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RESEARCH ARTICLE

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 freshwater (FW), in 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^- co-transporter (NCC), $Na^+/K^+/2Cl^-$ co-transporter (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 6 h 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.

KEY WORDS: Ion transporter, Osmoregulation, Prolactin, Rearing salinity, Tidal cycle, Tilapia

INTRODUCTION

The rigorous control of osmotic homeostasis is essential to life for most vertebrates. Teleost fish in freshwater (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 (cf. 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 (cf. Marshall and Grosell, 2006). Mozambique tilapia, *Oreochromis mossambicus* (Peters 1852), 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, 2006b, 2012c). Conversely, Mozambique tilapia transferred from FW to brackish water (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 as the primary site of net ion transport by the activities of highly specialized ionocytes (Kaneko et al., 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^- co-transporter (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 $Na^+/K^+/2Cl^-$ co-transporter (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 the 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-acclimated fish, while NKAα1b expression has been

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List of abbreviations

AQP3	aquaporin 3
BW	brackish water
CFTR	cystic fibrosis transmembrane conductance regulator
EF1α	elongation factor 1α
FW	fresh water
NCC	Na^+/Cl^- co-transporter
NHE3	Na^+/H^+ exchanger 3
NKA	Na^+/K^+-ATPase
NKCC	$Na^+/K^+/2Cl^-$ co-transporter
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

shown to be greater or unchanged in SW-acclimated fish when compared with FW-acclimated 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 et al., 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, 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, 2006b, 2012b; Inokuchi et al., 2008, 2009; Ouattara et al., 2009; Breves et al., 2010b,c,d, 2011; Velan et al., 2011). While the effects of a 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 rearing fish 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 measured 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, NKAα1a, NKAα1b, CFTR, AQP3 and NHE3 in fish reared in FW, SW or tidally changing salinities faced with acute salinity challenges.

RESULTS

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 days (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^{-1} to 2 ng ml^{-1} within 6 h. Plasma PRL levels of TF fish transferred to SW, in contrast, were not significantly different at any of the time points between 6 h and 7 days. When transferred to FW, plasma PRL in both SW- and TS-reared fish increased sharply by 48 h, but by 7 days, though still well above pre-transfer levels, plasma PRL had declined to levels that were similar to those of FW-reared fish (Fig. 1B).

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

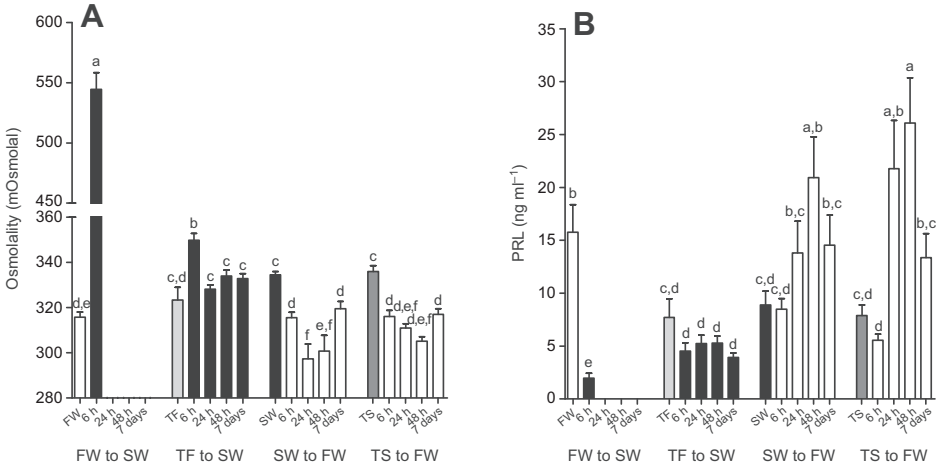

Fig. 1. Effects of acute salinity challenges on plasma osmolality and plasma prolactin in Mozambique tilapia (*Oreochromis mossambicus*). (A) Plasma osmolality; (B) plasma prolactin (PRL). Fish were transferred from freshwater (FW) to seawater (SW), a tidally changing salinity (TF, see Materials and methods and Fig. 6) to SW, SW to FW and a tidally changing salinity (TS) to FW, and were sampled 6 h, 24 h, 48 h and 7 days 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's LSD test, $P<0.05$).

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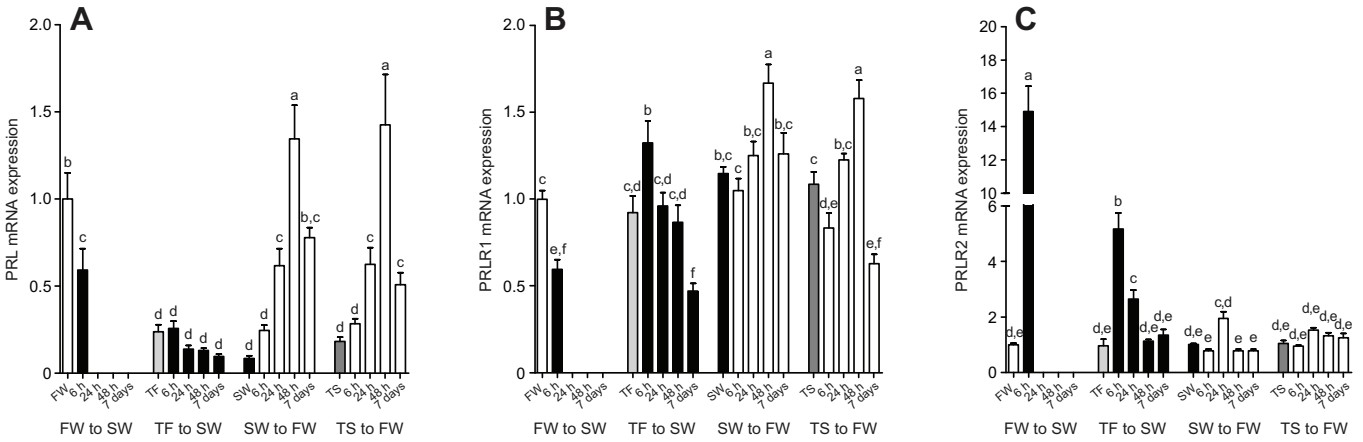

Fig. 2. Effects of acute salinity challenges on pituitary PRL and PRL receptor (PRLR) mRNA expression in Mozambique tilapia. (A) PRL mRNA expression; (B) PRLR1 mRNA expression; and (C) PRLR2 mRNA expression. Fish were transferred from FW to SW, TF to SW, SW to FW and TS to FW, and were sampled 6 h, 24 h, 48 h and 7 days 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's LSD test, $P<0.05$).

PRL expression in FW fish was 4-fold greater than that in TF and TS fish and was 10-fold greater than that 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 days to about half 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 days. By contrast, PRLR1 expression in TS fish transferred to FW fell to half steady-state FW and SW levels after 7 days (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 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).

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 at 6 h to levels that were similar to those of steady-state SW fish. The transfer of both SW and TS fish to FW produced an increase in PRLR1 expression with a peak at 24 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 and 48 h, with a return to initial TS levels after 7 days (Fig. 3B).

Significant effects of salinity rearing regime ($P<0.0001$) and time ($P<0.0001$) were observed on branchial NCC expression. The

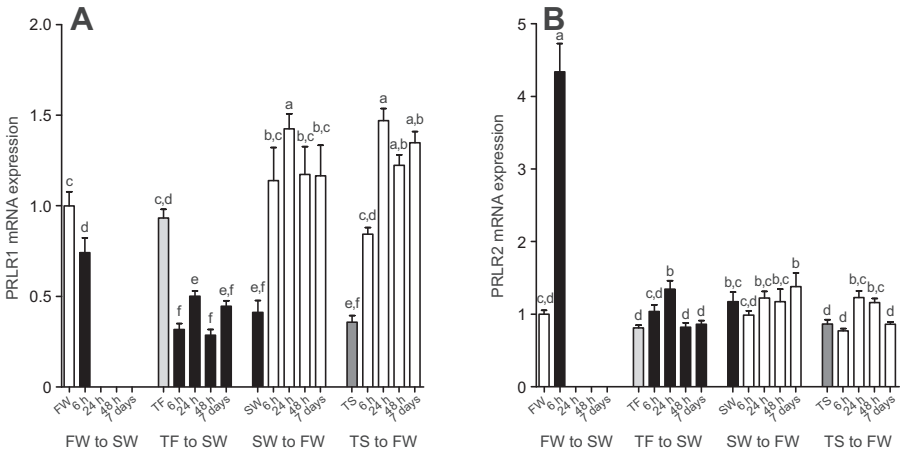

Fig. 3. Effects of acute salinity challenges on branchial PRLR mRNA expression in Mozambique tilapia. (A) PRLR1 mRNA expression; (B) PRLR2 mRNA expression. Fish were transferred from FW to SW, TF to SW, SW to FW and TS to FW, and were sampled 6 h, 24 h, 48 h and 7 days after the salinity transfers. Values are expressed as means±s.e.m. (*N*=10–20). Means not sharing the same letter are significantly different (two-way ANOVA, Fisher's LSD test, $P<0.05$).

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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 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 NKCC1a expression. The transfer of FW fish to SW produced a 3-fold increase in NKCC1a expression at 6 h. The transfer of TF fish to SW produced a 1.5-fold increase in NKCC1a expression that peaked at 24 h. The transfer of both SW and TS fish to FW brought about a fall in the expression of NKCC1a 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 NKAα1a expression. The transfer of FW fish to SW produced an 80% decline in NKAα1a expression at 6 h. NKAα1a expression in TF fish was 50% of that seen in FW fish before the start of the transfer, and by 48 h the expression in TF fish decreased to 1/10th of its initial expression levels. The transfer of both SW and TS fish to FW produced a 10-fold increase in NKAα1a expression by 24 h, which continued to increase through to 7 days after transfer. The peak expression of NKAα1a was greater in SW fish than in TS fish when both were transferred to FW (Fig. 4C).

Significant effects of salinity rearing regime (P<0.0001) were observed on branchial NKAα1b expression. The transfer of FW fish to SW brought about a fall in the expression of NKAα1b within 6 h. The transfer of TF fish to SW caused a significant reduction in NKAα1b expression, but not until 24 h after transfer. The transfer of SW fish to FW produced a 4-fold increase in NKAα1b expression at 24 h and at 7 days 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 NKAα1b at 6 h, a rise that was maintained through to 7 days (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 on 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 days. 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. 5B).

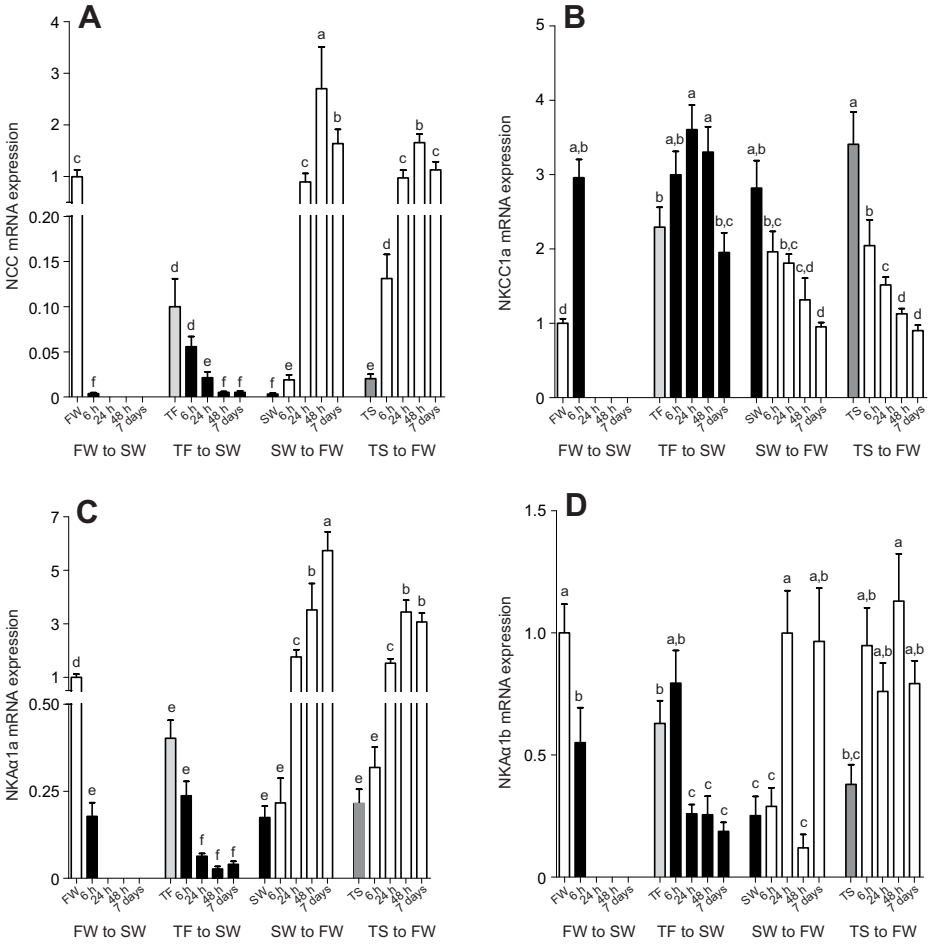

Fig. 4. Effects of acute salinity challenges on branchial expression of ion transporters and pumps in Mozambique tilapia. (A) NCC mRNA expression, (B) NKCC1a mRNA expression, (C) NKAα1a mRNA expression and (D) NKAα1b mRNA expression. Fish were transferred from FW to SW, TF to SW, SW to FW and TS to FW, and were sampled 6 h, 24 h, 48 h and 7 days 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's LSD test, P<0.05).

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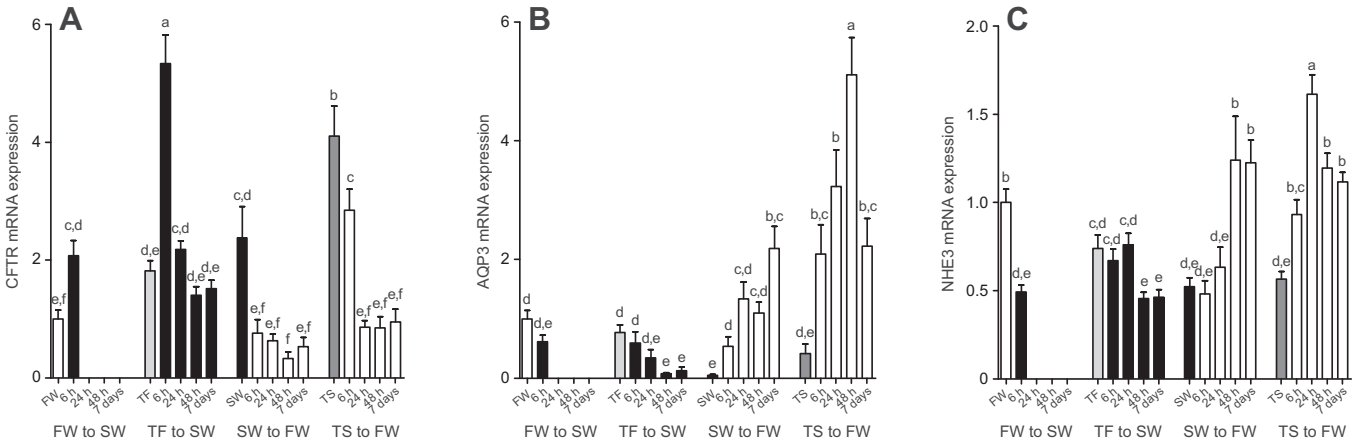

Fig. 5. Effects of acute salinity challenges on branchial expression of ion and water channels in Mozambique tilapia. (A) CFTR mRNA expression; (B) AQP3 mRNA expression; and (C) NHE3 mRNA expression. Fish were transferred from FW to SW, TF to SW, SW to FW and TS to FW, and were sampled 6 h, 24 h, 48 h and 7 days 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's LSD test, P<0.05).

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 a half in 6 h. The transfer of TF fish to SW was without significant effect on NHE3 expression at 6 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 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 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).

DISCUSSION

The principal objective of the present study was to compare endocrine and osmoregulatory responses of Mozambique tilapia reared in 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 and its receptors as well as on osmoregulatory elements of the gill. This was carried out 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 their 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, the 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 is dependent on the rate at which the fish 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, 2012b; Breves et al., 2010d). It has been shown through many studies that the survival threshold is between 450 and 550 mOsmolal (Yada et al., 1994; Seale et al., 2002, 2012b; Kajimura et al., 2004; Wang et al., 2009; Breves et al., 2010c,d). Tilapia reared in a tidally changing salinity and then exposed to SW for 7 days showed no mortality, suggesting that they were completely acclimated to SW. We have observed previously that the plasma osmolality of tilapia reared in a tidally changing salinity fluctuated between 320 and 345 mOsmolal in the FW and SW phases of the cycle, respectively (Moorman et al., 2014). Previous experiments have shown that 320 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 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., 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, 2006b, 2012a,b). Likewise, circulating plasma PRL levels in FW tilapia are higher than in SW tilapia (Yada et al., 1994; Seale et al., 2002, 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, 2006b, 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, the response of PRLR1 and PRLR2 expression differs

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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., 2010c, 2011). Furthermore, branchial PRLR1 mRNA 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, in contrast, 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 increased 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 days. 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 climbs 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., 2012b). 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 that 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). In the pituitary, however, 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 the 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 and reduces the mRNA expression of ion transporting proteins that would be maladaptive (Hiroi et al., 2005, 2008; Breves et al., 2010d; Tipsmark et al., 2011; Velan et al., 2011). NKA provides the driving force for ion transport within ionocytes. In salmon, NKAα1a expression is higher in FW than in SW and NKAα1b 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 NKAα1a expression in FW- than in SW-acclimated fish (Tipsmark et al., 2011; Moorman et al., 2014). Likewise, NKAα1a 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 NKAα1b expression, however, have not been as consistent among past studies in tilapia. While Tipsmark and co-workers (2011) found NKAα1b 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 NKAα1b expression was higher in FW fish than in SW fish (Tipsmark et al., 2011; Moorman et al., 2014). While differences in the approach between the two studies have been discussed (Moorman et al., 2014), the nature of this discrepancy remains uncertain, as in the current experiment the two 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, NKAα1a, NKAα1b and NHE3 decreased more rapidly in fish transferred from FW to SW than 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., 2010d). While branchial NKCC expression increased in fish transferred from FW to SW by 6 h, it took 24 h 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 the two 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 fish 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 (cf. Marshall and Singer, 2002), CFTR expression increased by 6 h following 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, in contrast, are slower and appear to be involved in a later response necessary for extended acclimation to a hyperosmotic environment.

AQP3 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*

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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 a 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 observation 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 the rearing conditions and life histories of each species is essential for 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.

MATERIALS AND METHODS

Fish rearing

Mozambique tilapia (*O. 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^{-1}) and stocked at a density of 100 fish per tank (mean mass, 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 12 h light:12 h dark cycle. Fish were fed crushed Silver Cup Flake food (Silver Cup, Harrietta, MI, USA) *ad libitum* daily. After 2 days in FW, six of the eight tanks were transitioned from FW to BW (10‰) over the course of 3 h, composed of SW (35‰ Kaneohe Bay, HI, USA) diluted with FW. After an additional 2 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 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 4 months. After 4 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 days after the transfer. TF fish and TS fish refer to fish sampled 30 min 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.

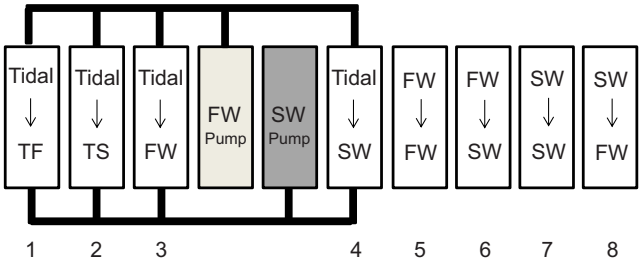

Fig. 6. Illustration of the tank setup for the experiment showing the initial salinity of the tank and the transferred condition. Fish were reared in FW, SW or a tidal environment (Tidal), which alternated between FW and SW every 6 h. The sampling conditions were FW, SW, and a tidal condition with the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

Treatments and sampling

At the time of sampling, fish were netted and anesthetized with 2-phenoxyethanol (0.3 ml l^{-1}). Blood was collected with a needle and syringe coated with sodium heparin (200 U ml^{-1}, 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.

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, USA). Using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), 500 ng of total RNA was reverse transcribed into cDNA. The qRT-PCR reactions 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% (elongation factor 1α, 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% (NKAα1a), 0.997 and 98.1% (NKAα1b), 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); NKAα1a and NKAα1b (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 µl) contained 7.5 µl of 2× Power SYBR Green PCR Master Mix (Life Technologies), 200 nmol l^{-1} 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.

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 RPD 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 (1993).

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 by Fisher's 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).

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Competing interests

The authors declare no competing or financial interests.

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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References

Agre, P., King, L. S., Yasui, M., Guggino, W. B., Ottersen, O. P., Fujiyoshi, Y., Engel, A. and Nielsen, S. (2002). Aquaporin water channels–from atomic structure to clinical medicine. *J. Physiol.* **542**, 3-16.

Ayson, F. G., Kaneko, T., Tagawa, M., Hasegawa, S., Grau, E. G., Nishioka, R. S., King, D. S., Bern, H. A. and Hirano, T. (1993). Effects of acclimation to hypertonic environment on plasma and pituitary levels of two prolactins and growth hormone in two species of tilapia, *Oreochromis mossambicus* and *Oreochromis niloticus*. *Gen. Comp. Endocrinol.* **89**, 138-148.

Berdan, E. L. and Fuller, R. C. (2012). Interspecific divergence of ionoregulatory physiology in killifish: insight into adaptation and speciation. *J. Zool.* **287**, 283-291.

Bern, H. A. (1983). Functional evolution of prolactin and growth hormone in lower vertebrates. *Am. Zool.* **23**, 663-671.

Borski, R. J., Hansen, M. U., Nishioka, R. S. and Grau, E. G. (1992). Differential processing of the 2 prolactins of the tilapia (*Oreochromis mossambicus*) in relation to environmental salinity. *J. Exp. Zool.* **264**, 46-54.

Breves, J. P., Hirano, T. and Grau, E. G. (2010a). Ionoregulatory and endocrine responses to disturbed salt and water balance in Mozambique tilapia exposed to confinement and handling stress. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **155**, 294-300.

Breves, J. P., Watanabe, S., Kaneko, T., Hirano, T. and Grau, E. G. (2010b). Prolactin restores branchial mitochondrion-rich cells expressing Na+/Cl– cotransporter in hypophysectomized Mozambique tilapia. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **299**, R702-R710.

Breves, J. P., Fox, B. K., Pierce, A. L., Hirano, T. and Grau, E. G. (2010c). Gene expression of growth hormone family and glucocorticoid receptors, osmosensors, and ion transporters in the gill during seawater acclimation of Mozambique tilapia, *Oreochromis mossambicus*. *J. Exp. Zool. A Ecol. Genet. Physiol.* **313A**, 432-441.

Breves, J. P., Hasegawa, S., Yoshioka, M., Fox, B. K., Davis, L. K., Lerner, D. T., Takei, Y., Hirano, T. and Grau, E. G. (2010d). Acute salinity challenges in Mozambique and Nile tilapia: differential responses of plasma prolactin, growth hormone and branchial expression of ion transporters. *Gen. Comp. Endocrinol.* **167**, 135–142.

Breves, J. P., Seale, A. P., Helms, R. E., Tipsmark, C. K., Hirano, T. and Grau, E. G. (2011). Dynamic gene expression of GH/PRL-family hormone receptors in gill and kidney during freshwater-acclimation of Mozambique tilapia. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **158**, 194-200.

Cutler, C. P. and Cramb, G. (2002). Branchial expression of an aquaporin 3 (AQP-3) homologue is downregulated in the European eel *Anguilla anguilla* following seawater acclimation. *J. Exp. Biol.* **205**, 2643-2651.

Dharmamba, M., Handin, R. I., Nandi, J. and Bern, H. A. (1967). Effect of prolactin on freshwater survival and on plasma osmotic pressure of hypophysectomized *Tilapia mossambica*. *Gen. Comp. Endocrinol.* **9**, 295-302.

Dharmamba, M., Mayer-Gostan, N., Maetz, J. and Bern, H. A. (1973). Effect of prolactin on sodium movement in *Tilapia mossambica* adapted to sea water. *Gen. Comp. Endocrinol.* **21**, 179-187.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.

Fiess, J. C., Kunkel-Patterson, A., Mathias, L., Riley, L. G., Yancey, P. H., Hirano, T. and Grau, F. G. (2007). Effects of environmental salinity and temperature on osmoregulatory ability, organic osmolytes, and plasma hormone profiles in the Mozambique tilapia (*Oreochromis mossambicus*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **146**, 252-264.

Fiol, D. F., Sanmarti, E., Sacchi, R. and Kültz, D. (2009). A novel tilapia prolactin receptor is functionally distinct from its paralog. *J. Exp. Biol.* **212**, 2007-2015.

Grau, E. G., Nishioka, R. S. and Bern, H. A. (1981). Effects of osmotic pressure and calcium ion on prolactin release in vitro from the rostral pars distalis of the tilapia *Sarotherodon mossambicus*. *Gen. Comp. Endocrinol.* **45**, 406-408.

Heijden, A., Verbost, P., Eygensteyn, J., Li, J., Bonga, S. and Flik, G. (1997). Mitochondria-rich cells in gills of tilapia (*Oreochromis mossambicus*) adapted to fresh water or sea water: quantification by confocal laser scanning microscopy. *J. Exp. Biol.* **200**, 55-64.

Helms, L. M. H., Grau, E. G. and Borski, R. J. (1991). Effects of osmotic pressure and somatostatin on the cAMP messenger system of the osmosensitive prolactin cell of a teleost fish, the tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* **83**, 111-117.

Hiroi, J. and McCormick, S. D. (2012). New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. *Respir. Physiol. Neurobiol.* **184**, 257-268.

Hiroi, J., McCormick, S. D., Ohtani-Kaneko, R. and Kaneko, T. (2005). Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na+/K+-ATPase, Na+/K+/2Cl– cotransporter and CFTR anion channel. *J. Exp. Biol.* **208**, 2023-2036.

Hiroi, J., Yasumasu, S., McCormick, S. D., Hwang, P.-P. and Kaneko, T. (2008). Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. *J. Exp. Biol.* **211**, 2584-2599.

Inokuchi, M., Hiroi, J., Watanabe, S., Lee, K. M. and Kaneko, T. (2008). Gene expression and morphological localization of NHE3, NCC and NKCC1a in branchial mitochondria-rich cells of Mozambique tilapia (*Oreochromis mossambicus*) acclimated to a wide range of salinities. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **151**, 151-158.

Inokuchi, M., Hiroi, J., Watanabe, S., Hwang, P.-P. and Kaneko, T. (2009). Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia. *J. Exp. Biol.* **212**, 1003-1010.

Kajimura, S., Hirano, T., Moriyama, S., Vakkuri, O., Leppaluoto, J. and Grau, E. G. (2004). Changes in plasma concentrations of immunoreactive ouabain in the tilapia in response to changing salinity: is ouabain a hormone in fish? Gen. *Comp. Endocrinol.* **135**, 90-99.

Kaneko, T., Watanabe, S. and Lee, K. M. (2008). Functional morphology of mitochondrion-rich cells in euryhaline and stenohaline teleosts. *Aqua BioSci. Monogr.* **1**, 1-62.

Madsen, S. S., Kiilerich, P. and Tipsmark, C. K. (2009). Multiplicity of expression of Na+,K+-ATPase alpha-subunit isoforms in the gill of Atlantic salmon (*Salmo salar*): cellular localisation and absolute quantification in response to salinity change. *J. Exp. Biol.* **212**, 78-88.

Madsen, S. S., Bujak, J. and Tipsmark, C. K. (2014). Aquaporin expression in the Japanese medaka (*Oryzias latipes*) in freshwater and seawater: challenging the paradigm of intestinal water transport? J. *Exp. Biol.* **217**, 3108-3121.

Magdeldin, S., Uchida, K., Hirano, T., Grau, E. G., Abdelfattah, A. and Nozaki, M. (2007). Effects of environmental salinity on somatic growth and growth hormone/insulin-like growth factor-I axis in juvenile tilapia *Oreochromis mossambicus*. *Fish. Sci.* **73**, 1025-1034.

Manzon, L. A. (2002). The role of prolactin in fish osmoregulation: a review. *Gen. Comp. Endocrinol.* **125**, 291-310.

Marshall, W. S. and Grosell, M. (2006). Ion transport, osmoregulation and acid-base balance. In *Physiology of Fishes* (ed. D. H. Evans and J. B. Claiborne), pp. 177-230. Boca Raton, FL: CRC Press.

Marshall, W. S. and Singer, T. D. (2002). Cystic fibrosis transmembrane conductance regulator in teleost fish. *Biochim. Biophys. Acta Biomembr.* **1566**, 16-27.

McCormick, S. D., Regish, A. M. and Christensen, A. K. (2009). Distinct freshwater and seawater isoforms of Na^+/K^+-ATPase in gill chloride cells of Atlantic salmon. *J. Exp. Biol.* **212**, 3994-4001.

Moorman, B. P., Inokuchi, M., Yamaguchi, Y., Lerner, D. T., Grau, E. G. and Seale, A. P. (2014). The osmoregulatory effects of rearing Mozambique tilapia in a tidally changing salinity. *Gen. Comp. Endocrinol.* **207**, 94-102.

Nagahama, Y., Nishioka, R. S., Bern, H. A. and Gunther, R. L. (1975). Control of prolactin secretion in teleosts, with special reference to *Gillichthys mirabilis* and *Tilapia mossambica*. *Gen. Comp. Endocrinol.* **25**, 166-188.

Ouattara, N., Bodinier, C., Negre-Sadargues, G., D'Cotta, H., Messad, S., Charmantier, G., Panfili, J. and Baroiller, J.-F. (2009). Changes in gill ionocyte morphology and function following transfer from fresh to hypersaline waters in the tilapia *Sarotherodon melanotheron*. *Aquaculture* **290**, 155-164.

Pickford, G. E. and Phillips, J. G. (1959). Prolactin, a factor in promoting survival of hypophysectomized killifish in fresh water. *Science* **130**, 454-455.

Pierce, A. L., Fox, B. K., Davis, L. K., Visitacion, N., Kitahashi, T., Hirano, T. and Grau, E. G. (2007). Prolactin receptor, growth hormone receptor, and putative somatolactin receptor in Mozambique tilapia: tissue specific expression and differential regulation by salinity and fasting. *Gen. Comp. Endocrinol.* **154**, 31-40.

Richards, J. G., Semple, J. W., Bystriansky, J. S. and Schulte, P. M. (2003). Na +/K+-ATPase (alpha-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J. Exp. Biol.* **206**, 4475-4486.

Sakamoto, T., Shepherd, B. S., Madsen, S. S., Nishioka, R. S., Siharath, K., Richman, N. H., Bern, H. A. and Grau, E. G. (1997). Osmoregulatory actions of growth hormone and prolactin in an advanced teleost. *Gen. Comp. Endocrinol.* **106**, 95-101.

Seale, A. P., Riley, L. G., Leedom, T. A., Kajimura, S., Dores, R. M., Hirano, T. and Grau, E. G. (2002). Effects of environmental osmolality on release of prolactin, growth hormone and ACTH from the tilapia pituitary. *Gen. Comp. Endocrinol.* **128**, 91-101.

Seale, A. P., Hirano, T. and Grau, E. G. (2006a). Osmoreception: a fish model for a fundamental sensory modality. In *Fish Endocrinol* (ed. M. Reinecke, G. Zaccone and B. G. Kapoor), pp. 419-440. Enfield, NH: Science Publishers.

Seale, A. P., Fiess, J. C., Hirano, T., Cooke, I. M. and Grau, E. G. (2006b). Disparate release of prolactin and growth hormone from the tilapia pituitary in response to osmotic stimulation. *Gen. Comp. Endocrinol.* **145**, 222-231.

Seale, A. P., Watanabe, S. and Grau, E. G. (2012a). Osmoreception: perspectives on signal transduction and environmental modulation. *Gen. Comp. Endocrinol.* **176**, 354-360.

Seale, A. P., Moorman, B. P., Stagg, J. J., Breves, J. P., Lerner, D. T. and Grau, E. G. (2012b). Prolactin177, prolactin188 and prolactin receptor 2 in the pituitary of the euryhaline tilapia, *Oreochromis mossambicus*, are differentially osmosensitive. *J. Endocrinol.* **213**, 89-98.

Seale, A. P., Watanabe, S., Breves, J. P., Lerner, D. T., Kaneko, T. and Grau, E. G. (2012c). Differential regulation of TRPV4 mRNA levels by acclimation salinity and extracellular osmolality in euryhaline tilapia. *Gen. Comp. Endocrinol.* **178**, 123-130.

Shepherd, B. S., Sakamoto, T., Hyodo, S., Nishioka, R. S., Ball, C., Bern, H. A. and Grau, E. G. (1999). Is the primitive regulation of pituitary prolactin (tPRL(177) and tPRL(188)) secretion and gene expression in the euryhaline tilapia (*Oreochromis mossambicus*) hypothalamic or environmental? *J. Endocrinol.* **161**, 121-129.

Stickney, R. R. (1986). Tilapia tolerance of saline waters: a review. *Prog. Fish Cult.* **48**, 161-167.

Tipsmark, C. K., Breves, J. P., Seale, A. P., Lerner, D. T., Hirano, T. and Grau, E. G. (2011). Switching of Na(+), K(+) -ATPase isoforms by salinity and prolactin in the gill of a cichlid fish. *J. Endocrinol.* **209**, 237-244.

Trewevas. (1983). *Tilapiine Fishes of the Genera Sarotherodon, Oreochromis and Danakilia*. Ithaca, NY: Comstock Publishing Associates.

Uchida, K., Kaneko, T., Miyazaki, H., Hasegawa, S. and Hirano, T. (2000). Excellent salinity tolerance of Mozambique tilapia (*Oreochromis mossambicus*): elevated chloride cell activity in the branchial and opercular epithelia of the fish adapted to concentrated seawater. *Zool. Sci.* **17**, 149-160.

Velan, A., Hulata, G., Ron, M. and Cnaani, A. (2011). Comparative time-course study on pituitary and branchial response to salinity challenge in Mozambique tilapia (*Oreochromis mossambicus*) and Nile tilapia (*O. niloticus*). *Fish Physiol. Biochem.* **37**, 863-873.

Wang, P.-J., Lin, C.-H., Hwang, L.-Y., Huang, C.-L., Lee, T.-H. and Hwang, P.-P. (2009). Differential responses in gills of euryhaline tilapia, *Oreochromis mossambicus*, to various hyperosmotic shocks. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **152**, 544-551.

Watanabe, S., Kaneko, T. and Aida, K. (2005). Aquaporin-3 expressed in the basolateral membrane of gill chloride cells in Mozambique tilapia *Oreochromis mossambicus* adapted to freshwater and seawater. *J. Exp. Biol.* **208**, 2673-2682.

Watanabe, S., Niida, M., Maruyama, T. and Kaneko, T. (2008). Na(+)/H(+) exchanger isoform 3 expressed in apical membrane of gill mitochondrion-rich cells in Mozambique tilapia *Oreochromis mossambicus*. *Fish. Sci.* **74**, 813-821.

Wigham, T., Nishioka, R. S. and Bern, H. A. (1977). Factors affecting *in vitro* activity of prolactin cells in the euryhaline teleost Sarotherodon mossambicus (*Tilapia mossambica*). *Gen. Comp. Endocrinol.* **32**, 120-131.

Wood, C. M. and Grosell, M. (2009). TEP on the tide in killifish (*Fundulus heteroclitus*): effects of progressively changing salinity and prior acclimation to intermediate or cycling salinity. *J. Comp. Physiol. B* **179**, 459-467.

Yada, T., Hirano, T. and Grau, E. G. (1994). Changes in plasma levels of the two prolactins and growth hormone during adaptation to different salinities in the Euryhaline Tilapia, *Oreochromis mossambicus*. *Gen. Comp. Endocrinol.* **93**, 214-223.