLR2 expression. PRLR2 mRNA expression in gill and pituitary of the Mozambique tilapia rises when environmental salinity is increased (Fiol et al., 2009; Seale et al., 2012b). This change has been suggested to confer an increased ability to adapt to a hyper-osmotic environment (Fiol et al., 2009). Consistent with this, I found here that PRLR2 expression increases in both the pituitary and gill when FW fish are transferred to SW. This response was less robust in fish transferred from a tidally-changing salinity, likely due to the lower plasma PRL concentration found in these fish (Moorman et al., 2014).

It has been shown that exposing tilapia to an osmotic challenge both increases the mRNA expression of appropriate ion transporting proteins while reducing the mRNA expression of ion transporting proteins that would be maladaptive (Hiroi et al., 2005; Hiroi et al., 2008; Breves et al., 2010d; Tipsmark et al., 2011; Velan et al., 2011). Consistent with their role in ion extrusion, and in agreement with previous research, I observed increases in NKCC1a and CFTR when FW fish were transferred to SW (Inokuchi et al., 2008; Breves et al., 2010d). Likewise, the transfer from FW to SW brought about a reduction in the mRNA expression of NCC, NHE3, and NKA α1a (Hiroi et al., 2005; Hiroi et al., 2008; Breves et al., 2010d; Tipsmark et al., 2011). In the current experiment, changes in mRNA expression occurred within 6 h following the beginning of the SW component of the tidal cycle. Using immunohistochemistry (IHC) I have observed that fish reared in a tidally-changing salinity environment maintained branchial ionocytes similar to those seen in fish reared in SW, regardless of whether the fish were sampled in the FW or SW phase of the tidal cycle (Moorman et al., 2014). In the current study, the mRNA expression of ion transporting proteins showed less dramatic changes in fish transferred from a tidally-changing salinity to SW than was observed in FW fish

transferred to SW, with the exception of CFTR. The increase in mRNA expression of CFTR was greater in fish transferred from a tidally-changing salinity to SW than in FW fish transferred to SW and the expression 6 h after the transfer was 2.5-fold greater. The increases in CFTR expression in response to increases in salinity agree with CFTR's role in ion extrusion, in line with the widely accepted model of osmoregulation (Evans et al., 2005; Hwang et al., 2011). There is a positive relationship between external salinity and CFTR mRNA expression in the gill (Li et al., 2014). In our previous study, I observed CFTR signals in the IHC images taken of the gills of tilapia reared in tidally varying salinity which were not present in the gills of FW fish (Moorman et al., 2014). This suggests that a rapid and robust increase in CFTR may be crucial for successful adaptation to SW.

In addition to the differences seen in CFTR expression an important finding of this experiment is the difference in the response of AQP3 gene expression between FW fish transferred to SW and fish reared in a tidally-changing salinity transferred to SW. A previous study showed that in the European eel, *Anguilla anguilla,* AQP3 is crucial for maintaining water balance in FW and that when fish are transferred to SW AQP3 expression in the gill is downregulated (Cutler and Cramb, 2002). Our current results support the notion that the inability to reduce AQP3 underlies the mortality observed following direct transfer from FW to SW. High AQP3 in a hyperosmotic environment could lead to excessive water loss, and consequent increase in plasma osmolality to lethal levels. By contrast, upon transfer of tidal fish to SW, blood osmolality does not rise to lethal levels and AQP3 gene expression is reduced.

Taking into account rearing conditions and life histories of each species is essential to determining the extent and plasticity of osmoregulatory ability. The importance of rearing history on osmoregulation has been suggested for killifish. Specifically, it has been shown that maintaining killifish in a tidally-changing salinity enables them to move to both higher and lower salinities than is usual during short times with little osmotic disturbance (Wood and Grosell, 2009). Our current results clearly demonstrate that the ability of Mozambique tilapia to adapt to osmotic challenges is dependent on their rearing salinity history and is facilitated by previous exposure to cyclical salinity variations. Applying the tilapia model to determine the ability of

euryhaline species to adapt to osmotic challenges will expand our understanding of how environmental salinity modulates osmoregulatory physiology over a fish's life history.

CHAPTER IV

Rearing Mozambique tilapia in tidally-changing salinities: effects on growth and the growth hormone/ insulin-like growth factor I axis

Abstract

The growth hormone (GH)/insulin-like growth factor (IGF) axis plays a central role in the regulation of growth in teleosts and has been previously shown to be affected by rearing tilapia in different salinities. This study was aimed at characterizing the effects of rearing tilapia in a tidally-changing salinity on growth and on the GH/IGF axis in the tilapia *Oreochromis mossambicus*. Tilapia were raised in fresh water (FW), seawater (SW), and in a tidally-changing environment for 4 months with growth being measured periodically throughout the experiment. The growth rate of fish reared in a tidally-changing environment was significantly higher than any of the other treatments. I compared plasma growth hormone (GH), plasma insulin like growth factor 1 (IGF-1), pituitary GH mRNA, and expression of IGF-I, IGF-II, and growth hormone receptor 2 (GHR2) in the muscle and liver of tilapia reared in FW, SW, and in tidally-changing salinity, which varied between FW (TF) and SW (TS) every 6 h. Plasma IGF-I was higher in FW and TS than in SW and TF tilapia. Pituitary GH expression was higher in TF and TS than in FW and SW tilapia. Hepatic gene expression of IGF-I and GHR2 in both the muscle and liver changed between FW and SW phases of the tidal cycle. This study indicates that rearing tilapia in a tidally-changing salinity does not cause endocrine parameters associated with growth to fluctuate between levels seen in FW or SW fish, but instead, leads to a unique pattern of expression which leads to enhanced growth.

1. Introduction

Growth in vertebrates is largely controlled through the coordinated activities of the growth hormone (GH)/insulin-like growth factor (IGF) axis. In teleosts, GH is involved in the regulation of several physiological processes such as growth, osmoregulation, metabolism, reproduction, development, and feeding behavior (Madsen and Bern, 1993; McLean et al., 1993; Duan, 1997; Shepherd et al., 1997; Kawauchi and Sower, 2006). GH and its mediator, insulin-like growth factor I (IGF-I), have also been shown to facilitate SW adaptation in several salmonids (McCormick et al., 1991; Sakamoto et al., 1991) and in the euryhaline tilapia, *Oreochromis mossambicus* (Yada et al., 1994; Sakamoto et al., 1997; Fruchtman et al., 2000). In the liver and muscle, GH can act directly by stimulating mitosis and differentiation among other cellular behaviors, and acts indirectly by initiating the production and release of IGF-I which has been linked to a variety of growth-promoting actions in most tissues (Butler and Le Roith, 2001; Wood et al., 2005; Fan et al., 2009; Duan et al., 2010). GH and IGFs act by binding to membrane receptors (Kajimura et al., 2004b; Pierce et al., 2007; Duan et al., 2010). Two GHR sequences in Mozambique tilapia, (*Oreochromis mossambicus*), have been discovered and denoted GHR1 and GHR2 (Kajimura et al., 2004b; Pierce et al., 2007). Phylogenetic analyses, tissue expression patterns, and regulation by GH of these two GHRs suggest that GHR2 encodes the primary GH receptor in Mozambique tilapia (Kajimura et al., 2004b; Pierce et al., 2007; Pierce et al., 2012).

Understanding how environmental factors like salinity play a role in growth and what effects they have on the GH/IGF axis is becoming increasingly important due to the prominent role of tilapia in aquaculture. According to the United Nations Food and Agriculture Organization tilapia ranks third in food fish production throughout the world with 4.2 million tons produced in 2012. For intensive aquaculture to be successful, it is important to understand how to increase growth, increase feed efficiency, and to understand how each individual factor can effect growth so that production can be maximized. Previous studies of growth and the GH/IGF axis in Mozambique tilapia have focused on fasting and refeeding, varying amount and protein content of feed, hormonal supplementation, and acclimation salinity (Ron et al., 1995; Shepherd et al., 1997; Riley et al., 2003; Sparks et al., 2003; Uchida et al., 2003; Fox et al., 2006; Magdeldin et al.,

2007; Pierce et al., 2007; Fox et al., 2010). Previous work focused on the effect of rearing salinity on growth in different steady-state environments have shown that Mozambique tilapia reared in SW grow faster than fish in other steady-state conditions (Kuwaye et al., 1993; Ron et al., 1995; Sparks et al., 2003). Evidence suggests that the faster growth seen in SW in these previous experiments is at least in part tied to activation of the GH/IGF system. Circulating GH and pituitary GH have been shown to increase in response to a transfer from FW to SW, and GH release has been found to increase in response to increases in extracellular osmolality *in vitro* (Borski et al., 1994; Seale et al., 2002; Seale et al., 2006b; Pierce et al., 2007; Breves et al., 2010c; Breves et al., 2010d). Evidence suggests that pituitary GH may be a better indicator of growth than circulating GH and IGF-I levels (Riley et al., 2002). Rearing fish in salinities that change cyclically is a currently unexplored paradigm for examining how the environment affects growth rates and the endocrine control of growth. The Mozambique tilapia (*Oreochromis mossambicus*) is a euryhaline teleost with a worldwide tropical distribution that originates from estuaries and near-shore rivers from the lower Zambezi River to the southeast coast of South Africa not generally more than a mile from the tidal ebb and flow (Trewevas, 1983). Hence, the examination of growth in tilapia that have been reared in tidally-changing salinities may better reflect their native conditions. The goal of this study is to determine if rearing Mozambique tilapia in a tidally-changing salinity can be used as a method to increase growth and enhance the activation of the GH/IGF axis which would be important due to due to the role of tilapia in aquaculture production. I hypothesize that by mimicking rearing salinity conditions that approximate those of their native environment tilapia with grow faster, and activate the GH/IGF system to a greater extent than compared with fish reared in steady-state salinities.

To assess whether euryhaline tilapia reared in a tidally-changing salinity show enhanced activation of the GH/IGF axis compared with tilapia reared in FW or SW. I examined endpoints associated with the GH/IGF axis in the plasma, pituitary, muscle, and liver. Specifically I characterized plasma levels of GH and IGF-I, pituitary GH mRNA expression, and expression of IGF-I, IGF-II, and GHR2 in the muscle and liver of tilapia reared under a tidally-changing environment, as compared with tilapia reared in FW and SW.

2. Materials and Methods

2.1 Fish Maintenance and treatments

Mozambique tilapia (*Oreochromis mossambicus*) yolk-sac fry were collected from broodstock tanks maintained in FW at the Hawaii Institute of Marine Biology (Kaneohe, HI). The fry were kept in 75 L glass aquaria supplied with circulating FW until yolk-sac absorption was complete. Fry were then combined into one 75 L aquarium containing FW. Two days after yolk-sac absorption they were distributed into five 75 L glass aquaria supplied with FW (3 L/min) and stocked at a density of 100 fry per tank (mean weight, 12±1 mg). Water temperature was maintained at 25±1°C in all tanks. The fish were exposed to a 12L:12D cycle. After two days in FW, three of the four tanks were transitioned from FW to BW over the course of 3 hours (10 ‰), composed of sea water (SW; 35 ‰ Kaneohe Bay, HI) diluted with FW. After an additional two days, one of the BW tanks was transitioned to SW and two other tanks were maintained under a tidally-changing salinity. As a result, the salinity in four tanks was adjusted to FW, SW, and two were maintained in a tidally-changing salinity.

Salinity in the tanks subjected to the tidally-changing salinity alternated between FW and SW every 6 h yielding a complete salinity transfer within 1.5 h. FW and SW flowing to the tanks with a tidally-changing salinity were controlled by two magnetic drive utility pumps (Pondmaster, Islandia, NY) that were connected to two electric timer outlets (Sylvania, Danvers, MA). The FW and SW pumps were set to switch every 6 h and were set 6 h out of phase. The fish were maintained in these conditions until sampling. Forty fish from each tank were sampled after four months. 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 Diet and Feeding

Fish were fed crushed Silver Cup Flake food (Silver Cup, Harrietta, MI) *ad libitum* daily. Silver Cup Flake feed (Silver Cup, Harrietta, Michigan) was crushed into a powder for feeding fry and placed into individual vials for each tank and weighed daily. The Silver Cup Flake feed consisted of not less than 41% protein, not less than 6% fat, not more than 3% fiber, and not more than 12% ash. An arbitrary but equal amount of feed that was able to be consumed by every tank in the first half hour was added to each tank from its corresponding vial. The fish were then given 30 min to eat the food. The tank was then visually inspected for uneaten feed and if no feed remained the fish were fed again. This was repeated until there was food remaining on the bottom of the tank by visual inspection after 30 minutes. The weight of the vial was recorded at the end of the day and subtracted from the weight of the vial at the beginning of the day to determine how much food was eaten. After two months fish were able to consume 2mm Trout Chow pellets and were given pellets instead of flake food. The method of feeding remained the same as with the flake food.

2.3 Sampling

To determine the differences in growth and survival between the treatments thought the experiment fish were weighed at 25 days after the start of the experiment and every 21 days after that until the end of the experiment. All of the fish were removed from the tank and individually weighed so that the weight and number of fish remaining in each tank could be assessed and to avoid any biases associated with sampling a subset of the tank.

After four months fish were sampled, fish were netted and anesthetized with 2-phenoxyethenol (0.3 ml/L). Blood was collected with a needle and syringe coated with sodium heparin (200 U/ml, Sigma-Aldrich, St. Louis, MO). Plasma was separated by centrifugation and stored at −80°C until later analyses. Fish were rapidly decapitated. Muscle and liver samples were harvested and then immediately frozen in liquid nitrogen and stored at −80°C prior to RNA extraction.

2.4 Feed Conversion Ratio

Feed conversion Ratio (FCR) was calculated for fish in each treatment at each time point using the following formula. *FCR = Total amount of dry feed consumed (g) / (W_f-W_i)*, where the wet weight gain of fish can be calculated by subtracting the weight at the beginning of the sampling period (W_i) from that of the end of the sampling period (W_f).

2.5 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from frozen muscle and liver samples using TRI Reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Using High Capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA), 500 ng of total RNA was reverse transcribed into cDNA. The quantitative real-time PCRs (qRT-PCR) were set up as previously described (Pierce et al., 2007). The mRNA levels of reference and target genes were determined by standard curves generated from 5-fold serial dilutions of cDNA transcribed from FW muscle mRNA for muscle samples and FW liver mRNA for liver samples as specified by StepOne Software v2.0 (Life Technologies). The R^2 values and amplification efficiencies for standard curves were as follows, respectively: 0.993 and 98.2% (EF1α), 0.996 and 93.7% (GH), 0.981 and 88.3% (IGF-I), 0.997 and 86.3% (IGF-II), and 0.995 and 83.4% (GHR2). 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 cDNA. The following cycling parameters were employed: 2 min at 50°C, 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min using the StepOnePlus real-time PCR system (Applied Biosystems). The mRNA levels of elongation factor 1α (EF1α) were used to normalize that of target genes because it showed no effect of treatment. Data are expressed as fold-change from FW values. Primer pairs have been previously described for GH (Magdeldin et al., 2007), IGF-I (Pierce et al., 2007), IGF-II (Davis et al., 2008), GHR2 (Pierce et al., 2007), and EF1α (Breves et al., 2010a).

2.6 Plasma Parameters

Plasma levels of GH were measured by homologous radioimmunoassay (Ayson et al., 1993). Plasma IGF-I levels were measured from 25ul of plasma that was extracted with 100ul of acid–ethanol (Shimizu et al., 1999). Plasma levels of IGF-I were measured by a radioimmunoassay employing recombinant salmon IGF-I standard and anti-barramundi IGF-I antiserum (GroPep, Adelaide, Australia), as validated by (Shimizu et al., 2000).

2.7 Statistical Analysis

Statistical analyses were conducted using a one-way analysis of variance (ANOVA) with salinity rearing regime as the independent variable. Significant main effects of salinity regime ($P < 0.05$) were followed up by the Bonferroni multiple comparison test. Data are expressed as means ± S.E.M. Data were tested and met assumptions of normality and equal variance. Statistical calculations were performed using a statistical software program, Prism 5.0 (GraphPad, La Jolla, CA).

3. Results

3.1 Growth and efficiency

The effects of salinity on the growth of the tilapia are shown in Fig. 11. Tilapia reared in a tidally-changing environment grew significantly faster than fish reared in FW or SW. Differences in growth between the treatments were visible within the first 25 days of the experiment and persisted through the end of the experiment.

There was a significant difference in feed conversion ratio between 26 and 46 days with SW fish having the highest feed conversion ratio, tidal fish having the lowest FCR, and FW fish having an FCR not significantly different from either of the other two treatments (Fig. 12).

3.2 Plasma osmolality, GH, IGF-I, and pituitary GH expression

Plasma osmolality was 319-323 mOsmolal in FW and TF fish and 341-344 mOsmolal in SW and TS fish (Fig. 13A). Plasma GH was higher in TF fish than in FW and SW fish (Fig 13B). Plasma IGF-I in tilapia sampled in FW and at the end of the SW phase of the tidal cycle (TS) was 1.2 ng/ml while plasma IGF-I was significantly lower in SW fish and fish at the end of the FW phase of the tidal cycle (TF) which had 0.75ng/ml (Fig. 13C). Pituitary GH mRNA expression was lowest in FW fish, intermediate in SW fish, and highest in fish sampled in TF and TS (Fig 13D).

3.3 Muscle and Liver gene expression of IGF-I, IGF-II, and GHR2

IGF-I expression in muscle was significantly higher in SW fish than in FW fish. There was no significant difference in IGF-I expression between the two phases of the fish reared in tidally-changing conditions which were intermediate to FW and SW fish (Fig. 14A). There were no significant differences in the expression of IGF-II in the muscle of tilapia in any of the sampled conditions (Fig. 14B). GHR2 expression in the muscle of FW fish and SW fish did not differ significantly while GHR2 expression in TF fish was 2.5-fold greater than in TS fish (Fig. 14C).

Hepatic expression of IGF-I of TF fish was 2-fold greater than that in TS fish. Hepatic IGF-I expression did not differ significantly between FW and SW fish (Fig. 15A). Hepatic IGF-II expression was 2-fold greater in SW fish than in FW fish. Hepatic IGF-II expression was also 2-fold greater in TF fish than in TS fish (Fig. 15B). Hepatic GHR2 expression was 2-fold greater in TF fish than in TS fish. There was no significant difference in the hepatic GHR2 expression between FW and SW fish. (Fig. 15C)

Fig. 11. The effects of rearing condition on the growth of the tilapia, *Oreochromis mossambicus*, during 1-109 days post yolk-sac absorption. Values are expressed as means ± S.E.M. (n = 200-600 for the first 3 time points and n= 75-225 for the last 3 time points). Means not sharing the same letter are significantly different (one-way ANOVA, Bonferroni multiple comparison test, $P < 0.05$). On day 25 fish reared in a tidally-changing salinity were significantly larger than FW or SW fish.

Figure 11

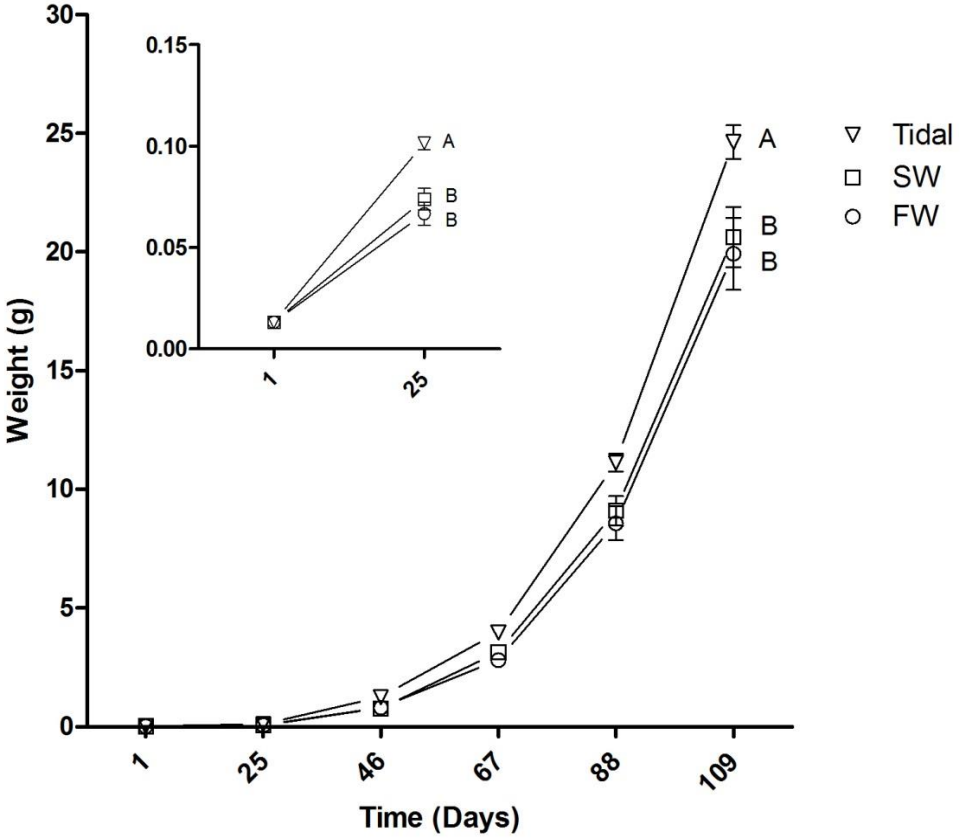

30
25
20
15
10
5
0
Weight (g)
0.15
0.10
0.05
0.00
A
B
B
1
25
A
B
B
1
25
46
67
88
109
Time (Days)
Tidal
SW
FW

Fig. 12. The effects of rearing condition on the feed conversion ratio of the tilapia, *Oreochromis mossambicus*, during 1-109 days post yolk-sac absorption. Values are expressed as means ± S.E.M. (n = 4-12). Means not sharing the same letter are significantly different from the other treatments at the same time point (one-way ANOVA, Bonferroni multiple comparison test, $P < 0.05$).

Figure 12

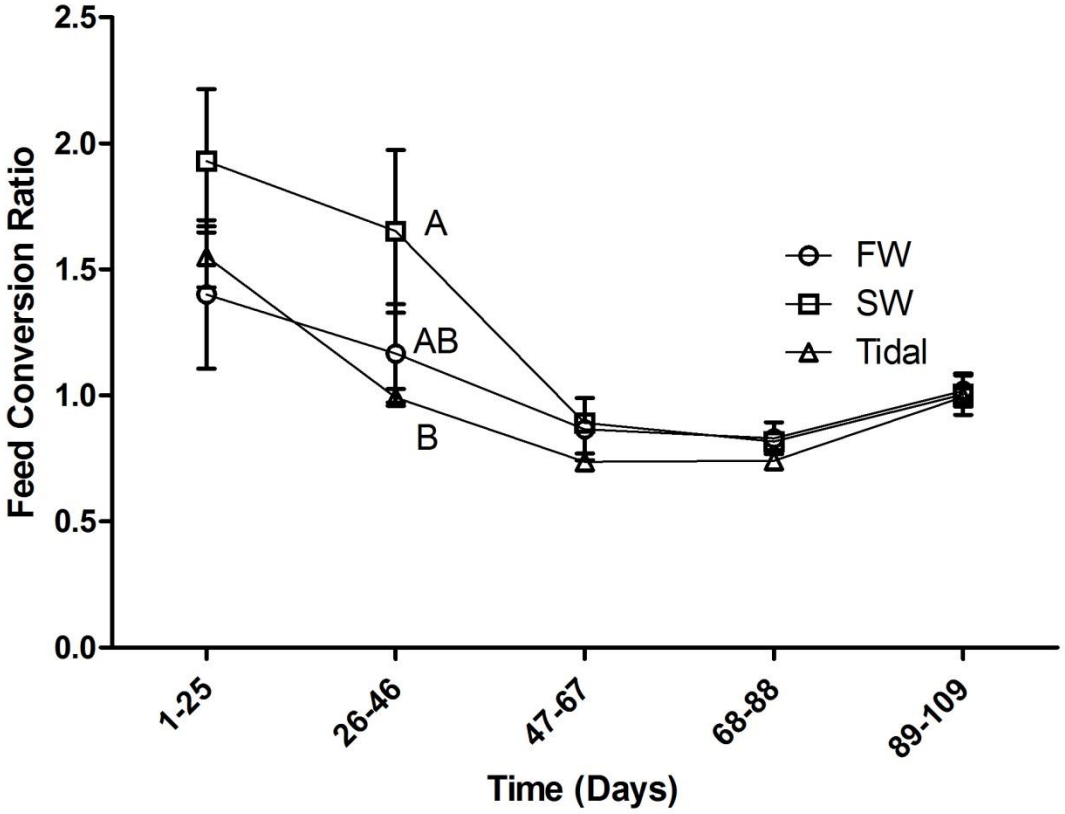

2.5
2.0
1.5
1.0
0.5
0.0
Feed Conversion Ratio
A
AB
B
FW
SW
Tidal
1-25
26-46
47-67
68-88
89-109
Time (Days)

Fig. 13. Effects of rearing condition on plasma osmolality (A), plasma GH (B), plasma IGF-I (C), and pituitary GH mRNA expression (D) 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 = 35-40). Means not sharing the same letter are significantly different (one-way ANOVA, Bonferroni multiple comparison test, $P < 0.05$).

Figure 13

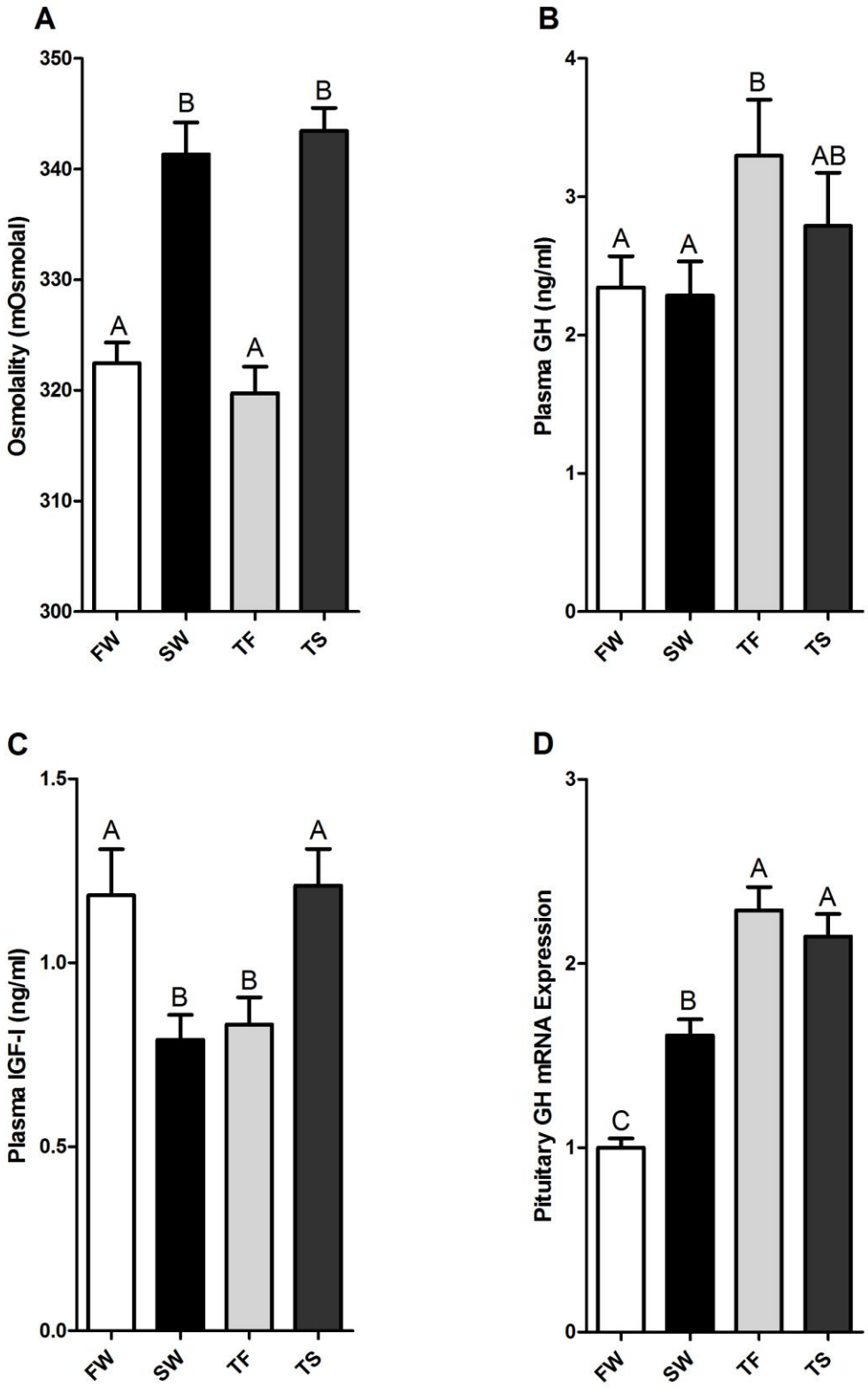

A
Osmolality (mOsmolal)
350
340
330
320
310
300
A
B
A
B
FW
SW
TF
TS
B
Plasma GH (ng/ml)
4
3
2
1
0
A
A
B
AB
FW
SW
TF
TS
C
Plasma IGF-I (ng/ml)
1.5
1.0
0.5
0.0
A
B
B
A
FW
SW
TF
TS
D
Pituitary GH mRNA Expression
3
2
1
0
C
B
A
A
FW
SW
TF
TS

Fig. 14. Effects of rearing condition on muscle mRNA expression of IGF-I (A), IGF-II (B), and GHR2 (C) 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 = 35-40). Means not sharing the same letter are significantly different (one-way ANOVA, Bonferroni multiple comparison test, $P < 0.05$).

Figure 14

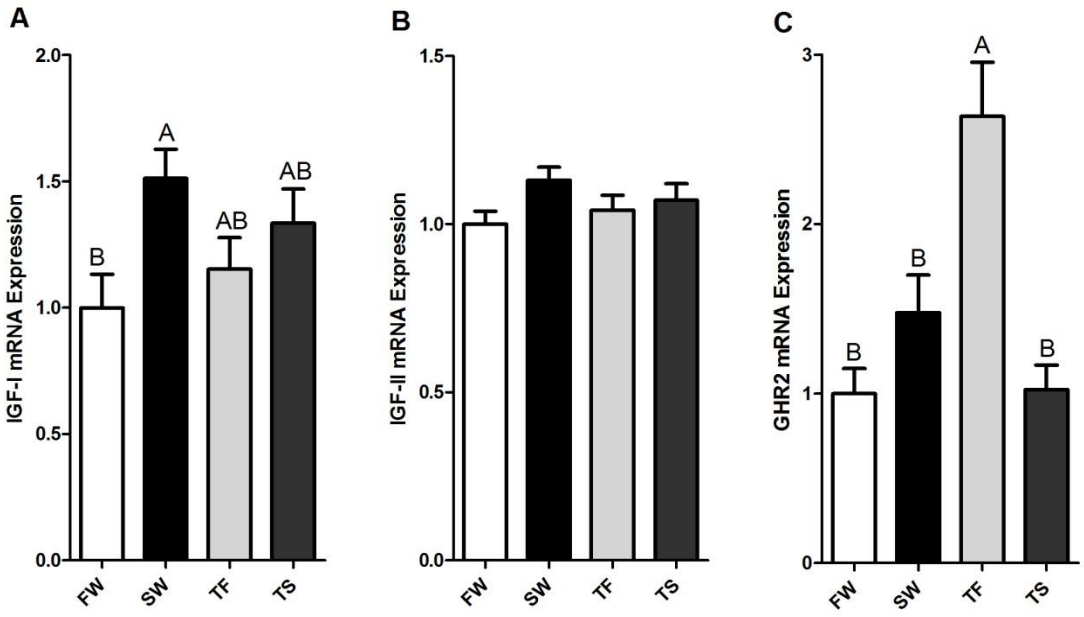
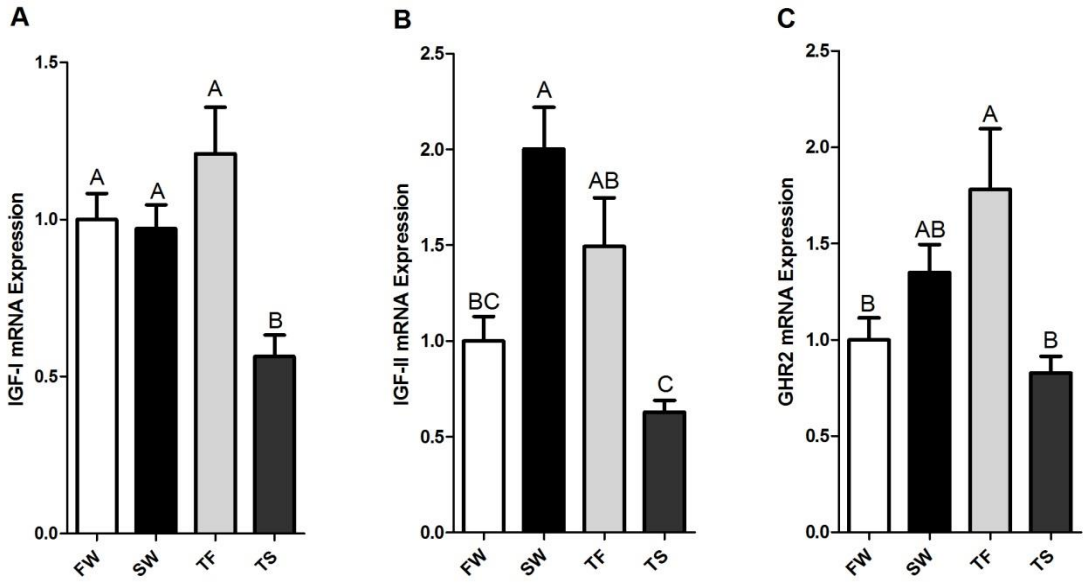

A
IGF-I mRNA Expression
2.0
1.5
1.0
0.5
0.0
B
A
AB
AB
FW
SW
TF
TS
B
IGF-II mRNA Expression
1.5
1.0
0.5
0.0
FW
SW
TF
TS
C
GHR2 mRNA Expression
3
2
1
0
B
B
A
B
FW
SW
TF
TS

Fig. 15. Effects of rearing condition on hepatic mRNA expression of IGF-I (A), IGF-II (B), and GHR2 (C) 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 = 35-40). Means not sharing the same letter are significantly different (one-way ANOVA, Bonferroni multiple comparison test, $P < 0.05$).

Figure 15


A
IGF-I mRNA Expression
1.5
1.0
0.5
0.0
A
A
A
B
FW
SW
TF
TS
B
IGF-II mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
BC
A
AB
C
FW
SW
TF
TS
C
GHR2 mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
B
AB
A
B
FW
SW
TF
TS

4. Discussion

The purpose of this study was to characterize patterns of growth and in elements of the GH/IGF axis in Mozambique tilapia reared in a tidally-changing environment compared with those observed in fish raised in FW and SW. I reared the euryhaline tilapia, *O. mossambicus*, in an environment characterized by salinity changes which varied with a tidal cycle to assess growth and the GH/IGF axis in an experimental paradigm that models their natural environment. Our major findings were that: 1) fish reared in a tidal environment grew faster than fish in FW or SW; and 2) with the exception of plasma GH and IGF-II expression in the muscle, all of the parameters measured showed a different pattern of expression between TF and TS fish compared with FW and SW fish. To our knowledge, this is the first time that growth and the GH/IGF axis have been studied in Mozambique tilapia reared in a tidally-changing salinity.

Previous experiments with Mozambique tilapia have demonstrated differences in growth between fish reared in different salinities. In six to seven month long experiments it has been shown that SW fish grow 2-times faster than FW fish, but significant differences were not seen in the first 4 months (Kuwaye et al., 1993; Ron et al., 1995). In the current study, I saw a significantly higher growth rate in fish reared in a tidally-changing environment compared with fish reared in FW or SW. Previous studies have shown that in a single salinity transfer, growth is slower when fish are transferred from SW to FW, at least for up to 21 days (Riley et al., 2003; Magdeldin et al., 2007). One major difference between the experiments is that in the current experiment fish were fed *ad libitum* and the amount of feed eaten was measured where as in the experiments by Kuwaye and Ron fish were fed a percentage of their body weight (Kuwaye et al., 1993; Ron et al., 1995). The main difference with the feeding method used in these previous experiments is that it is unclear whether the fish are being overfed or underfed, due to the feed being given by a percentage of the body weight. When comparing the growth of different treatments in experiments conducted by Kuwaye, Ron, and Sparks it is difficult to tell whether the differences are due to differences in the amount of feed consumed or a more efficient use of the food when a measure of feed efficiency is not presented

(Kuwaye et al., 1993; Ron et al., 1995; Sparks et al., 2003). Previous studies have shown that there is a high metabolic cost to osmoregulation in a variety of teleosts (Boeuf and Payan, 2001). I examined FCR in the current experiment to determine whether the differences observed in growth were due to differences in the amount of feed consumed or differences in the efficiency with which the fish were able to convert the feed into body weight. I found that the difference in growth among the treatments was not due to differences in the amount of feed consumed because the fish reared in a tidally-changing salinity had significantly lower FCR between 26 to 46 days (Fig. 12).

Differences in plasma GH levels due to salinity have shown inconsistent results in previous experiments (Riley et al., 2003; Pierce et al., 2007; Breves et al., 2011). In the current experiment I found no significant difference in plasma GH levels between FW and SW fish, but plasma GH was significantly higher in TF fish. Previous studies have made the case that pituitary GH expression is a better indicator of growth and the current experiment, along with others, show that pituitary GH expression is consistently higher in tilapia maintained in SW compared with those maintained in FW (Riley et al., 2003; Magdeldin et al., 2007; Breves et al., 2010c). Our results indicate that fish collected in both the end of 6 h in the FW phase and at end of 6 h in the SW phase of the tidal cycle had greater pituitary GH gene expression than those acclimated to steady-state SW or FW ($P< 0.0001$)(Fig. 13 D). Interestingly, GHR2 expression in muscle and liver was higher in fish collected at the end of TF than at end of TS of the tidal cycle (Fig. 14C and Fig. 15C). This pattern suggests that the sensitivity to GH in target tissues, such as muscle and liver, oscillates and is controlled by environmental salinity in a pulsatile fashion that may underlie the elevated growth in fish reared under a tidal regimen.

IGF-I mediates many of GH's growth-promoting actions in teleosts (McCormick et al., 1991; cf. Duan, 1997). Our study indicates that IGF-I mRNA in the muscle is significantly higher in SW fish than in FW fish. Unlike SW and FW fish whose muscle IGF-I mRNA vary with salinity, there was no significant difference between TF and TS fish. Unlike IGF-I expression in the muscle, there was no significant difference in hepatic expression of IGF-I in FW and SW fish and the hepatic expression of IGF-I was significantly different between TF and TS fish. It has been shown that IGF-I mRNA in hepatocytes is stimulated by GH (Pierce et al., 2011).

In summary, the rearing paradigm for Mozambique tilapia employed in this study experimentally simulated the conditions found in their common native habitat and provided a model with which to study the growth and the GH/IGF axis of euryhaline fish under naturally occurring dynamic salinity conditions. Mozambique tilapia reared under tidally-changing salinities grew faster than fish reared in either FW or SW and patterns of expression seen in elements of the GH/IGF axis are unique to fish reared in a tidally-changing salinity. The results of this study suggest that the tidal paradigm may provide a useful method of increasing the rate of growth without increasing the amount of feed required to attain that higher rate of growth.

CHAPTER V

CONCLUSIONS AND FINAL REMARKS

I conducted two 4-month grow-out trials in duplicate from which I obtained data that was used to address my two main objectives which were to understand the mechanisms of osmoregulation and growth in Mozambique tilapia reared in a tidally-changing salinity regimen.

First, I described the effects of different rearing salinities on PRL gene expression and plasma levels as well as on the gene expression of key ion transporters in branchial ionocytes, which are key targets for PRL's action. PRL has been shown to be vital for FW osmoregulation in euryhaline teleosts examined to date (cf. Manzon, 2002). Consistent with its role in maintaining ion balance, PRL reduces water permeability and increases ion uptake, at least in part, by upregulating NCC mRNA expression in the gills (Breves et al., 2010b). Another key component of osmoregulation in FW is the water channel AQP3 which has been shown to be involved in the movement of water across plasma membranes in the gills (Watanabe et al., 2005). It has been shown that the release of PRL from the pituitary is directly regulated by decreases in extracellular osmolality that accompany FW acclimation (Wigham et al., 1977; Grau et al., 1981; Helms et al., 1991; Borski et al., 1992; Seale et al., 2002; Seale et al., 2006b; Seale et al., 2012b; Seale et al., 2012a). In addition, circulating levels of PRL are higher in FW tilapia than SW tilapia (Yada et al., 1994; Seale et al., 2002; Seale et al., 2006b). When tilapia are moved from FW to SW, plasma PRL levels decrease; conversely, tilapia moved from SW to FW exhibit rapid increases in plasma PRL (Yada et al., 1994; Seale et al., 2002; Seale et al., 2006b; Seale et al., 2012b). My measurements of the steady-state plasma PRL levels and pituitary PRL gene expression in FW and SW are consistent with those of previous studies, showing an inverse relationship between PRL and plasma osmolality. I hypothesized that fish in the FW and SW phases of the tidal cycle would show a similar response in PRL levels compared with fish reared in FW and SW respectively, but plasma PRL levels in both TF and TS fish did not change with plasma osmolality or with environmental salinity, and were similar to the range observed in fish maintained in steady-state salinities (Chapter II). This result suggests that the inverse relationship

between osmolality and PRL does not apply to fish reared in a tidally changing salinity environment. Also, the finding that PRL levels did not change until fish were transferred to FW for at least 24 h suggests that as long as the tidal cycle is maintained, changes in circulating PRL may not be critical for survival, but future studies using hypophysectomy techniques would be needed to determine the necessity of PRL in a tidally-changing salinity.

The main findings of this study with respect to ion transporters and the gill are: 1) that fish in a tidally-changing salinity maintain gene expression of proteins involved in ion uptake at higher levels than steady-state SW fish, but much lower than those of FW fish, whereas the transcripts of ion extrusion-promoting molecules were found to be at levels which were similar to those of SW fish; and 2) in both phases of the tidal cycle, ionocytes in the gill of tidally-reared fish most closely resemble the ionocytes of SW fish. The results of both gene expression measurements and immunohistochemistry indicated that fish reared in a tidally-changing salinity maintain SW-type ionocytes in both phases of the tidal cycle. This is consistent with the similarity in trans-epithelial potential between killifish, *Fundulus heteroclitus*, raised in SW and a tidal environment (Wood and Grosell, 2009). Both TF and TS fish maintain ionocytes similar to that of SW fish and are adapted for ion extrusion, except for the dramatic decrease of CFTR in TF fish. A possible explanation for low CFTR immunoreactivity in TF is that the membrane trafficking of the CFTR protein is salinity dependent (Marshall and Singer, 2002). The low abundance of CFTR is important for survival in FW since fish need to actively uptake Cl- ions and those same ions would be lost by passive diffusion through CFTR channels. The rapid decrease in CFTR is likely part of the reason that TF fish are able to survive in FW for 6 h with very few FW-ionocytes. In addition to the changes in CFTR, I saw a fluctuation in branchial AQP3 expression between the two phases of the tidal cycle which suggest that it is important to change water permeability even if fish are only transferred to a different salinity for 6 h. These conclusions led to the work performed in Chapter III which focused on the effect that rearing condition had on the ability of tilapia to adapt to osmotic challenges.

Following the findings from the previous chapter I compared the osmoregulatory physiology of fish reared in different salinity regimes after they were transferred to either

FW or SW for 6 hours, 1 day, 2 days, or 7 days. The focus of this chapter was to compare the endocrine and osmoregulatory responses of Mozambique tilapia reared in either FW, SW, or in salinity that varied with tidal frequency between FW and SW when faced with an osmotic challenge. I found that PRL significantly increased only after TS fish had been held in FW for 24 h, a pattern that was paralleled in PRL gene expression in the pituitary. This suggests that there is a threshold between 6 h and 24 h post-FW exposure for which an elevation in plasma PRL becomes necessary to maintain osmotic balance.

I hypothesized that the similarities in ionocytes and mRNA expression of key ion transporters, pumps, and channels would provide tilapia reared in a tidally-changing salinity an enhanced ability to adapt to osmotic challenges compared with fish reared in FW or SW. It has been shown that exposing tilapia to an osmotic challenge both increases the mRNA expression of appropriate ion transporting proteins while reducing the mRNA expression of ion transporting proteins that would be maladaptive (Hiroi et al., 2005; Hiroi et al., 2008; Breves et al., 2010d; Tipsmark et al., 2011; Velan et al., 2011). Consistent with their role in ion extrusion, and in agreement with previous research, I observed increases in NKCC1a and CFTR when FW fish were transferred to SW (Inokuchi et al., 2008; Breves et al., 2010d). Likewise, the transfer from FW to SW brought about by decreases in the mRNA expression of NCC, NHE3, AQP3, and NKA α1a (Hiroi et al., 2005; Hiroi et al., 2008; Breves et al., 2010d; Tipsmark et al., 2011). In the experiment performed in Chapter III, the mRNA expression of proteins involved in ion and water transport showed less dramatic changes in fish transferred from a tidally-changing salinity to SW than was observed in FW fish transferred to SW, with the exception of CFTR. The increase in mRNA expression of CFTR was greater in fish transferred from a tidally-changing salinity to SW than in FW fish transferred to SW. The increases in CFTR expression in response to increases in salinity are consistent with CFTR's role in ion extrusion and with the widely accepted model of osmoregulation (Evans et al., 2005; Hwang et al., 2011). This suggests that a rapid and robust increase in CFTR may be crucial for successful adaptation to SW. Another important difference between FW fish transferred to SW which did not survive and TF fish transferred to SW which did survive was the change in AQP3 expression. While AQP3 decreased upon transfer from TF to SW, it did not change in fish transferred from FW to SW that did not

survive. The inability to decrease gene expression of AQP3 likely led them to excessive water loss, and the observed increase in plasma osmolality. By contrast, the blood osmolality of tidal fish did not rise to lethal levels upon transfer to SW, possibly due to the decrease in AQP3 gene expression. A limitation of this study was that I looked at mRNA data only for many of the endpoints in this study. Although increases in mRNA are likely related to increases in protein abundance and activity, additional studies using IHC or other techniques to measure protein levels and their cellular localization will be required to determine whether the responses are consistent between mRNA expression and protein abundance and activity.

Taking into account rearing conditions and life histories of each species is essential to determining the extent and plasticity of osmoregulatory ability. The results clearly demonstrate that the ability of Mozambique tilapia to adapt to osmotic challenges is dependent on their rearing salinity history and is facilitated by previous exposure to cyclical salinity variations. Applying the tilapia model to assess the ability of euryhaline species to adapt to osmotic challenges will expand our understanding of how environmental salinity modulates osmoregulatory physiology over a fish's life history.

From the samples obtained in the two chapters above, I analyzed growth, FCR, and endocrine parameters of growth for fish reared in FW, SW, and in a tidally-changing salinity. The high energetic cost of osmoregulation in fishes means that it is closely linked to the metabolic capacity for growth (Boeuf and Payan, 2001). This link is by no means trivial, inasmuch as tilapias are among the most important aquacultured fish worldwide. The results gained in Chapter IV of this dissertation demonstrated that rearing Mozambique tilapia in a tidally-changing salinity environment increased growth rates relative to fish reared in a steady-state salinity. Previous studies which have examined plasma GH levels in tilapia reared in different salinities have found varying results which suggests that plasma GH levels may be a poor indicator of growth (Riley et al., 2003; Pierce et al., 2007; Breves et al., 2011). Although plasma GH does not appear to vary in a consistent manner with rearing salinity, the study addressed in Chapter IV, along with others, show that pituitary GH expression is consistently higher in tilapia maintained in SW compared with those maintained in FW (Riley et al., 2003; Magdeldin et al., 2007; Breves et al., 2010c). The results indicate that fish collected in both the end of 6 h in the

FW phase and at end of 6 h in the SW phase of the tidal cycle had greater pituitary GH gene expression than those acclimated to steady-state SW or FW. IGF-I mediates many of GH's growth promoting actions in teleosts (McCormick et al., 1991; cf. Duan, 1997). This study found that IGF-I mRNA in the muscle is significantly higher in SW fish than in FW fish. Unlike SW and FW fish whose muscle IGF-I mRNA vary with salinity there was no significant difference between TF and TS fish. Mozambique tilapia reared under tidally-changing salinities grew faster than fish in the other salinity treatments with a lower FCR. The increased growth in fish reared in a tidally-changing salinity compared with fish in FW and SW are likely due to increases in plasma GH, pituitary GH mRNA expression, and GHR expression in the muscle and liver (Chapter IV). Optimization of rearing strategies for tilapiine species requires understanding the links between ionoregulatory demands and growth.

In summary, I have described in this dissertation the effects of rearing Mozambique tilapia in a tidally-changing salinity. During this process I have reported: 1) the similarity between the ionocytes and mRNA expression patterns of ion transporters, pumps, and channels between fish in both the FW and SW phases of the tidal cycle and fish reared in SW; 2) the enhanced ability of fish reared in a tidally-changing salinity to adapt to hyperosmotic challenges compared with their steady-state FW counterparts; 3) evidence that tilapia reared in a tidally-changing salinity grow faster than fish reared in FW or SW and that this difference may be related to a more efficient use of feed; and 4) the enhanced growth seen in this study is also associated with an upregulation of key components of the GH/IGF axis. These studies have provided a framework for future research geared at understanding the role of a tidally-changing environment on the physiology of tilapia and other euryhaline species.

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