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

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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. We 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; 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., 2010c). 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; Ouattara et al., 2009; Breves et al., 2010d; Breves et al., 2010e; Breves et al., 2010c; 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, we 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, we 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 pattern in greater depth, we 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, we 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, we measure a variety of osmoregulatory endpoints in the pituitary and gill. Specifically, we 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. Results

2.1 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. 1A). 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. 1B).

2.2 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. 2A). 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. 2B).

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were also observed on pituitary PRLR2 expression. The transfer of 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. 2C).

2.3 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. 3A).

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. 3B).

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. 4A).

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. 4B).

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. 4C).

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. 4D).

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. 5A).

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 branchial AQP3 expression was greater in TS fish transferred to FW than 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. 5C).

3. 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 environment, significantly improves the ability to survive the transfer to SW compared with that of FW-reared tilapia; 2) tilapia reared in either SW or a tidal salinity regime easily adapt when transferred to FW; 3) When transferred to FW, both SW and tidally-reared tilapia, show similar patterns of change in the branchial mRNA expression of effectors of ion transport measured; and 4) unlike the other effectors of ion transport measured, CFTR and AQP3 mRNA expression increased and decreased, respectively, 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., 2010e; 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., 2004; Wang et al., 2009; Breves et al., 2010d; Breves et al., 2010e; 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. We 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., 2010c).

PRL is essential 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., 2010c). 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). Recently, we found that Mozambique tilapia reared in tidally-changing salinities do not change plasma PRL with each phase of the tidal cycle. 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, we 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.

We 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, that the response of PRLR1 and PRLR2 expression differs not only according to the direction of salinity transfer, but also between the gill and pituitary. Previous studies have shown that branchial PRLR1 expression is stimulated when fish are transferred from SW to FW and suppressed when fish are transferred from FW to SW (Breves et al., 2010d; Breves et al., 2011). Furthermore, branchial *prlr1* levels decrease after hypophysectomy in SW but not in FW, suggesting that *prlr1* expression is regulated, at least in part, by the pituitary (Breves et al., 2010b). The expression of PRLR2, on the other hand, follows an inverse pattern, where increases are seen in gill and PRL cells under hyperosmotic conditions (Fiol et al., 2009). In the current study, FW fish transferred to SW decreased pituitary PRLR1 expression, while TF fish transferred to SW transiently increase PRLR1 expression at 6 h before decreasing. Conversely, in transfers from both SW and TS to FW, there was a rise in PRLR1 expression at 48 h before subsiding to pre-transfer levels, or lower, by 7 d. By contrast, in gill, the response of PRLR1 expression to transfers to FW and SW were more pronounced than that in pituitary. This difference in expression pattern may be related to the differences in the role of PRL between the pituitary, where it is produced, and gill, a main target for the regulation of effectors of ion transport.

In both pituitary and gill, PRLR2 mRNA expression increased in FW and TF fish transferred to SW, although the response from fish reared in a tidally changing environment was less prominent. This range of responses could be tied to the changes seen in plasma osmolality, which climb to much higher levels following FW to SW transfers than from TF to SW. We have previously found that PRLR2 expression in dispersed PRL cells is directly proportional to increases in medium osmolality (Seale et al., 2012). The significance of this tight regulation by extracellular osmolality may be tied to the involvement of this PRLR isoform in the cellular remodeling of ionocytes required during SW acclimation. Two splice variants have been described for PRLR2, a short non-functional variant and a long functional variant (Fiol et al., 2009). It was proposed that the transient increase in PRLR2 after fish are transferred from FW to SW provides an increase in osmotolerance which supports cell survival during the critical time when the gill epithelium is being restructured for the change in direction of ion transport (Fiol et al., 2009). On the other hand, in the pituitary, it has been suggested that PRL secretion may be

subject to negative feedback from PRL (Nagahama et al., 1975). Based on the differential regulation of pituitary PRLR1 and PRLR2 by extracellular osmolality, we have previously proposed that autocrine regulation of PRL cell activity through PRLRs, may serve to fine tune the effects of PRL (Seale et al., 2012b). Thus, similar to the role of PRLR2 suggested in gill, the increase in PRLR2 expression in tilapia PRL cells in response to hyperosmotic conditions could be a mechanism to maintain low plasma PRL levels in a hyperosmotic environment.

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., 2010e; Tipsmark et al., 2011; Velan et al., 2011). NKA provides the driving force for ion transport within ionocytes. In salmon, NKA α1a expression is higher in FW than in SW and NKA α1b expression is higher in SW than in FW (Madsen et al., 2009; McCormick et al., 2009). This pattern of salinity-dependent NKA isoform expression was also described in tilapia (Tipsmark et al., 2011). Consistent with previous experiments in tilapia, our current experiment showed higher NKA α1a expression in FW- than in SW-acclimated fish (Tipsmark et al., 2011; Moorman et al., 2014). Likewise, NKA α1a expression increased when fish were transferred from SW to FW and decreased when fish were transferred from FW to SW (Tipsmark et al., 2011; Moorman et al., 2014). Changes in NKA α1b expression, however, have not been as consistent among past studies in tilapia. While Tipsmark and co-workers (2011) found NKA α1b expression in the gill to increase by 1 day in fish that were transferred from FW to SW, our previous study found that steady-state branchial NKA α1b expression was higher in FW fish than in SW fish (Tipsmark et al., 2011; Moorman et al., 2014). While differences in the approach between both studies have been discussed (Moorman et al., 2014), the nature of this discrepancy remains uncertain, as in the current experiment both NKA isoforms changed following a similar pattern.

Previous experiments have shown that mRNA expression of NHE3 is higher in FW than in SW fish, which is consistent with its role in ion uptake (Inokuchi et al., 2008; Watanabe et al., 2008; Moorman et al., 2014). We observed that branchial mRNA expression of NCC, NKA α1a, NKA α1b, and NHE3 decreased more rapidly in fish transferred from FW to SW than in from TF to SW. This suggests that the rapid decrease in mRNA expression of effectors of ion uptake was not enough to maintain survival during acute hyperosmotic challenge. By contrast, a close

examination of the response patterns of branchial mRNA expression of CFTR and AQP3 may underlie the failure of tilapia to survive an acute transfer to SW.

Consistent with their role in ion extrusion, and in agreement with previous research, we observed increases in NKCC1a and CFTR when FW fish were transferred to SW (Inokuchi et al., 2008; Breves et al., 2010e). While branchial NKCC expression increased in fish transferred from FW to SW by 6 h, it took 24h for a significant rise in NKCC expression to be observed in fish transferred from TF to SW. This could be partially explained by the lower baseline expression levels of NKCC in fish in FW relative to those in TF. We have also shown that NKCC immunofluorescence is maintained at similar intensities between both phases of the tidal cycle and close to those observed in SW fish (Moorman et al., 2014). At any rate, NKCC expression levels were similar between FW and TF fish transferred to SW, but only the fish that were reared in tidal conditions survived the transfer. By contrast, branchial CFTR expression changed rapidly and dramatically in from both steady-state and tidal salinities, and in both directions of transfer, indicating a critical role in acclimating to acute changes in salinity. Consistent with its role in providing a conduit for Cl^- extrusion in hyperosmotic environments (c.f. (Marshall and Singer, 2002), CFTR expression increased by 6 h following a transfer from FW and TF to SW and decreased by 6 h following a transfer from SW and TS to FW. These early responses in CFTR transcription are consistent with our previous immunofluorescence observations showing that CFTR signal intensity changed between the two phases of the tidal cycle (Moorman et al., 2014). Together, these data suggest that changes in CFTR expression elicit early responses that are immediately important when facing osmotic challenges and hence crucial for surviving a hyperosmotic challenge. Changes in NKCC, on the other hand, are slower and appear to be involved in a later response necessary for extended acclimation to a hyperosmotic environment.

Aquaporin 3 is a member of a family of integral membrane pore proteins that facilitate water transport across cell membranes (Agre et al., 2002). AQP3 has been localized in the basolateral membrane of ionocytes in the gill, which is consistent with its proposed role in cellular volume regulation and as an osmosensor (Watanabe et al., 2005; Madsen et al., 2014). A study using the European eel, *Anguilla anguilla*, has shown that AQP3 is crucial for maintaining water balance in FW and that when fish are transferred to SW, branchial AQP3 expression is downregulated (Cutler and Cramb, 2002). A study of Japanese medaka, *Oryzias latipes*, showed

that branchial mRNA expression of AQP3 was higher in FW than in SW fish (Madsen et al., 2014). High branchial AQP3 expression in a hyperosmotic environment, therefore, could lead to excessive water loss, and consequent increase in plasma osmolality to lethal levels. Similarly, the inability of tilapia to reduce AQP3 during an acute transfer from FW to SW could have contributed to the failure to lower plasma osmolality and subsequent mortality. By contrast, upon transfer of tidal fish to SW, AQP3 gene expression became reduced and plasma osmolality did not rise to lethal levels. While this characterization is limited to transcriptional regulation, it provides insight into future studies aimed at characterizing the contribution of aquaporins to salt and water balance during salinity acclimation.

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.

4. Materials and Methods

4.1 Fish Rearing

Mozambique tilapia (*Oreochromis mossambicus*) yolk-sac larvae 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. The fish 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 fish 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) *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 over the course of 3 h 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. 6). 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.

4.2 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.

4.3 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., 2010c); 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.

4.4 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

493 homologous radioimmunoassay (RIA) as described by Ayson and colleagues (Ayson et al.,
494 1993).
495
496 *4.5 Statistical Analysis*
497
498 Statistical analyses were conducted by two-way analysis of variance (ANOVA) with
499 salinity rearing regime and time as the independent variables. Significant main effects of salinity
500 rearing regime and time ($P<0.05$) were followed up by the Fisher LSD test. Data are expressed as
501 means ± S.E.M. When necessary, individual values were log-transformed to meet assumptions of
502 normality and equal variance. Each replicate experiment was analyzed individually and after
503 determining that the results were consistent between the two experiments the data were
504 combined. Statistical calculations were performed using a statistical software program, Prism 6.0
505 (GraphPad, La Jolla, CA, USA).
506

SYMBOLS AND ABBREVIATIONS

AQP3 = Aquaporin 3

BW = Brackish water

CFTR = Cystic fibrosis transmembrane conductance regulator

EF1α = Elongation Factor 1α

FW = Fresh water

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

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COMPETING INTERESTS

No competing interests are declared.

AUTHOR CONTRIBUTIONS

B.P.M. conducted the experiments, analyzed the data, and wrote the manuscript. A.P.S. conducted experiments and assisted with writing the manuscript. All authors were involved in the conception and design of the experiments, data interpretation, and critical revisions of the manuscript.

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Figure Legends:

Figure 1: **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 2: **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 3: **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, $P < 0.05$).

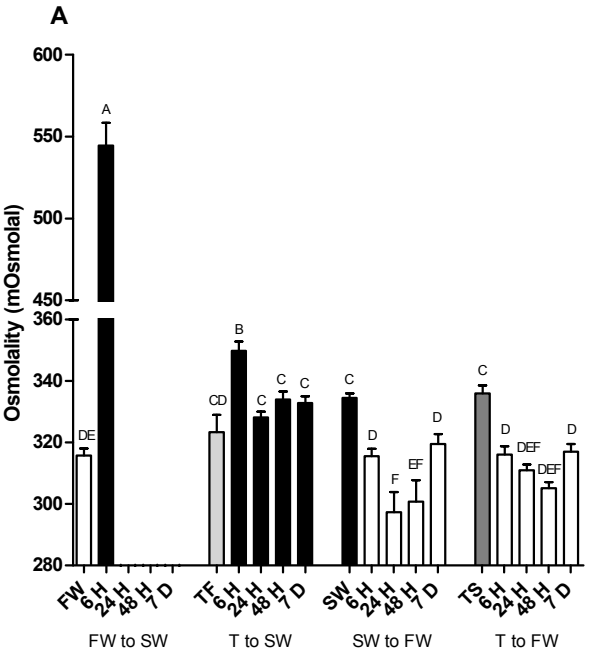
Figure 4: **Effects of acute salinity challenges on branchial NCC mRNA expression (A), NKCC1a mRNA expression (B), NKA α1a mRNA expression (C), and NKA α1b mRNA expression (D) in Mozambique tilapia (*Oreochromis mossambicus*) transferred from fresh water (FW) to seawater (SW), a tidally-changing salinity 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 5: **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 6: **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 1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24 H
48 H
7 D
FW to SW
T to SW
SW to FW
T to FW

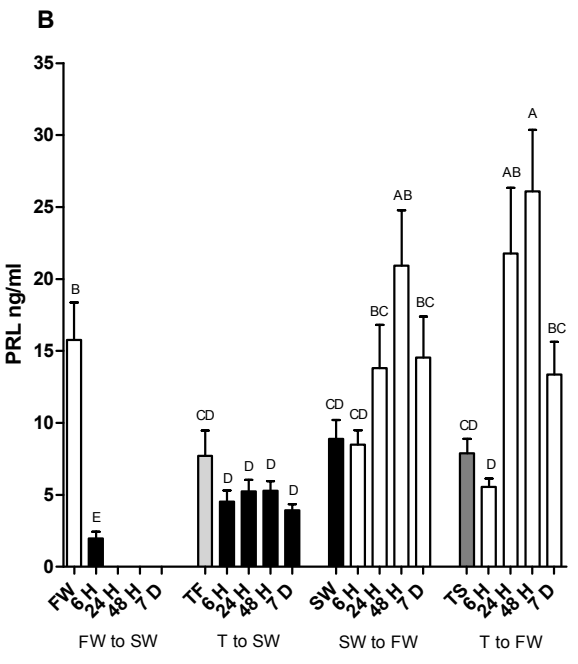
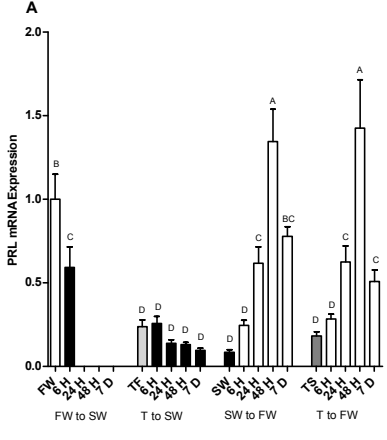
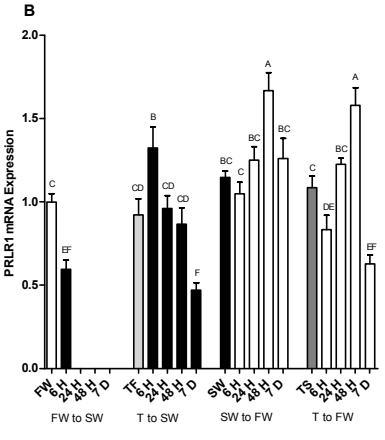

B
PRL ng/ml
35
30
25
20
15
10
5
0
B
E
CD
D
D
D
D
CD
CD
BC
AB
BC
CD
D
AB
A
BC
FW
6 H
24 H
48 H
7 D
TF
6 H
24 H
48 H
7 D
SW
6 H
24 H
48 H
7 D
TS
6 H
24 H
48 H
7 D
FW to SW
T to SW
SW to FW
T to FW

Figure 2


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
2.0
1.5
1.0
0.5
0.0
FW to SW
T to SW
SW to FW
T to FW

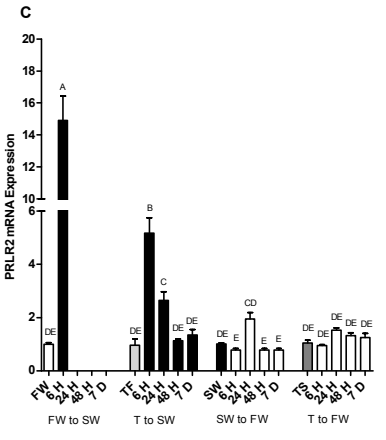
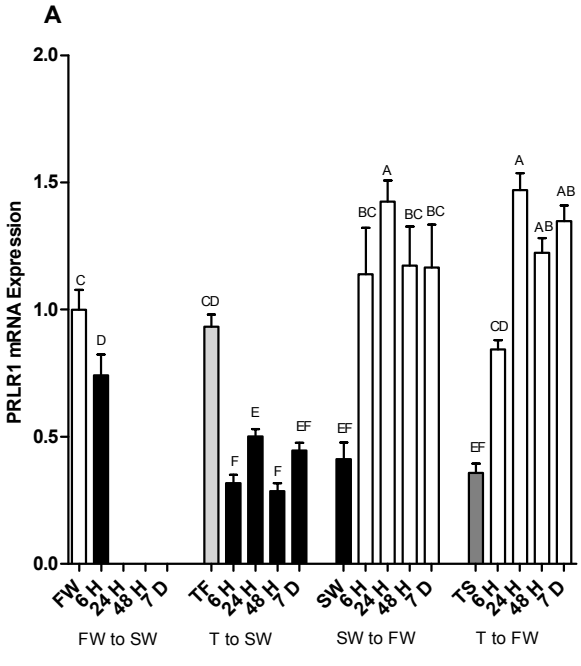

C
PRLR2 mRNA Expression
20
18
16
14
12
10
6
4
2
0
FW to SW
T to SW
SW to FW
T to FW

Figure 3


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

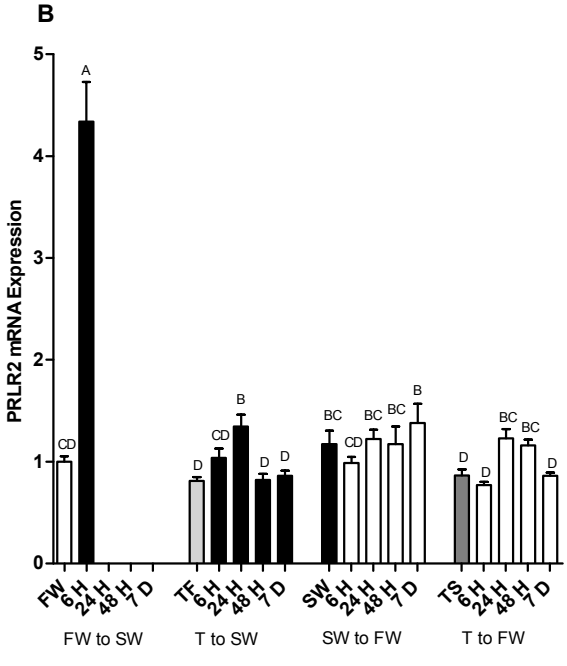
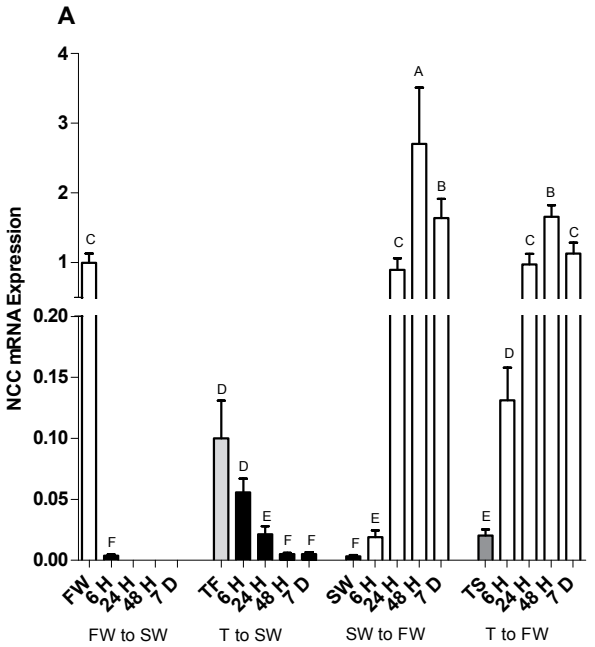
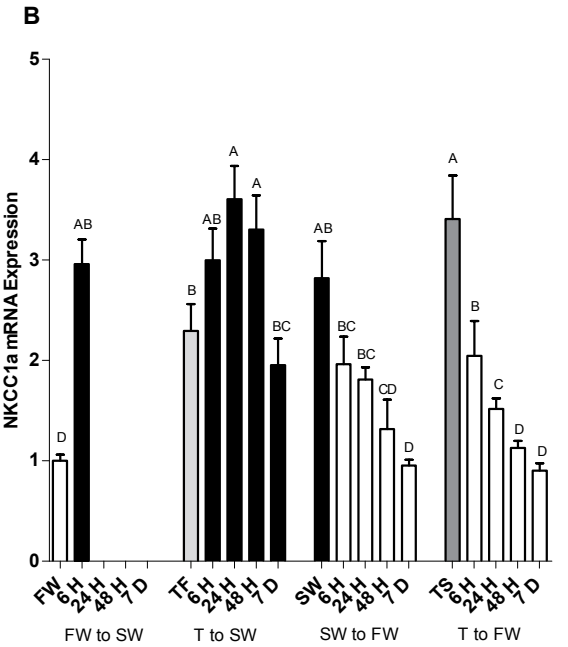
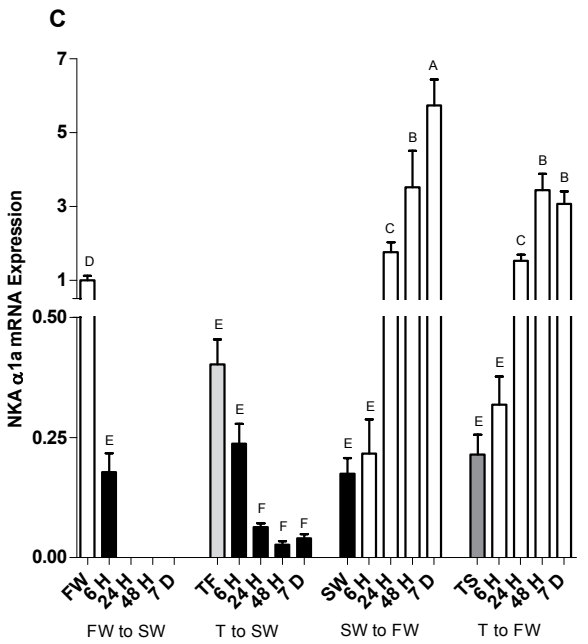

B
PRLR2 mRNA Expression
5
4
3
2
1
0
A
CD
D
CD
B
D
D
BC
CD
BC
BC
B
D
D
BC
BC
D
FW
6 H
24 H
48 H
7 D
TF
SW
TS
FW to SW
T to SW
SW to FW
T to FW

Figure 4


A
NCC mRNA Expression
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
NKCC1a mRNA Expression
FW to SW
T to SW
SW to FW
T to FW


C
NKA α1a mRNA Expression
FW to SW
T to SW
SW to FW
T to FW

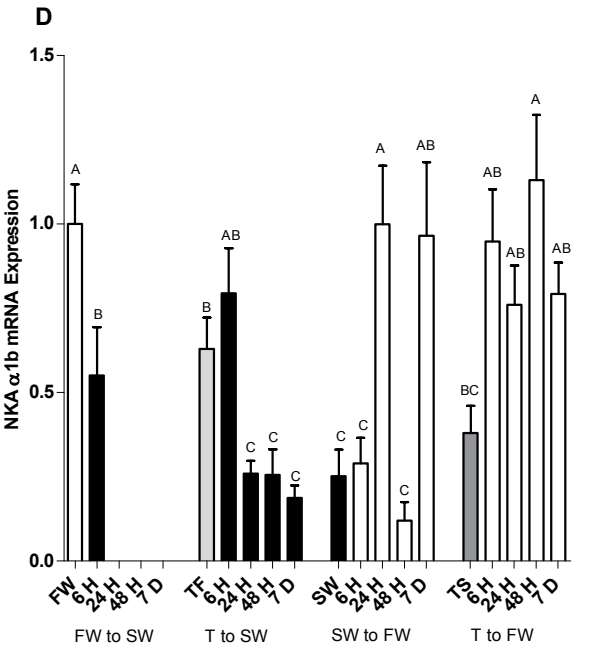
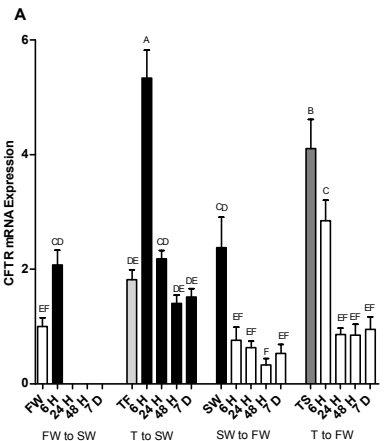
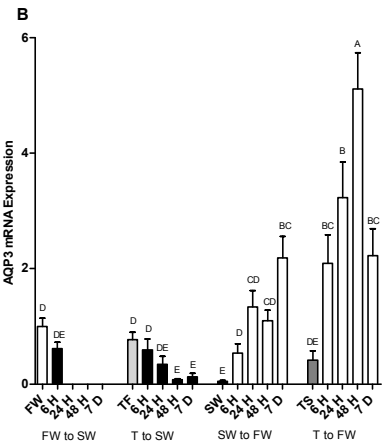

D
NKA α1b mRNA Expression
FW to SW
T to SW
SW to FW
T to FW

Figure 5


A
CFTR mRNA Expression
6
4
2
0
EF
CD
DE
A
CD
DE
DE
CD
EF
EF
F
EF
B
C
EF
EF
EF
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
AQP3 mRNA Expression
6
4
2
0
D
DE
D
D
DE
E
E
E
D
CD
CD
BC
DE
BC
B
B
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

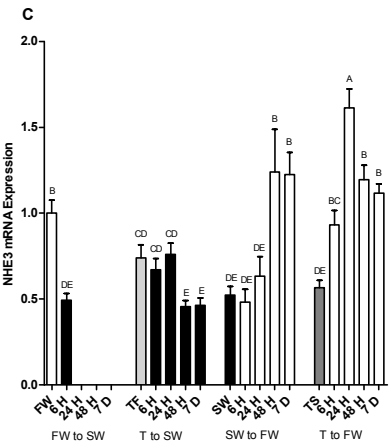
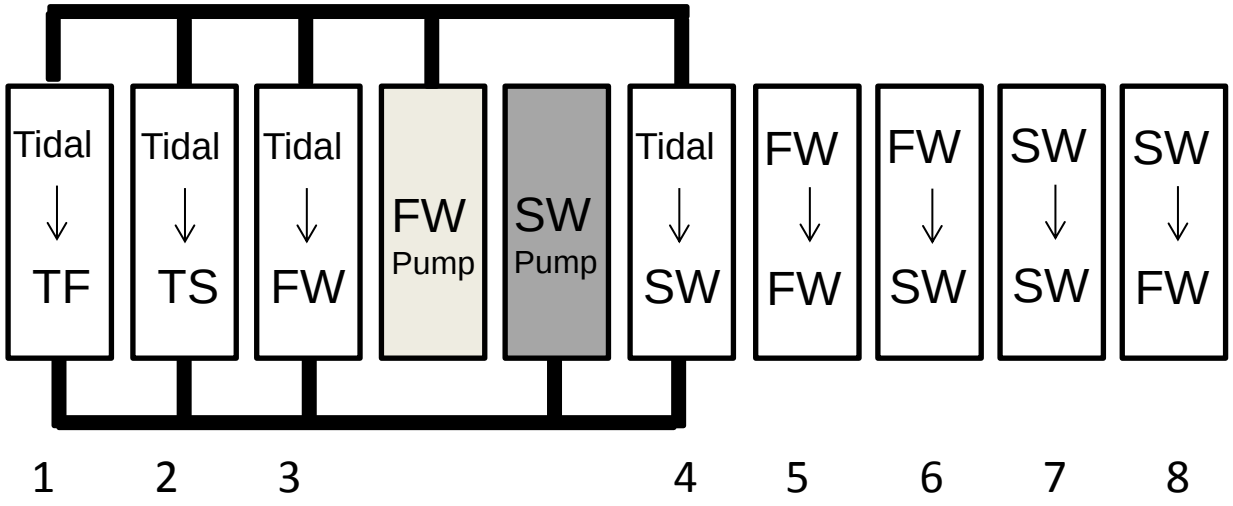

C
NHE3 mRNA Expression
2.0
1.5
1.0
0.5
0.0
B
DE
CD
CD
CD
E
E
DE
DE
DE
B
B
DE
BC
A
B
B
FW
6 H
24 H
48 H
7 D
TF
SW
TS
FW to SW
T to SW
SW to FW
T to FW

Figure 6


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