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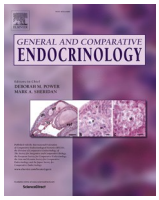

Prolactin signaling in the highly osmotolerant Mozambique tilapia, *Oreochromis mossambicus*☆

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ABSTRACT

Teleost fishes maintain hydromineral balance through the hormonal regulation of epithelial ion transport. In the euryhaline, and remarkably osmotolerant Mozambique tilapia (*Oreochromis mossambicus*), two prolactin (Prl) isoforms, Prl_{188} and Prl_{177}, are released from the pituitary in response to hyposmotic stimulation to promote branchial ion absorption. Prl_{188} and Prl_{177} bind two Prl receptors (Prlrs), Prlr1 and Prlr2, which are expressed in the pituitary and key ionoregulatory organs, including the gill. To understand how Prl signaling operates across a range of salinities that reflect their scope for osmotolerance, we exposed Mozambique tilapia to conditions spanning from fresh water (FW; < 0.1‰) to triple-strength seawater (3x SW; 105‰). In the pituitary, prl_{188}/prl_{177} and *prlr1/prlr2* ratios decreased as salinity increased, dropping to levels in 3x SW that were less than 10% of those in FW. Branchial *prlr1/prlr2* ratios also decreased with increased salinity, in parallel with effectors of branchial ion uptake, including Na^+/Cl^- *cotransporter 2* and Na^+/K^+ *ATPase-α1a*. While the changes in ratios reflect differences in the reductions of both prl_{188} and prl_{177}, they also reflect downregulation of *prlr1* and upregulation of *prlr2* with increasing salinity. Gene transcripts encoding mediators of ion extrusion, such as $Na^+/K^+/2Cl^-$ *cotransporter 1a* and *cystic fibrosis transmembrane conductance regulator 1,* were elevated by high-salinity conditions. Our findings indicate that when Mozambique tilapia are exposed to hypersaline conditions, they shift Prl signaling toward Prl_{177} and Prlr2 relative to Prl_{188} and Prlr1. In turn, the combinatorial nature of isoform-specific responses linked to Prl signaling contributes to both the euryhalinity and exceptional osmotolerance of Mozambique tilapia.

1. Introduction

The ability of euryhaline fishes to maintain hydromineral balance across disparate environmental salinities enables them to utilize diverse habitats. Because they can survive in salinities ranging from fresh water (FW) to seawater (SW), and in some cases under hypersaline conditions, euryhaline teleosts are studied to elucidate the physiological mechanisms underlying osmotolerance (Gonzalez, 2012). The modulation of osmoregulatory capacities following changes in environmental salinity is generally orchestrated by a combination of central and peripheral pathways that involve direct osmosensing and endocrine signaling (Evans and Kultz, 2020; Kültz, 2012; Sakamoto and McCormick, 2006; Seale et al., 2012c). The euryhaline Mozambique tilapia (*Oreochromis mossambicus*) is a highly osmotolerant teleost capable of surviving salinities up to 120‰ (Stickney, 1986) and serves as a model for studying hormonal responses to salinity challenges (Seale and Breves, 2022). In tilapia and other euryhaline species, prolactin (Prl) is crucial for FW acclimation because it promotes the active uptake of environmental ions while reducing their passive loss (Hirano, 1986; Breves et al., 2014). Notably, in tilapia, pituitary Prl cells are directly osmosensitive, serving as a model to study the cellular and molecular mechanisms that mediate osmoreception (Grau and Helms (1990); Seale et al., 2005; Seale et al., 2020).

Two Prl isoforms, Prl_{188} and Prl_{177}, are produced and released from

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the same cells in the pituitary of Mozambique tilapia (Specker et al., 1985; Specker et al., 1993). Consistent with their promotion of hyperosmoregulation, circulating levels of both Prls rise when fish are transferred from SW to FW and fall when transferred from FW to SW (Yada et al., 1994). *In vitro*, the release of Prl from dispersed cells and *rostral pars distalis* (RPD) organoids is inversely related to extracellular osmolality and depends on the entry of extracellular Ca^{2+} (Seale et al., 2003; Seale et al., 2012b). Likewise, prl_{188} and prl_{177} gene expression is stimulated by hyposmotic conditions, both *in vivo* and *in vitro*. While Prl_{188} and Prl_{177} were initially found to exert similar osmoregulatory effects (Specker et al., 1985), subsequent studies showed they differ in their osmosensitivity. The relative quantities of Prl_{188} and Prl_{177} in the RPDs of tilapia acclimated to FW, SW, and during acclimation from one salinity to the other indicate that they are regulated distinctly. Specifically, the ratio of Prl_{188} to Prl_{177} is greater than 1 in fish acclimated to FW and less than 1 in SW-acclimated fish (Borski et al., 1992). This observation was subsequently confirmed not only for plasma Prls in fish undergoing salinity transitions, but also for Prls released from pituitary cells incubated in different medium osmolalities (Seale et al., 2012a). These patterns correspond with the greater responsiveness of Prl_{188} and prl_{188} to hyposmotic stimulation *in vitro*, compared with Prl_{177} and prl_{177} (Malintha et al., 2023b). Accordingly, the transcription factors that bind the prl_{188} and prl_{177} promoters are influenced by environmental salinity themselves (Malintha et al., 2023a; Seale et al., 2020).

Tilapia Prls function via two Prl receptors (Prlrs), Prlr1 and Prlr2, which are expressed in the pituitary and key osmoregulatory organs (Fiol et al., 2009; Pierce et al., 2007; Sandra et al., 2000; Seale et al., 2014). The *prlrs* differ in their transcriptional responses to 'one-way' salinity changes. Branchial *prlr1* expression increases when fish move from SW to FW (Breves et al., 2011), while *prlr2* increases when fish move from FW to SW (Fiol et al., 2009). Even when tilapia are exposed to a tidal regime in which salinity fluctuates between FW and SW every 6 h, *prlr1* and *prlr2* are oppositely regulated (Moorman et al., 2014; Seale et al., 2019). Furthermore, *prlr1*, but not *prlr2*, expression was increased in incubated gill filaments exposed to Prl_{188} and Prl_{177} (Inokuchi et al., 2015). Since pituitary *prlr* expression, especially *prlr2*, is sensitive to extracellular osmotic conditions (Seale et al., 2012a), the autocrine/paracrine effects of both Prls are also influenced by environmental salinity.

The salinity tolerance of Mozambique tilapia has been studied through various experimental paradigms, including transfers from FW to SW, SW to FW, as well as under tidal conditions (Seale et al., 2024). Despite facing continuously changing salinities, Mozambique tilapia subjected to a tidal regime maintain plasma osmolality between ~320 mOsm/kg during the FW phase of the cycle and ~340 mOsm/kg during the SW phase (Moorman et al., 2015). When "California" Mozambique and Wami tilapia hybrids (*O. mossambicus* x *O. urolepis hornorum*) were gradually exposed to hypersaline conditions, plasma osmolality remained relatively stable below salinities of 60‰, then increased after the fish were exposed to salinities up to 95‰ (Sardella and Brauner, 2008; Sardella et al., 2004). The rise in plasma osmolality was accompanied by an increase in branchial Na^+/K^+-ATPase activity (Sardella and Brauner, 2008), indicating that enhanced Na^+ and Cl^- secretion by ionocytes is crucial for tolerating hypersalinities (Uchida et al., 2000). Operating as the primary sites for active transport of Na^+ and Cl^-, ionocytes of the branchial epithelium are key targets for both endocrine and environmental regulation of ion-transport processes (Breves and Shaughnessy, 2024). In tilapia, FW-type ionocytes responsible for ion uptake express *Na^+/Cl^- cotransporter 2* (*ncc2*; *slc12a10*) and *Na^+/K^+-ATPase α-1a* (*nkaα1a*) (Miyanishi et al., 2026); these genes are upregulated in response to Prl and downregulated by hyperosmotic conditions (Breves et al., 2010; Hiroi et al., 2008; Inokuchi et al., 2015; Tipsmark et al., 2011). In contrast, genes encoding the ion transporters and channels that facilitate Na^+ and Cl^- secretion by SW-type ionocytes, such as *$Na^+/K^+/2Cl^-$ cotransporter 1a* (*nkcc1a*; *slc12a2a*) and *cystic fibrosis transmembrane conductance regulator 1* (*cftr1*), are upregulated in hyperosmotic environments (Hiroi et al., 2008; Li et al., 2014). In turn, the expression of FW- and SW-type ionocyte-related gene transcripts can offer functional context when describing how Prl signaling responds to hypersaline conditions.

We hypothesized that a gradual decline in prl_{188}/prl_{177} and *prlr1/prlr2* ratios would be associated with acclimation to hypersaline conditions in Mozambique tilapia. In turn, we exposed tilapia to salinities that reflect its remarkably broad osmotolerance and measured pituitary expression of prl_{188}, prl_{177}, *prlr1,* and *prlr2* in FW (< 0.1‰), SW (35‰), 2x SW (70‰), and 3x SW (105‰) conditions. We then surveyed branchial Prl signaling alongside markers of ionoregulatory capacity by analyzing the expression of *prlr1*, *prlr2*, *ncc2*, *nkaα1a*, *nkcc1a*, and *cftr1*.

2. Methods

2.1. Animals

Male Mozambique tilapia were reared at the Tuahine Aquaculture Research and Education Center (TAREC; Seale et al., 2025) at the University of Hawai'i at Mānoa for 7 months in FW prior to the hypersaline challenge experiment. Fish were kept in outdoor 400-L tanks under natural photoperiod and fed 1.5 mm Steelhead Fry pellets (Skretting, Tooele, UT) once daily up to 1% initial mean body weight. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the University of Hawai'i.

2.2. Experimental design

Forty adult (63.8 ± 16.2 g) male Mozambique tilapia were divided into 4 tanks (10 fish per tank), supplied with 400 L of FW (0.1 ± 0.1‰, municipal water), and kept at 24 ± 2 °C for 5 days. The tanks corresponded to four salinity treatments: FW (< 0.1‰), SW (35‰), 2x SW (70‰), and 3x SW (105‰). Except for the FW treatment, the salinity of all tanks was raised by adding sea salt (Instant Ocean, Blacksburg, VA) to the FW. During the trial, all tanks were kept static, with daily water changes of up to 10% of their volume. Water quality was monitored daily for salinity and weekly for ammonia (<2 mg/L), nitrite (<2 mg/L), nitrate (20 mg/L), and dissolved oxygen (4.95 ± 1.26 mg/L).

At the onset of the experiment, the salinity of all tanks except the FW controls was raised from 0 to 20‰ over 2 days by replacing 10% of the volume with 200‰ artificial SW. To acclimate tilapia to the target salinities, the water in the SW, 2x SW, and 3x SW treatment tanks was gradually replaced with 100-180‰ SW, increasing salinity by ~3‰ per day until the target salinities were reached. The periods required to acclimate fish to SW, 2x SW, and 3x SW were therefore 7, 19, and 31 days, respectively. All fish were kept at their respective target salinities prior to sampling at day 35.

2.3. Sampling

At the time of sampling, fish were netted from their respective tanks and anesthetized with 2-phenoxyethanol (0.3 mL/L, Sigma-Aldrich, St. Louis, MO). Following anesthesia, fish were weighed, and blood was collected from the caudal vasculature using a needle and syringe coated with sodium heparin (200 U/mL; Sigma-Aldrich). Fish were then euthanized by rapid decapitation; pituitaries and gill filaments from the second gill arch (left side) were excised, frozen in liquid nitrogen, and stored at −80 °C. After centrifuging blood samples at 10,000 rpm for 5 min, plasma was removed, and osmolality was measured with a vapor pressure osmometer (Wescor 5520 Vapro; Logan, UT).

2.4. Quantitative real-time PCR (qPCR)

Total RNA was extracted from pituitary and gill samples using TRI Reagent following the manufacturer's protocol (MRC, Cincinnati, OH). Total RNA (90 ng) was reverse transcribed using a cDNA reverse

transcription kit (Cat. No.: 4368814; Thermo Fisher Scientific, Waltham, MA). The levels of reference and target genes were determined using the relative quantification method, in which relative expression levels and PCR efficiencies were calculated from standard curves produced from serial dilutions of pituitary or gill cDNA using a StepOnePlus real-time qPCR system (Thermo Fisher Scientific). Briefly, qPCR reactions (15 μL) contained SYBR Green PCR Master Mix (Cat. No.: 4367659; Applied Biosystems, Warrington, UK), 200 nmol/L forward and reverse primers, and 1 μL of cDNA. The PCR cycling parameters were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Gene-specific primers for prl_{188}, prl_{177}, *prlr1*, *prlr2*, *ncc2*, *nkcc1a*, and *cftr1* have been previously described (Breves et al., 2010; Inokuchi et al., 2008; Magdeldin et al., 2007; Moorman et al., 2014; Pierce et al., 2007). *Elongation factor 1α* (*ef1α*) was used as the reference gene to normalize target gene levels (Breves et al., 2010) after confirming its stable expression across treatment groups. All primer pairs are listed in Table 1. The relative expression of target genes was calculated following the Pfaffl method (Pfaffl, 2001). Data are expressed as the mean ratio of target gene normalized by the reference gene ± standard error of the mean (S.E.M.).

2.5. Statistical analyses

Analyses of plasma osmolality and relative mRNA expression in response to salinity treatment were conducted by one-way ANOVA. Significant effects ($P < 0.05$) were followed up by protected Fisher's LSD test. Assumptions for one-way ANOVA were confirmed using the Shapiro-Wilk test for normality and the Brown-Forsythe test for equal variances. When necessary, data were log-transformed to satisfy normality and homogeneity of variance assumptions. Data are expressed as means ± S.E.M. Statistical analyses were performed using Prism 10 (GraphPad Prism 10, La Jolla, CA).

3. Results

3.1. Effects of hypersalinity on plasma osmolality

There was a significant effect of acclimation salinity on plasma osmolality (Fig. 1). While no difference in plasma osmolality was observed between fish acclimated to FW and SW (~300 mOsm/kg), the osmolality of fish acclimated to 2x SW and 3x SW rose to ~ 340 and ~ 440 mOsm/kg, respectively.

Table 1
Primer sequences used in quantitative real-time PCR.

Target Gene	Primer Sequence (5′ to 3′)	Accession No.	Reference
ef1a	F: TAAAACCCTGCCTGACTTCC R: AATCCTCATTAGCCCCAAAA	AB075952	Breves et al., 2010
prl_{188}	F: GGCCACTCCCCATGTTTAAA R: GGCATAATCCCAGGAGGAGAC	X93280	Magdeldin et al., 2007
prl_{177}	F: TGGTTTGGCTCTTTTAACACAGTG R: AGACAATGAGGAGTCACAGAGATTTTAC	M27011	Magdeldin et al., 2007
prlr1	F: TGGGTCAGCTACAACATCACTGT R: GGATGGGGCTTGACAATGTAGA	EU999785	Pierce et al., 2007
prlr2	F: GCCCTTGGGAATACATCTTCAG R: GTGCATAGGGCTTCACAATGTC	EU999783	Breves et al., 2010
ncc2	F: CCGAAAGGCACCCTAATGG R: CTACACTTGCACCAGAAGTGACAA	EU518934	Inokuchi et al., 2008
nkaα1a	F: AACTGATTTGGTCCCTGCAA R: ATGCATTTCTGGGCTGTCTC	GR644771	Tipsmark et al., 2011
nkcc1a	F: GGAGGCAAGATCAACAGGATTG R: AATGTCCGAAAAGTCTATCCTGAACT	AY513737	Inokuchi et al., 2008
cftr1	F: CATGCTCTTCACCGTGTTCT R: GCCACAATAATGCCAATCTG	AB601825	Moorman et al., 2014

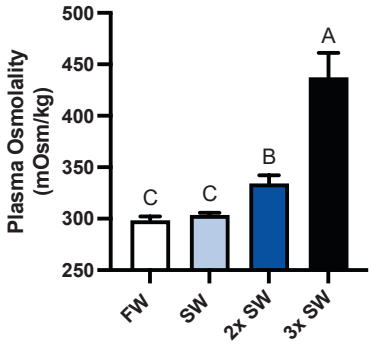

Fig. 1. Plasma osmolality of fish transferred from FW to SW, 2x SW, and 3x SW. Bars represent means ± S.E.M. ($n = 10$). Means not sharing the same letter are significantly different (one-way ANOVA, protected Fisher's LSD test, $P < 0.05$).

3.2. Effects of hypersalinity on pituitary prls and prlrs

The pituitary expression patterns of prl_{188}, prl_{177}, and their ratios in tilapia acclimated to hypersaline conditions are provided in Fig. 2A–C. prl_{188} expression was highest in fish acclimated to FW. In SW, 2x SW, and 3x SW, prl_{188} was downregulated to 1/13, 1/84, and 1/490 of FW values, respectively (Fig. 2A). prl_{177} expression was also highest in FW-acclimated fish and downregulated in SW, 2x SW, and 3x SW to 1/6, 1/11, and 1/27 of FW values, respectively. prl_{188}/prl_{177} ratios were inversely related to salinity. In FW, pituitary prl_{188}/prl_{177} ratios were 1.1, whereas in SW, 2x SW, and 3x SW they were 0.59, 0.20, and 0.06, respectively (Fig. 2C).

The pituitary expression patterns of *prlr1*, *prlr2*, and their ratios in tilapia acclimated to hypersaline conditions are provided in Fig. 2D–F. Similar to prl_{188} and prl_{177}, *prlr1* expression was highest in fish acclimated to FW compared with those in higher salinities. *prlr1* was downregulated to 1/1.5 in SW, 1/2.4 in 2x SW and 1/3.4 in 3x SW relative to FW values (Fig. 2D). By contrast, *prlr2* expression was lowest in FW-acclimated fish and became upregulated in SW, 2x SW, and 3x SW by 1.4-, 1.3-, and 2.5-fold, respectively (Fig. 2E). Similar to prl_{188}/prl_{177} ratios, *prlr1/prlr2* ratios were also inversely related to salinity. In FW, pituitary *prlr1/prlr2* ratios were 3.3, whereas in SW, 2x SW, and 3x SW they were 1.7, 1.0, and 0.4, respectively (Fig. 2F).

3.3. Effects of hypersalinity on branchial prlrs and effectors of ion transport

The branchial expression patterns of *prlr1*, *prlr2*, and their ratios in tilapia acclimated to hypersaline conditions are provided in Fig. 3. Similar to the pituitary, branchial *prlr1* expression was highest in fish acclimated to FW compared with those in higher salinities. The expression of *prlr1*, however, was similar between SW, 2x SW, and 3x SW (Fig. 3A). Conversely, branchial *prlr2* expression was lowest in FW- and SW-acclimated fish and was upregulated by 2.9- and 5.9-fold in 2x SW, and 3x SW, respectively (Fig. 3B). Similar to pituitary *prlr1/prlr2* ratios, the branchial *prlr1/prlr2* ratio was also inversely related to salinity. In FW, branchial *prlr1/prlr2* ratios were 21.8, whereas in SW, 2x SW, and 3x SW they were 8.1, 2.7, and 1.2, respectively (Fig. 3C).

The branchial expression patterns of *ncc2*, *nkaα1a*, *nkcc1a*, and *cftr1* in tilapia acclimated to hypersaline conditions are provided in Fig. 4. Relative to levels in FW-acclimated fish, *ncc2* expression was downregulated with a rise in salinity; *ncc2* was reduced to 1/41, 1/110, and 1/1042 of FW values in SW, 2x SW, and 3x SW, respectively (Fig. 4A). Likewise, *nkaα1a* expression was highest in fish acclimated to FW compared with those transferred to higher salinities, but expression was similar between SW, 2x SW, and 3x SW (1/7.6, 1/26, and 1/7.6 relative to FW values, respectively; Fig. 4B). In contrast, *nkcc1a* was upregulated by hypersalinity, rising by 14-, 90-, and 305-fold in SW, 2x SW, and 3x SW-acclimated fish, respectively (Fig. 4C). Similarly, compared with

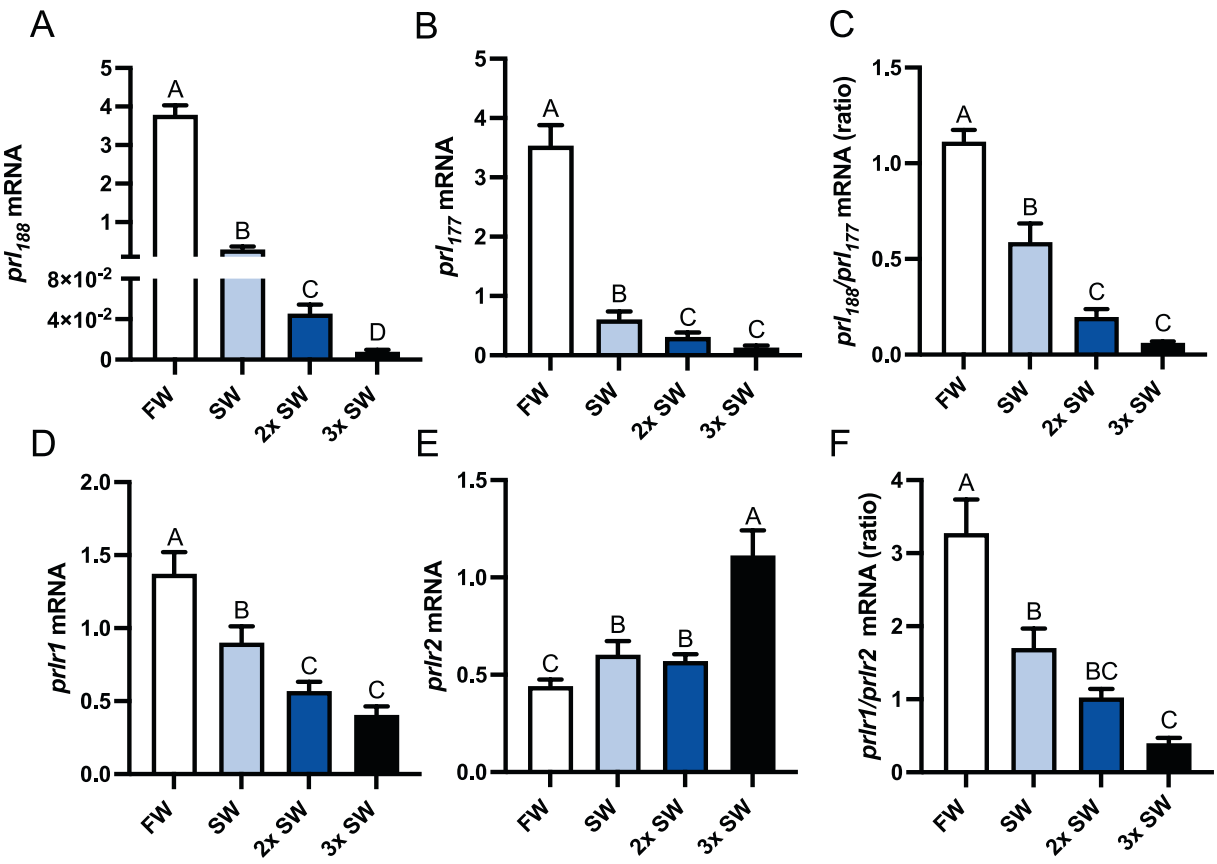

Fig. 2. Pituitary expression of prl_{188} (A), prl_{177} (B), prl_{188}/prl_{177} ratios (C), *prlr1* (D), *prlr2* (E), and *prlr1/prlr2* ratios (F) in Mozambique tilapia transferred from FW to SW, 2x SW, and 3x SW. Data are expressed as the means of the target gene normalized by the reference gene ± S.E.M ($n = 10$). Means not sharing the same letter are significantly different (one-way ANOVA, protected Fisher's LSD test, $P < 0.05$).

FW-acclimated fish, all treatments exhibited elevated *cftr1*; *cftr1* increased by 39-, 118-, and 233-fold in fish held in SW, 2x SW, and 3x SW, respectively (Fig. 4D).

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4. Discussion

This study characterized the effects of hypersaline conditions on the gene expression of *prls*, *prlrs*, and effectors of ion transport in Mozambique tilapia. A gradual salinity acclimation approach was adopted to investigate the responses of Prl isoforms, their receptors, and key regulators of ion transport to hypersaline conditions. Given the complex nature of Prl_{188}, Prl_{177}, Prlr1, and Prlr2 responses to salinity challenges in Mozambique tilapia (Fiol et al., 2009; Seale et al., 2020; Specker et al., 1985), the use of ratios between Prl isoforms has facilitated interpretation of regulatory patterns and provided insight into their concerted responses to salinity challenges (Borski et al., 1992; Seale et al., 2012a). Here, we found that not only did prl_{188}/prl_{177} ratios decrease in SW relative to FW, but they continued to decline in hypersaline conditions. *prlr1/prlr2* ratios showed a similar trend, while known effectors of ion uptake and extrusion were strongly down- and upregulated, respectively, with increasing salinity. Importantly, however, while ratios of *prls* and *prlrs* generally decreased in response to a rise in salinity, they do not reflect similar directional changes across individual transcripts. Notably, *prlr2* increased following a rise in salinity, in contrast to a decrease in *prlr1* and both *prls*. Together, these results reveal an osmoregulatory response pattern involving Prl signaling that relies on isoform-specific responses to hypersaline conditions.

The osmoregulatory actions of Prl following hyposmotic challenges have been firmly established in teleost fishes. The requirement of Prl for the survival of euryhaline fish in FW was established using multiple approaches, including hypophysectomy, extirpation, and auto-transplantation of the RPD to an ectopic site (Pickford and Phillips, 1959; Shepherd et al., 1999). In fact, Prl is such a powerful osmoregulatory factor in Mozambique tilapia that its direct response to hyposmotic conditions is considered more direct and potent than other osmotic responses mediated by the brain or other hormonal systems (see Seale et al., 2013). Importantly, for an endocrine system to play such a vital regulatory role in tolerating one extreme of possible osmotic conditions (i.e., low salinity), it must be strongly attenuated at the other extreme (i.e., high salinity).

Congeneric species inhabiting habitats with distinct salinity ranges provide insight into the underpinnings of salinity tolerance. For example, when Mozambique tilapia are transferred directly to brackish water (20‰), plasma osmolality temporarily rises without exceeding 400 mOsm/kg, while circulating Prl is lowered by 24 h (Yamaguchi et al., 2018). On the other hand, Nile tilapia (*O. niloticus*), which are less tolerant of elevated salinity, are unable to maintain osmolality below 450 mOsm/kg in brackish water, and their Prl cells are less responsive to changes in extracellular osmolality *in vitro* (Yamaguchi et al., 2018). In Mozambique tilapia, Prl cells are highly sensitive to extracellular osmolalities ranging from 250 to 450 mOsm/kg (Seale et al., 2006), further indicating the importance of direct osmotic responses by Prl cells in helping them acclimate to environmental salinity. Despite their tolerance of hypersaline conditions, adult Mozambique tilapia cannot survive a direct transfer from FW to SW unless they have been pre-acclimated to an intermediate salinity (Inokuchi et al., 2021; Moorman et al., 2015). Hence, a gradual increase in salinity (3‰ per day) was applied in this study, enabling full survival at 105‰ by 31 days. After salinity reached 105‰ in the 3x SW group, 3 mortalities were observed

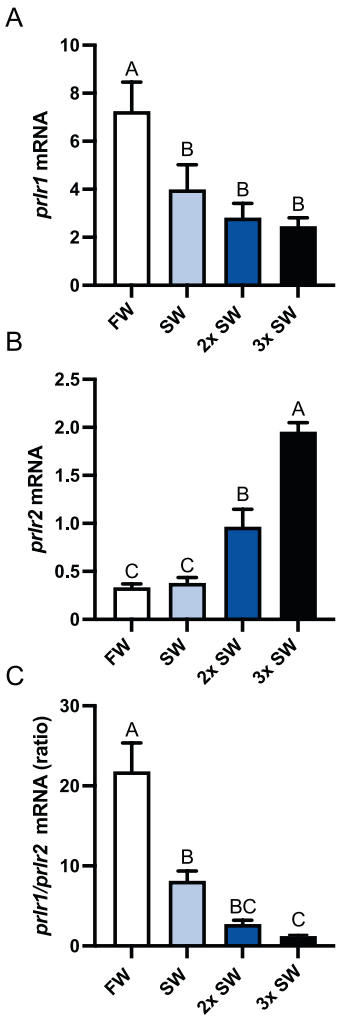

Fig. 3. Branchial expression of *prlr1* (A), *prlr2* (B), and *prlr1/prlr2* ratios (C) in Mozambique tilapia transferred from FW to SW, 2x SW, and 3x SW. Data are expressed as the mean of the target gene normalized by the reference gene ± S.E.M ($n = 10$). Means not sharing the same letter are significantly different (one-way ANOVA, protected Fisher's LSD test, $P < 0.05$).

between days 32 and 35. Likely a consequence of the gradual rise in salinity, no differences in plasma osmolality were observed between FW and SW fish. Typically, fish transferred to SW with only one acclimation step in brackish water are slightly hyperosmotic compared to those kept in FW (~340 vs. ~320 mOsm/kg; Seale and Breves, 2022). Nonetheless, as salinity rose to 65 and 105‰, plasma osmolality correspondingly rose to ~340 and ~440 mOsm/kg, respectively. This pattern, in which plasma osmolality rises gradually with exposure to ~65‰, then increases sharply at higher salinities, was also observed in "California" Mozambique tilapia (Sardella et al., 2004), indicating progressive challenges in maintaining osmotic balance in hypersaline environments. These challenges, along with changes in homeostatic energy allocation and protein networks, have been specifically characterized in the "pejus" and "pessimum" zones of salinity thresholds above 75‰ (Root and Kültz, 2024).

Aligned with Prl's hyperosmoregulatory actions, transcriptional suppression of both prl_{188} and prl_{177} was observed at higher salinities. Interestingly, the transcriptional suppression was more pronounced for prl_{188} than for prl_{177}, as reflected by the consistent decrease in prl_{188}/prl_{177} ratios at higher salinities. This observation suggests distinct roles for both Prls, with the actions of Prl_{177} (e.g., somatotropic activity) favored at higher salinities (Borski et al., 1992; Seale et al., 2012a; Shepherd et al., 1997). The prl_{188} and prl_{177} genes contain upstream binding sites with distinct regulatory elements, and hypo- and hyperosmotic stimuli differentially activate some of their predicted transcription factor modules (Malintha et al., 2023a; Seale et al., 2020). For example, the prl_{188}-specific transcription factor module, comprising pituitary-specific transcription factor 1 and octamer (OCT1_PIT1), is highly activated in FW, whereas the prl_{177}-specific transcription factor module, composed of specificity protein 1 transcription factors (SP1F_SP1F), is activated in SW-acclimated fish (Malintha et al., 2023a). PIT1 is encoded by the *pou1f1* and *pou2f1b* genes, whose expression is inversely correlated with extracellular osmolality (Malintha et al., 2023a). The osmotic regulation of *pou1f1* and *pou2f1b* is more pronounced in fish acclimated to SW than in those acclimated to FW (Malintha et al., 2023a). This regulatory complexity in the transcription and activation of specific transcription factors further corroborates the intrinsically osmosensitive nature of *prl*-specific transcription factor modules in tilapia Prl cells. These layers of transcriptional control underscore the high osmosensitivity of Prl cells from FW-acclimated fish and the multiple regulatory mechanisms that rapidly and differentially inhibit secretion of Prl_{188} and Prl_{177} during the transition from FW to higher salinities.

A single Prl receptor was originally shown to bind both Prls in Nile tilapia, with higher affinity for Prl_{188} than for Prl_{177} (Auperin et al., 1994). A partial sequence of this receptor (Prlr1) was cloned in Mozambique tilapia (Prunet et al., 2000), and subsequently, two Prlr genes were reported in seabream (*Sparus aurata*) (Huang et al., 2007). A second Prl receptor, Prlr2, was identified in Mozambique tilapia and, in a heterologous expression system, shown to activate distinct downstream signaling pathways and to respond differently to Prl isoforms, in contrast to Prlr1 (Fiol et al., 2009). Moreover, HEK293 cells expressing *prlr2*, but not those transfected with *prlr1*, showed increased tolerance to hyperosmotic stress (Fiol et al., 2009). In Mozambique tilapia, *prlr2* expression in RPDs and dispersed Prl cells increased in direct response to hyperosmotic conditions; *prlr1* expression was unchanged following osmotic stimulation (Seale et al., 2012a). Consistent with the disparate responses of *prlr1* and *prlr2* to changes in extracellular osmolality in both the gill and pituitary (Breves et al., 2011; Seale et al., 2012a), we observed downregulation of *prlr1* and upregulation of *prlr2* as fish transitioned to hypersaline conditions. In particular, the ratios of pituitary *prlr1/prlr2*, which were above 3 in FW, gradually declined with increasing salinity, reaching 0.4 in 3x SW. The differential regulation of *prlrs* enables the fine-tuning of autocrine pathways in the pituitary, which differ between Prl_{188} and Prl_{177} and can be modulated by environmental osmolality (Yamaguchi et al., 2016). In the gill, the shift in *prlr1/prlr2* ratios was even more pronounced, from nearly 22 in FW to 1.2 in 3x SW. The physiological implications of this salinity-dependent shift in the relative abundance of *prlr* isoforms have been previously discussed (Breves et al., 2011; Inokuchi et al., 2015; Moorman et al., 2014; Moorman et al., 2015; Seale et al., 2012a). Two splice variants of Prlr2 have been described: a short, nonfunctional variant and a long, functional variant (Fiol et al., 2009). The short variant may sequester functional Prlrs by forming heterodimers with long-form Prlrs, thereby attenuating Prl-triggered signal transduction pathways (Fiol et al., 2009). Therefore, the relative inhibition of *prlr1* and stimulation of *prlr2* in hypersaline conditions may dampen the otherwise maladaptive effects of Prl (such as promoting ion-uptake pathways) at high salinities. When Mozambique tilapia experience frequent salinity changes between FW and SW, they maintain low circulating Prl levels, similar to those observed in SW-acclimated fish, while modulating branchial expression of *prlr1* and *prlr2* to align Prl sensitivity with environmental conditions (Seale et al., 2019). Having two receptors that respond to salinity in opposite directions enables the fine-tuning of responses to circulating Prls. The combined isoform-specific responses of prl_{188}, prl_{177}, *prlr1*, and *prlr2*, therefore, contribute to a strategy that supports the rapid acclimation of tilapia to changing salinities. Further functional and spatial studies of Prl signaling are needed to distinguish between the actions of Prl_{188} and Prl_{177}, their differential affinities for Prlr1 and Prlr2, and the

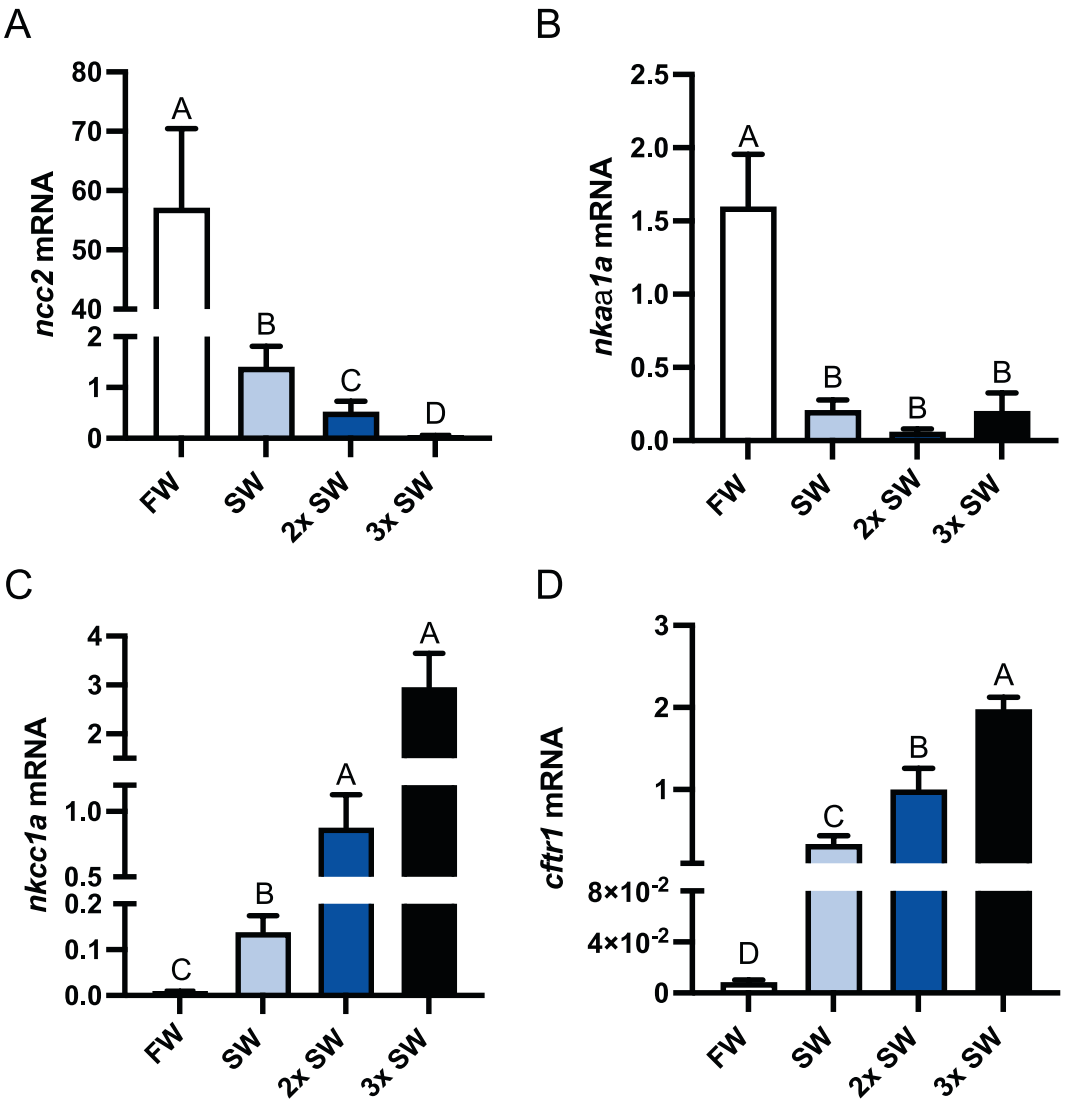

Fig. 4. Branchial expression of *ncc2* (A), *nkaα1a* (B), *nkcc1a* (C), and *cftr1* (D) in Mozambique tilapia transferred from FW to SW, 2x SW, and 3x SW. Data are expressed as the means of the target gene normalized by the reference gene ± S.E.M ($n = 10$). Means not sharing the same letter are significantly different (one-way ANOVA, protected Fisher's LSD test, $P < 0.05$).

identities of the cells mediating responses to shifts in environmental salinity.

Prl's transcriptional regulation of branchial effectors of ion uptake in fishes has been well documented (Breves and Shaughnessy, 2024). Consistent with their expression in FW-type ionocytes (Hiroi et al., 2008; Tipsmark et al., 2011; Miyanishi et al., 2026), we observed that *ncc2* and *nkaα1a* were highest in FW and then declined as fish transitioned to hypersaline conditions. The expression of *ncc2*, which is especially sensitive to both Prls and extracellular osmolality (Breves et al., 2010; Inokuchi et al., 2015), was markedly reduced in fish held at 3x SW. In contrast, both *nkcc1a* and *cftr1* were upregulated as salinity increased, reflecting the ion-secretory processes mediated by their encoded proteins in SW-type ionocytes (Hiroi et al., 2005). Branchial *nkcc1a* and *cftr1* levels continued to rise in fish acclimated to salinities exceeding SW, becoming upregulated by over 200-fold in 3x SW. Although the current data clearly support robust transcriptional regulation of the Prl system under hypersaline conditions, other endocrine systems, including those utilizing cortisol, thyroid hormones, and growth hormone (Breves and Shaughnessy, 2024), likely support acclimation to hypersaline environments by regulating SW-type ionocytes that express *nkcc1a* and *cftr1*.

In conclusion, our study shows that genes encoding Prls, their receptors, and the ion transporters they control are transcriptionally regulated, enabling Mozambique tilapia to tolerate hypersaline environments. The combined responses of prl_{188}, prl_{177}, *prlr1*, and *prlr2*, as reflected in salinity-induced shifts in their ratios, indicate how Prl signaling is modulated during the acclimation of tilapia, and potentially other euryhaline species, to extreme hypersaline environments.

CRediT authorship contribution statement

Andre P. Seale: Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ke Cao:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simran A. Singh:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Brooke Scalambrino:** Writing – review & editing, Methodology. **Ryan J.A. Chang:** Writing – review & editing, Methodology. **Tyler R. Goodearly:** Writing – review & editing, Methodology. **Reilly S. Merlo:** Writing – review & editing, Methodology. **Jason P. Breves:** Writing – review & editing, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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