Comparative Biochemistry and Physiology, Part A 278 (2023) 111356

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Osmosensitive transcription factors in the prolactin cell of a euryhaline teleost

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ARTICLE INFO

Editor: Michael Hedrick

Keywords:
Fish
Osmoreceptor
Prolactin
Transcription factor
Osmosensitive
Osmoregulation
Endocrinology
Gene transcription

ABSTRACT

In euryhaline fish, prolactin (Prl) plays a key role in freshwater acclimation. Prl release in the *rostral pars distalis* (RPD) of the pituitary is directly stimulated by a fall in extracellular osmolality. Recently, we identified several putative transcription factor modules (TFM) predicted to bind to the promoter regions of the two *prl* isoforms in Mozambique tilapia, *Oreochromis mossambicus*. We characterized the effects of extracellular osmolality on the activation of these TFMs from RPDs, *in vivo* and *in vitro*. OCT1_PIT1 01, CEBP_CEBP 01 and BRNF_RXRF 01 were significantly activated in freshwater (FW)- acclimated tilapia RPDs while SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 were significantly activated in seawater (SW)- counterparts. Short-term incubation of SW- acclimated tilapia RPDs in hyposmotic media (280 mOsm/kg) resulted in activation of CAAT_AP1F 01, OCT1_CEBP 01, AP1F_SMAD 01, GATA_SP1F 01, SORY_PAX6 01 and CREB_EBOX 02, EBOX_AP2F 01, EBOX_MITF 01 while hyperosmotic media (420 mOsm/kg) activated SORY_PAX3 02 and AP1F_SMAD 01 in FW- tilapia. Short-term incubation of dispersed Prl cells from FW- acclimated fish exposed to hyperosmotic conditions decreased *pou1f1*, *pou2f1b*, *stat3*, *stat1a* and *ap1b1* expression, while *pou1f1*, *pou2f1b*, and *stat3* were inversely related to osmolality in their SW- counterparts. Further, in Prl cells of SW- tilapia, *creb3l1* was suppressed in hyposmotic media. Collectively, our results indicate that multiple TFMs are involved in regulating *prl* transcription at different acclimation salinities and, together, they modulate responses of Prl cells to changes in extracellular osmolality. These responses reflect the complexity of osmosensitive molecular regulation of the osmoreceptive Prl cell of a euryhaline teleost.

1. Introduction

The maintenance of osmotic homeostasis for life in complex organisms necessitates appropriate mechanisms to detect and direct hydromineral balance. A first step in detecting changes in extracellular osmolality involves the activation of osmosensitive and osmoreceptive cells and tissues (Bourque and Oliet, 1997; Kültz, 2012; Seale et al., 2006a; Seale et al., 2012a; Wells, 1998). In euryhaline fish, release of the pituitary hormone prolactin (Prl) increases following a fall in extracellular osmolality, a response required for freshwater (FW) acclimation (Ball and Ingleton, 1973; Dharmamba and Nishioka, 1968; Grau et al., 1981; Pickford and Phillips, 1959; Seale et al., 2002). The osmoregulatory actions of Prl are largely driven by the stimulation of ion uptake in epithelial tissues such as gill, kidney and intestine (Breves et al., 2011, 2014; Hirano, 1986; Inokuchi et al., 2015; Seale et al., 2014). The euryhaline Mozambique tilapia, (*Oreochromis mossambicus*) is a tractable model to study osmoreception, largely due to the morphological arrangement of Prl cells, which comprise >99% of the *rostral pars distalis* (RPD) of the pituitary (Nishioka et al., 1988.; Seale et al., 2006a; Seale et al., 2005). Tilapia Prl cells secrete two isoforms of Prl, Prl_{177} and Prl_{188}, which are encoded by separate genes (Rentier-Delrue et al., 1989; Specker et al., 1985; Yamaguchi et al., 1988). Both Prls exert hyperosmoregulatory functions by binding their cognate receptors, Prlr1 and Prlr2 in target tissues (Fiol et al., 2009). Despite similar ion-retaining actions (Specker et al., 1985), Prl_{188} release and prl_{188} mRNA expression respond more robustly to hyposmotic stimulation than Prl_{177} and prl_{177} (Inokuchi et al., 2015; Seale et al., 2012b).

When exposed to a fall in extracellular osmolality, tilapia Prl cells

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https://doi.org/10.1016/j.cbpa.2022.111356
Received 12 October 2022; Received in revised form 14 December 2022; Accepted 14 December 2022
Available online 16 December 2022

respond through an aquaporin-3 (Aqp3)-dependent increase in cell volume followed by rapid uptake of extracellular Ca^{2+} through transient receptor potential vanilloid (Trpv4) mechanosensitive channels, thereby triggering the release of stored Prl (Seale et al., 2003a, 2003b; Watanabe et al., 2009, 2012,; Weber et al., 2004). The acclimation salinity history of the fish plays a role in modulating the responsiveness of Prl cells to changes in extracellular osmolality. For example, Prl cells of Mozambique tilapia acclimated to FW are larger, and contain more secretory granules and stored Prl than those of fish acclimated to SW (Borski et al., 1992; Dharmamba and Nishioka, 1968). Likewise, mRNA levels of both *prl*s are ~30-fold higher in fish acclimated to FW than those in SW (Seale et al., 2012b). The implications for environmental regulation are distinct: in Prl cells of FW-acclimated fish, *prl* mRNA levels are less sensitive to further osmotic stimulation than in Prl cells of SW-acclimated fish, presumably because those levels are already very high in FW fish (Seale et al., 2012a). By contrast, hyposmotically-induced Prl release from Prl cells of FW-acclimated fish *in vitro* is more robust than that of SW fish (Seale et al., 2006a, 2012a; 2002).

While previous studies employing the tilapia Prl cell model to investigate osmoreception have focused on hyposmotically-induced Prl release, less is known about the underlying molecular mechanisms that regulate prl_{177} and prl_{188} genes following osmotic stimulation. Studies on the transcriptional regulation of the *prl* gene have shown similarities between tilapia and other vertebrates in promoter regions that bind transcription factors (TFs), such as Pit 1 (Nelson et al., 1988; Poncelet et al., 1996; Sohm et al., 1998). Pit 1 is mainly encoded by the *pou1f1* gene and has shown to be a central regulator of cellular differentiation and function in the pituitary, including Prl cells of both mammals and in fish (Howard et al., 2009; Sobrier et al., 2016; Wang et al., 2017). Through *in silico* analysis of the promoter regions of tilapia *prl* genes, we recently identified a number of putative TFs, in addition to Pit1, and TF modules (TFMs) predicted to play a role in the regulation of prl_{177} and prl_{188} expression (Seale et al., 2020). Whether these TFs and TFMs are activated during osmotic stimulation, however, remain to be determined. Based on the predicted prl_{177} and prl_{188} promoter binding sequences, we designed a customized TF activation assay to identify the osmotic sensitivity of the TFs and TFMs up to ~1600 bp upstream of prl_{177} and prl_{188} genes. First, we compared the activation of TFMs from RPDs of FW- and SW- acclimated Mozambique tilapia. Then, we compared the activation of TFMs from RPDs of FW- and SW- acclimated fish exposed to hyperosmotic and hyposmotic stimuli *in-vitro*, respectively. Last, we incubated dispersed Prl cells from both FW- and SW-acclimated tilapia in static incubation systems to determine how medium osmolality affects the transcription of TFs shown to be most activated by changes in extracellular osmolality and previously identified as being required for *prl* transcription. We hypothesized that TFs regulating osmotically-induced changes in prl_{177} and prl_{188} expression in the tilapia Prl cell, were themselves osmosensitive, especially those that are responsive to hyposmotic stimulation, a potent trigger of Prl secretion.

2. Materials and methods

2.1. Animals

Mature Mozambique tilapia (*O. mossambicus*) of mixed sexes and sizes were obtained from stocks maintained at the Hawai'i Institute of Marine Biology, University of Hawai'i (Kaneohe, HI) and at Mari's Garden (Mililani, HI). Fish were reared in outdoor tanks with a continuous flow of FW or SW under natural photoperiod and fed to satiety once a day with trout chow pellets (Skretting, Tooele, UT). Fish were anesthesized with 2-phenoxyethanol (0.3 mL/L, Sigma Aldrich, St. Louis, MO) and euthanized by rapid decapitation. All experiments were conducted in accordance with the ARRIVE guidelines and approved by the Institutional Animal Care and Use Committee, University of Hawai'i.

2.2. Experiment 1: Comparison between RPDs of FW- and SW-acclimated fish

The effects of environmental salinity on the activation of TFs predicted to bind prl_{177} and prl_{188} promoter regions were determined by comparing RPDs of fish acclimated to FW and SW. Six FW-acclimated Mozambique tilapia of mixed sex weighing 300–1200 g and six SW-acclimated tilapia of mixed sex weighing 180–400 g were anaesthetized with a 0.3 mL/L dose of 2-phenoxyethanol. Blood was collected from the caudal vasculature by a heparinized needle and syringe (200 U/mL, Sigma–Aldrich). Plasma was separated by centrifugation and stored at −80 °C until later analyses. Fish were decapitated and their pituitaries were sampled. RPDs were dissected from pituitary glands and stored at −80 °C prior to nuclear protein extraction and subsequent TF activation profiling.

2.3. Experiment 2: Static incubations of RPDs

The effects of short-term changes in extracellular osmolality (12*h*) on the activation of TFs predicted to bind prl_{177} and prl_{188} promoter regions were determined by incubating RPDs of fish acclimated to either FW or SW in hyper- or hyposmotic media, respectively. A total of 24 FW- and SW-acclimated Mozambique tilapia (*O. mossambicus*) of mixed sex weighing 200–1200 g were used for static RPD incubations *in vitro*. Following euthanasia, RPDs were dissected from the pituitary glands of each fish and placed individually into a single well of a 48-well plate containing 500 μL of isosmotic medium (330 mOsm/kg) (Yamaguchi et al., 2016). The incubation media contained 120 mM NaCl, 4 mM KCl, 0.81 mM $MgSO_4$, 0.99 mM $MgCl_2$, 2 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 1.34 mM Na_2HPO_4, 2.1 mM $CaCl_2$, 10 mM HEPES, 2.77 mM glucose, 2 mM glutamine, 100 IU/mL penicillin, 76.3 IU/mL streptomycin, and milli-Q water. After preincubation for 2 h at 26 °C, the RPDs were rinsed once with isosmotic medium. The RPDs from FW- acclimated fish were incubated in 500 μL of isosmotic medium (330 mOsm/kg) or hyperosmotic medium (420 mOsm/kg), while the RPDs from SW- acclimated fish were incubated in 500 μL of isosmotic medium (330 mOsm/kg) or hyposmotic medium (280 mOsm/kg). A total of six RPDs (three from males and three from females) per salinity group were incubated for 12 h at 26 °C under saturated humidity. At the end of the incubation, the RPDs were collected and stored at −80 °C prior to nuclear protein extraction and subsequent TF activation profiling.

2.4. Experiment 3: Static incubations of dispersed Prl cells

To detrmine the effects of extracellular osmolality on the gene expression of TFs that were shown to be highly expressed in Prl cells (Seale et al., 2020) and exhibit salinity-dependent TF activation, Prl cells from fish acclimated to either FW or SW were incubated in media spanning a range of osmolalities. A total of 60 FW- and SW-acclimated Mozambique tilapia (*O. mossambicus*) of mixed sex weighing 60–300 g were used in static incubations of dispersed Prl cells as previously described (Seale et al., 2012b). Briefly, RPDs dissected from either FW- or SW-acclimated tilapia were pooled in PBS (0.02 M, 330 mOsm/kg) and treated with 0.125% (wt/vol) trypsin (Sigma-Aldrich) for 30 min on a gyratory platform set at 120 rpm to allow for complete cell dissociation. The cells were centrifuged for 5 min at 1200 rpm and the supernatant decanted and discarded; cells were resuspended and triturated in trypsin inhibitor (0.125% wt/vol; Sigma-Aldrich) to terminate the trypsin treatment. Cells were washed with PBS twice and then resuspended in isosmotic medium. Cell viability was determined by trypan blue exclusion test and yield was estimated with a hemocytometer. Dispersed Prl cells were plated (4–6 × 10^4 cells/well on 96 well plate) and preincubated in isosmotic medium for 1 h. The cells were then incubated in media spanning a range of osmolalities (280, 300, 330, 355 and 420 mOsm/kg) for 6 h under saturated humidity (Seale et al., 2012b). At the end of the 6 h incubation, 200 μL of TRI Reagent (MRC,

Cincinnati, OH) was added to each well. The mixture of cells and TRI Reagent was then transferred to 1.5 mL tubes and stored at −80 °C prior to RNA extraction and gene expression analyses.

2.5. Radioimmunoassay

Prl_{177} and Prl_{188} levels in the collected plasma samples were measured by homologous radioimmunoassay (RIA) using the primary antibodies developed in rabbit against Prl_{177} and Prl_{188} (anti- Prl_{177} and anti-Prl_{188}) and secondary antibody raised in goat against rabbit IgG (anti-rabbit IgG) as previously described and validated (Ayson et al., 1994; Yada et al., 1994; Yamaguchi et al., 2016). Dilutions employed for anti-Prl_{177}, anti-Prl_{188} and anti-rabbit IgG were 1:8000, 1:35000 and 1:100 respectively.

2.6. Nuclear protein extraction and quantification

A commercial nuclear extraction kit (Signosis, Santa Clara, CA) was used for the preparation of nuclear extracts following the manufacturer's protocol with minor modifications. For each treatment, two RPDs were combined (one male and one female) to represent one mixed-sex sample of nuclear extract. Briefly, the manufacturer's protocol for cell culture was followed with an extra step to add 1× Buffer 1 and repeat the incubation on a shaking platform to ensure a better separation between cytoplasm and nuclear proteins. Also, an overnight freeze-thawing step at −80 °C was included to maximize the release of nuclear proteins. At the end of the procedure, the harvested nuclear protein extract was quantified using a Pierce BCA protein assay kit following the manufacturer's protocol (Thermo scientific, Rockford, IL). The concentrations of nuclear protein ranged between 200 and 900 μg/mL and were used to normalize each sample. The nuclear protein samples were stored at −80 °C until further analysis.

2.7. Transcription factor activation profiling assay

A custom-made TF activation profiling plate array (Signosis, Santa Clara, CA) was used to determine the activated TFs in the nuclei of tilapia Prl cells. Twenty six predicted TFMs up to 1600 bp upstream of the promoter regions of prl_{177} and prl_{188} were included in this screening (Fig. 2). The sequences of the predicted binding sites of each TFM in the prl_{177} and prl_{188} promoter regions were obtained through *in-silico* analysis using MatInspector: Matrix family library V.11.0 (Genomatix, Munich, Germany) (Seale et al., 2020) and used to design biotinylated probes in the customized TF activation plate (Table 1). The TF activation assay was conducted using 5 μg of nuclear protein per sample according to the manufacturer's protocol with minor modifications. Briefly, two additional washing steps were carried out to assure the complete removal of free probes from the TF-DNA complex and the incubation period with Streptavidin-HRP was reduced from 40 to 20 min to avoid background noise. Luminescence was read in a luminometer (Synergy Lx, BioTek, Santa Clara, CA) with gain set at 100 photomultiplier tube amplification units (PMT) and without the use of filters to avoid emission cutoff. The activity of ETSF_ETSF 06, a TFM that did not vary significantly among plates or treatments (one-way ANOVA, $P > 0.05$), was used to normalize TFM activation. Values are expressed as relative activity, defined as the ratio between target TFM and ETSF_ETSF 06.

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from Prl cells frozen in TRI Reagent following the manufacturer's protocol and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The levels of reference and target genes were determined by the relative quantification method using a StepOnePlus real-time qPCR system (Thermo Fisher Scientific). The qPCR reaction mix (15 μL) contained Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 200 nmol/L forward and reverse primers, and 1 μL of

Table 1
Predicted putative TFM consensus binding sites on prl_{177} and prl_{188} promoter regions.

Region number	Promoter sequence (5′-3′)	Strand position	Predicted TFM	Description
1	tgagtGGGCggtggggt	(+)	SP1F_SP1F 06, SP1F_SP1F 09	Stimulating protein 1, ubiquitous zinc finger transcription factor
2	cggtggGGTGgggttaa	(+)	SP1F_SP1F 06, SP1F_SP1F 09	GC box elements
3	cagactgtGCAAaat	(−)	CEBP_CEBP 01	CCAAT/enhancer binding protein (C/EBP), epsilon
4	tgagCCAAtgaagaa	(+)	CAAT_AP1F 01	Cellular and viral CCAAT box
5	atttGGAAaattgtgtgtt	(−)	NFAT_GATA 01	Nuclear factor of activated T-cells 5
6	caatagtAAACatctta	(+)	FKHD_NF1F 01	Hepatocyte nuclear factor 3 (alpha, beta) (FOXA1, FOXA2)
7	agaaaaCAATaaatataaagagt	(+)	SORY_PAX3 02	Sox-5
8	agctGGAAacttataaaaa	(+)	NFAT_AP1F 01	Nuclear factor of activated T-cells 5
9	cgtGTCTgacc	(−)	SMAD_AP1F 01, SMAD_MITF 01	Sma- and Mad-related proteins
10	gcaggttcACGTgtctgacca	(−)	CREB_EBOX 02	X-box-binding protein 1
11	tcagaCACGtgaacctg	(+)	CREB_EBOX 02, EBOX_AP2F 01, EBOX_MITF 01	MAX binding protein
12	aggtTCACgtgtctg	(−)	SMAD_MITF 01	Coordinated Lysosomal Expression and Regulation (CLEAR) elements bound by TFEB
13	tattcaGTCAatt	(+)	NFAT_AP1F 01	Transcription factor Jun-B
14	ggctttgAATGgatgcaacagg	(−)	SORY_PAX3 02	HMG box-containing protein 1
15	ttcaGATAaggag	(−)	GATA_AP1F 02	GATA binding factors
16	tagtcgccagagacGAAAccaacaa	(+)	IRFF_STAT 01	Interferon regulatory factors
17	atCATGtcatttgtc	(+)	OCT1_CEBP 01	Octamer binding protein
18	aagtgaCAAAagacaaatgacat	(−)	SORY_PAX6 01	SOX/SRY-sex/testis determinig and related HMG box factors
19	tgtGTCTgtcc	(+)	AP1F_SMAD 01	Vertebrate SMAD family of transcription factors
20	aagtgaCTCAatc	(−)	AP1F_SMAD 01	AP1, Activating protein 1
21	tatgaataaaaTAATtaca	(−)	BRNF_RXRF 01	Brn POU domain factors
22	ttattTTATtcataa	(+)	OCT1_PIT1 01	GHF-1 pituitary specific pou domain transcription factor
23	ttactgttGCAAtga	(−)	MYBL_CEBP 01	Ccaat/Enhancer Binding Protein
24	cagaatcaGGAAaaaacattt	(+)	ETSF_ETSF 06	Human and murine ETS1 factors
25	ggttGATAaggtg	(−)	GATA_SP1F 01	GATA binding factors
26	actgtgtgcTAATtatcaa	(−)	PBXC_PDX 01	Pancreatic and intestinal homeodomain transcription factor

All consensus sequences are shown in the 5′-3′ orientation of (+) or (−) strands. Red letters appear in a position where the matrix exhibits a high conservation profile (ci-value>60), and capital letters denote the core sequences used by MatInspector. The remaining letters represent the consensus *prl* sequence. Region numbers 1–14 represent TFMs in prl_{177} promoter region while 14–26 represent TFMs in prl_{188} promoter region.

cDNA. PCR cycling parameters were as follows: 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. Target gene transcripts were identified using the previously reported TF transcriptome data (Seale et al., 2020) and selected based on abundance and osmotically-induced activation. New primers were designed using the Primer3 software (Kõressaar et al., 2018) and their specificities were confirmed by melt curves. Primer sequences are listed in Table 2. The geometric mean of three reference genes (*ef1-α*, *18S*, and *β-actin*) was used to normalize target genes. Data are expressed as mean fold change ± SEM ($n = 8$) from the isosmotic treatment (330 mOsm/kg).

2.9. Statistics

Data representing the activation of TFs from steady-state salinity comparisons and from static incubations of RPDs were analyzed by Student's *t*-test. Data from static incubations of Prl cells were analyzed by one-way ANOVA. Significant effects of medium osmolality were followed up by protected Fisher's LSD test. When necessary, data were log-transformed to satisfy normality and homogeneity of variance requirements prior to statistical analysis. All statistics were performed using Prism 9 (GraphPad, La Jolla, CA) and data are reported as means ± SEM.

3. Results

3.1. Effects of acclimation salinity on plasma Prl

Plasma Prl_{177} and Prl_{188} levels in tilapia acclimated to FW and SW are shown in Fig. 1. Both, Prl_{177} and Prl_{188} levels were ~ 20-fold higher in tilapia acclimated to FW than those in SW (Fig. 1A and B).

3.2. Effects of acclimation salinity on transcription factor activation

Transcription factor activation assays in FW- and SW- acclimated Mozambique tilapia were developed based on predicted binding sites of TFs and TFMs to cis-regulatory elements of prl_{177} and prl_{188} promoter regions (Fig. 2).

The activation of TFMs in RPDs of fish acclimated to FW and SW is presented in order, from lowest to highest relative activity in FW (Fig. 3). The threshold for activation of a TFM was set at a relative activity of 1.5, based on a previous study (Ding et al., 2020). OCT1_PIT1 01 was the highest activated TFM in FW-acclimated tilapia. Activation of

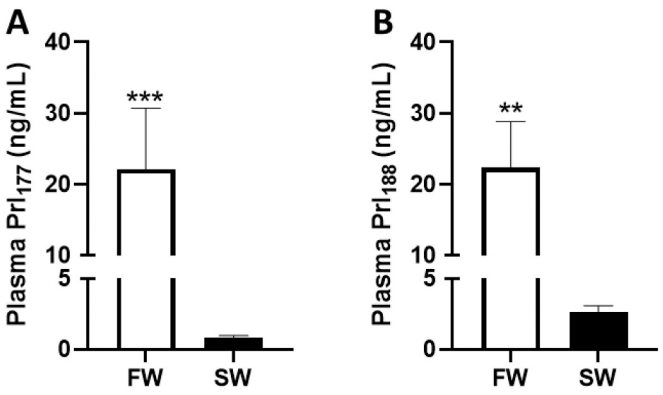

Fig. 1. Effects of acclimation salinity on plasma levels of Prl_{177} (A) and Prl_{188} (B) in Mozambique tilapia. Clear and solid bars represent fish acclimated to FW and SW, respectively. Data are expressed as mean ± SEM ($n = 9$–10). The effect of acclimation salinity was analyzed by Student's *t*-test; $^{**}p < 0.01$, $^{***}p < 0.001$.

OCT1_PIT1 01, BRNF_RXRF and CEBP_CEBP 01 in FW-acclimated tilapia was higher than that of SW-acclimated fish. By contrast, the activation of SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 was higher in tilapia acclimated to SW compared with those in FW. NFAT_GATA 01, SORY_PAX3 02, SP1F_SP1F 06, SP1F_SP1F 09, PBXC_PDX 01 and GATA_SP1F were not activated in FW-acclimated fish, while NFAT_GATA 01 and NFAT-AP1F 01 in the distal region of prl_{177} promoter were not activated in SW-acclimated fish.

3.3. Effects of extracellular osmolality on transcription factor activation in vitro

To characterize the activation of TFMs by a hyposmotic stimulus, RPDs from SW-acclimated tilapia were incubated in either isosmotic (330 mOsm/kg) or hyposmotic media (280 mOsm/kg) for 12 h prior to nuclear extraction and subsequent TFM profiling (Fig. 4). The activation of TFMs is presented in the order of activity in hyposmotic media. The activation of CAAT_AP1F 01, OCT1_CEBP 01, AP1F_SMAD 01, GATA_SP1F 01 and SORY_PAX6 01 was higher in RPDs of SW-acclimated tilapia incubated in hyposmotic media than the RPDs incubated in isosmotic media. The TFMs NFAT_GATA 01, SORY_PAX3 02, FKHD_NF1F 01, SP1F_SP1F 06, SP1F_SP1F 09, NFAT_AP1F 01,

Table 2
Gene specific primers used for qPCR.

Gene	Primer sequence (5′-3′)	R^2	Efficiency %	Accession number	Reference
18 S	F: GCTACCACATCCAAGGAAGGC R: TTCGTCACTACCTCCCCGAGT	0.998	90.5	AF497908	(Magdeldin et al., 2007)
ef1a	F: AGCAAGTACTACGTGACCATCATTG R: AGTCAGCCTGGGAGGTACCA	0.995	100.1	AB075952	(Breves et al., 2010)
β-actin	F: CTCTTCCAGCCTTCCTTCCT R: ACAGGTCCTTACGGATGTCG	0.998	95.3	FN673689	(Tipsmark et al., 2011)
pou1f1	F: GGCAATGCTCTCAGCAACAC R: GCATCTCCTGTGCTGCCAT	0.988	94.6	XM_019352661.2	(Seale et al., 2020)
stat3	F: TATCTGCGTTACCCCGTGTC R: TTTGTGCCTGGGAATCCGTT	0.994	104.6	XM_013269621.3	(Seale et al., 2020)
creb3l1	F: CAGTTTAACAGCGGAGAAACTCTA R: GGTCACCTGAGAAAGGCACATT	0.993	95.9	XM_005460642.4	(Seale et al., 2020)
stat1a	F: ACCATCAGAGGCTGCTGAAC R: CAGCCTGGACGGATGAACTT	0.989	78.3	XM_005452305.4	(Seale et al., 2020)
pou2f1b	F: GGGGACAGATTGCTGGAGTA R: AGCTTCAGCCAAGTCATCGT	0.922	160.0	XM_025903751.1	Newly designed
cebpb	F: CACATTCACACACCGGAGAC R: CCTGTGAAGCGTACCGTTTT	0.992	82.3	XM_003438913.5	Newly designed
nfatc1	F: GCCGCTGTAGCTTTAAGTGG R: ACACTGAGGCGAGCTCAAAT	0.935	84.1	XM_003447265.5	Newly designed
ap1b1	F: CACTGACAGCCTGGAGTGAA R: CTCATTGACTTCTGCCACGA	0.961	100.0	XM_005473361.4	Newly designed

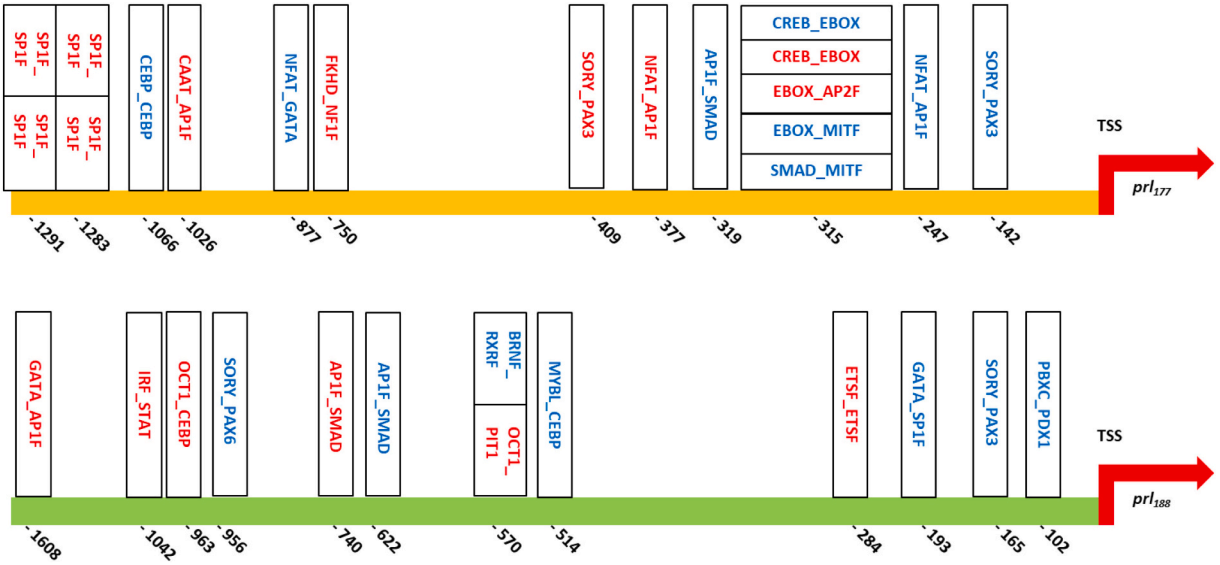

Fig. 2. Transcription factor modules (TFM) predicted in prl_{177} (yellow band) and prl_{188} (green band) promoter regions up to −1.6 kb (adapted from Seale et al., 2020, with author's permission) and used to design specific probes employed in the TF/TFM activation profiling assay. TFMs predicted to bind to the (−) strand are indicated by blue text and the TFMs predicted to bind to the (+) strand are indicated by red text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

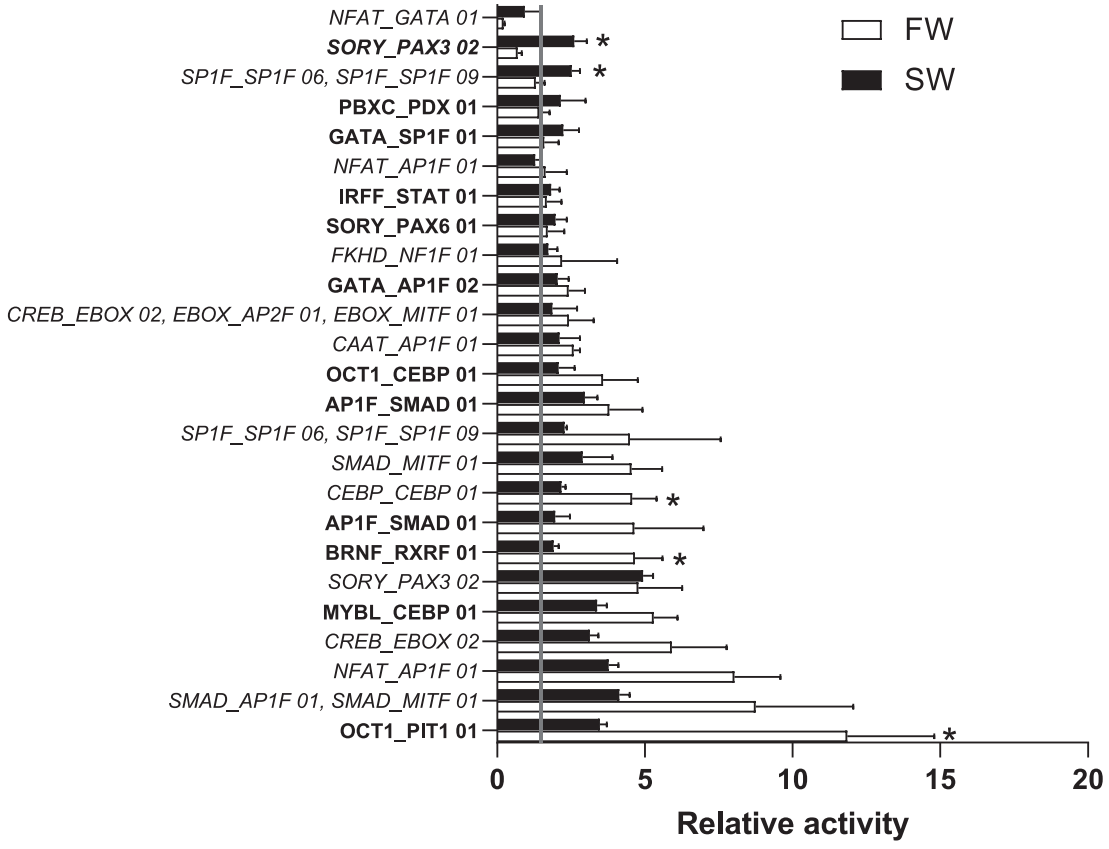

Fig. 3. Relative activation of predicted TFMs in RPDs of FW- and SW- acclimated tilapia. Clear and solid bars represent fish acclimated to FW and SW, respectively, and expressed as mean ± SEM. Data represents the relative activation of each TFM to the activity of ETSF_ETSF 01 and are shown in ascending order of activity in FW. TFMs predicted to bind to prl_{177} promoter region are written in italics and the TFMs predicted to bind to prl_{188} promoter region are written in bold letters. TFMs common to both *prls* are written in bold italics. The grey solid line shows the 1.5× activity relative to ETSF_ETSF 01. * Denotes significant differences in activities between FW and SW acclimated fish at $p < 0.05$, by Student's t-test ($n = 3$).

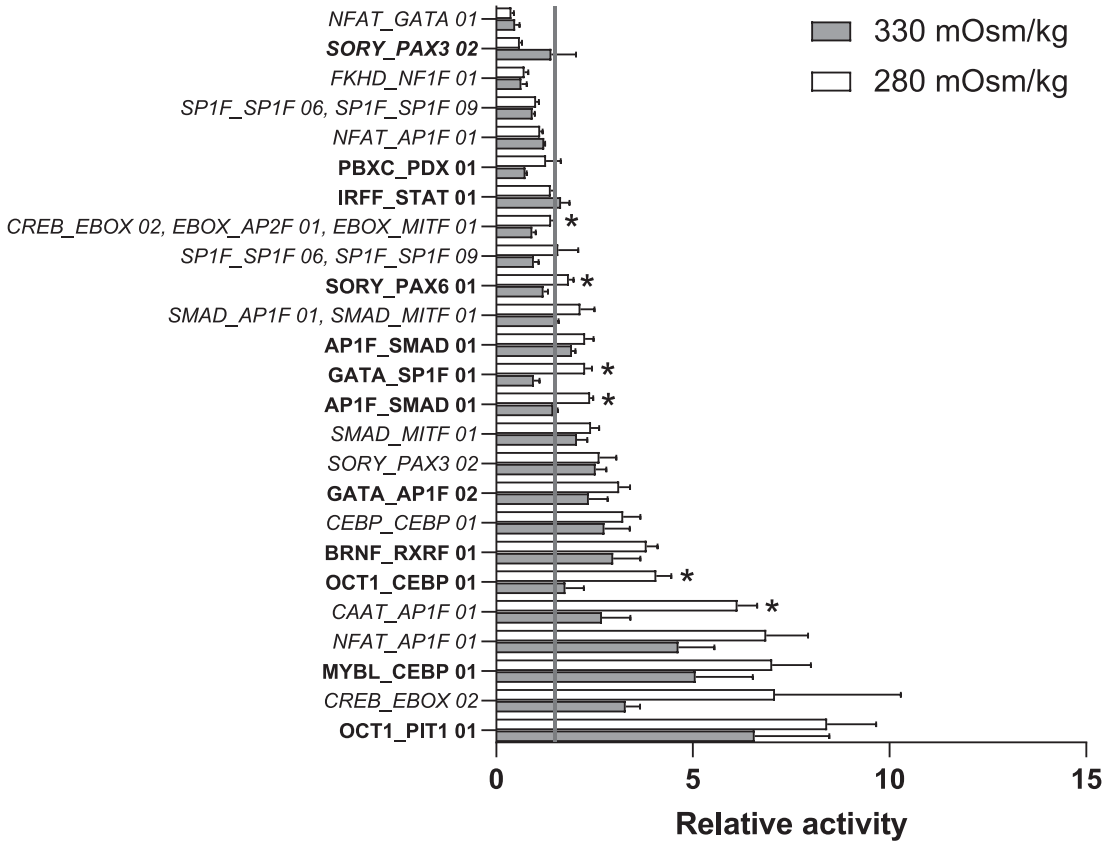

Fig. 4. Relative activation of predicted TFMs in RPDs of SW- acclimated tilapia incubated in hyposmotic (280 mOsm/kg) and isosmotic (330 mOsm/kg) media for 12 h. Clear and grey bars represent RPDs of SW fish incubated in hyposmotic and isosmotic media, respectively, and expressed as mean ± SEM. Data represents the relative activation of each TFM to the activity of ETSF_ETSF 01 and are shown in ascending order of activity in hyposmotic media. TFMs predicted to bind to the prl_{177} promoter region are written in italics and the TFMs predicted to bind to prl_{188} promoter region are written in bold letters. TFMs common to both *prls* are written in bold italics. The grey solid line shows the 1.5× activity compared to ETSF_ETSF 01. * Denotes significant differences in activities between RPD's incubated in hyposmotic and isosmotic media at $p < 0.05$, by Student's t-test ($n = 3$).

PBXC_PDX 01 and IRFF_STAT 01 were not activated in hyposmotic conditions. Even though the relative activity was below 1.5, CREB_EBOX 02, EBOX_AP2F 01, EBOX_MITF 01 had higher activity in hyposmotic media than in isosmotic media.

The incubation of RPDs from FW-acclimated fish in hyperosmotic media (420 mOsm/kg), was used to probe for hyperosmotically-induced activation of TFMs. They are listed in the order of activity in hyperosmotic media (Fig. 5). Activation of SORY_PAX3 02 and AP1F_SMAD 01 was elevated in hyperosmotic conditions. NFAT_AP1F 01, FKHD_NF1F 01, SP1F_SP1F 06, SP1F_SP1F 09 and IRFF_STAT 01 remained inactive when the RPDs were incubated in hyperosmotic media.

3.4. Effects of extracellular osmolality on mRNA expression of TF transcripts

The mRNA levels of the TF transcripts in Prl cells from FW- and SW-acclimated tilapia incubated in a range of osmolalities are shown in Figs. 6 and 7. In FW- acclimated tilapia Prl cells, *pou1f1* (Fig. 6A), *pou2f1b* (Fig. 6B) and *stat3* (Fig. 6C) levels were suppressed by hyperosmotic media compared with the levels in 280 mOsm/kg. Also, the expressions of *stat1a* (Fig. 6D) and *ap1b1* (Fig. 6H) were suppressed at 355 mOsm/kg. All of the TF mRNA levels were similar in FW-acclimated tilapia Prl cells incubated in 280 mOsm/kg and 330 mOsm/kg media. Incubation osmolality did not affect the expression of *creb3l1* (Fig. 6E), *cebpb* (Fig. 6F) or *nfatc1* (Fig. 6G).

In SW-acclimated tilapia Prl cells, *pou1f1* (Fig. 7A), *pou2f1b* (Fig. 7B) and *stat3* (Fig. 7C) mRNA levels were inversely related to incubation osmolality. Incubation in 420 mOsm/kg media inhibited the expression of *nfatc1* (Fig. 7G) compared with its expression in hyposmotic conditions. The expression of *creb3l1* (Fig. 7E) was inhibited at 280 mOsm/kg media while 355 mOsm/kg media upregulated the expression of *ap1b1* relative to other incubation osmolalities (Fig. 7H). Incubation osmolality did not have any effect on the expressions of *cebpb* (Fig. 7F), or *stat1a* (Fig. 7D).

4. Discussion

The present study examined the osmosensitivity of TFs and TFMs in Prl cells of Mozambique tilapia, an established euryhaline fish model for osmoreception studies. Our findings indicate that a range of TFMs are activated in both hypo- and hyperosmotic conditions and in accordance with acclimation salinity. In this discussion, we will consider each group of TFMs separately and discuss their osmosensitive characteristics. TFMs activated in FW- acclimated fish largely coincided with those activated by a hyposmotic stimulus *in-vitro*; the reverse was observed in SW-acclimated fish, where the most highly activated TFMs were similar to those activated by hyperosmotic conditions. Most notably, PIT1_OCT1 was robustly activated in fish in FW compared with those in SW, and their transcripts, *pou1f1* and *pou2f1b*, were inversely related to extracellular osmolality *in-vitro*. The osmotic response patterns in the activation of other TFMs were also similar to those observed in the expression of their corresponding transcripts. This study, therefore, lays the foundation for characterizing the salinity dependence and osmotically-induced activation of TFs in teleost fishes.

Consistent with their roles in promoting ion absorption and retention

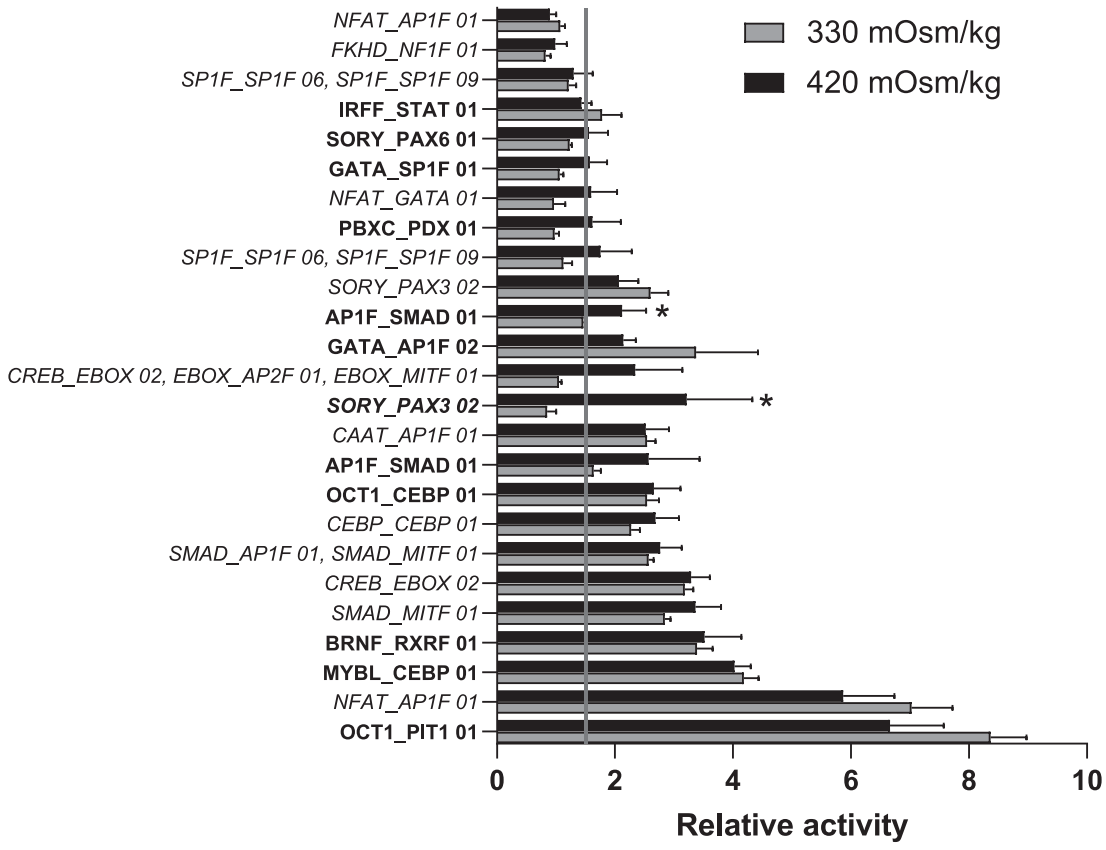

Fig. 5. Relative activation of predicted TFMs in RPDs of FW-acclimated tilapia incubated in hyperosmotic (420 mOsm/kg) and isosmotic (330 mOsm/kg) media for 12 h. Black and grey bars represent RPDs of FW fish incubated in hyperosmotic and isosmotic media, respectively, and expressed as mean ± SEM. Data represents the relative activation of each TFM to the activity of ETSF_ETSF 01 and are shown in ascending order of activity in hyperosmotic media. TFMs predicted to bind to the prl_{177} promoter region are written in italics and the TFMs predicted to bind to prl_{188} promoter region are written in bold letters. TFMs common to both *prls* are written in bold italics. The grey solid line shows the 1.5× activity compared to ETSF_ETSF 01. * Denotes significant differences in activities between RPD's incubated in hyperosmotic and isosmotic media at $p < 0.05$, by Student's t-test ($n = 6$).

across osmoregulatory epithelia, the mRNA expression of prl_{177} and prl_{188} and release of their gene products, Prl_{177} and Prl_{188}, are inversely related to extracellular osmolality (Seale et al., 2003a, 2006b, 2012b; Yada et al., 1994). Accordingly, both pituitary mRNA (Seale et al., 2012b) and circulating levels of Prls are higher in tilapia acclimated to FW than those acclimated to SW and our results confirm this (Seale et al., 2006b; Fig. 1). Here, we characterized the osmotic responses of TFs previously predicted to bind promoter regions of both tilapia *prl* genes (Seale et al., 2020). To monitor the simultaneous activation of multiple TFs, specific probes corresponding to 26 consensus sequences of TFM-DNA binding sites in the promoter regions of prl_{177} and prl_{188} were used for designing a customized TF profiling array (Fig. 2, Table 1). First, the activation of TFMs in RPDs of FW- and SW- acclimated fish were compared. The highest activated TFM observed in RPDs of FW-acclimated tilapia was OCT1_PIT1 01 (Fig. 3). Both TFs, OCT1 and PIT1, are members of the POU (Pit-Oct-Unc) family of TFs which are encoded by *pou2f1* and *pou1f1* genes respectively (Malik et al., 2018). OCT1_PIT1 01, CEBP_CEBP 01 and BRNF_RXRF 01 were highly activated in FW-acclimated tilapia. Conversely, SORY_PAX3 02, a common TFM that can bind to the promoter regions of both *prls* and SP1F_SP1F 06, SP1F_SP1F 09 were most highly activated in SW- acclimated tilapia.

PIT1 is considered a key TF in the regulation of *prl* transcription in vertebrates, including fishes such as rainbow trout (*Oncorhynchus mykiss*),carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*) (Argenton et al., 1996; Kausel et al., 2006; Poncelet et al., 1996). In fact, there is significant homology between tilapia and mammalian binding sites for PIT1 (Poncelet et al., 1996). Previous truncation analyses of the tilapia prl_{188} promoter employing a luciferase reporter assay revealed that the region containing PIT1 was required for the trancriptional activation of *prl* (Poncelet et al., 1996). Here, we show a novel result, where the activation of the PIT1_OCT1 01 TFM, predicted to bind ~570 bp upstream of prl_{188}, is increased in Mozambique tilapia acclimated to FW when compared with fish in SW. Interestingly, the absence of a OCT1_PIT1 01 binding site in the promoter region of prl_{177} could underlie the observed regulatory differences between Prl isoforms, such as discrepancies between their circulating levels and the enhanced sensitivity and magnitute of Prl_{188} release in response to hyposmotic stimulation in tilapia (Seale et al., 2006b). Thus, the high activation of PIT1 in FW is in agreement with the maintenance of sustained prl_{188} transcription and elevated synthesis and secretion of Prl_{188} in FW-acclimated fish.

BRNF_RXRF 01 was also highly activated in FW- acclimated fish. The TF, BRN is considered as another member in the POU domain and it is found to be regulated by retinoic acid (Turner et al., 1994). Similarly, retinoic acid is reported to regulate retinoid X receptors (RXR) (Allenby et al., 1993). This common regulator of expression might be a possible reason for BRN and RXR to bind together and act as a TFM. Also, BRN transcripts are reported to be involved in *pit1* expression and lead towards elevated *prl* expression (Toda et al., 2008). This could be the reason behind the significant activation of this TFM, BRNF_RXRF 01 observed in FW- acclimated fish.

The TFMs SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 were predominantly activated in SW-acclimated tilapia. Specifically, the activation of SORY_PAX3 02 was 3-fold higher in SW- than in FW-acclimated fish. PAX3 has been reported to contain regions capable of confering both activation and inhibition of gene transcription (Chalepakis et al., 1994). Our previous analysis indicated the presence of

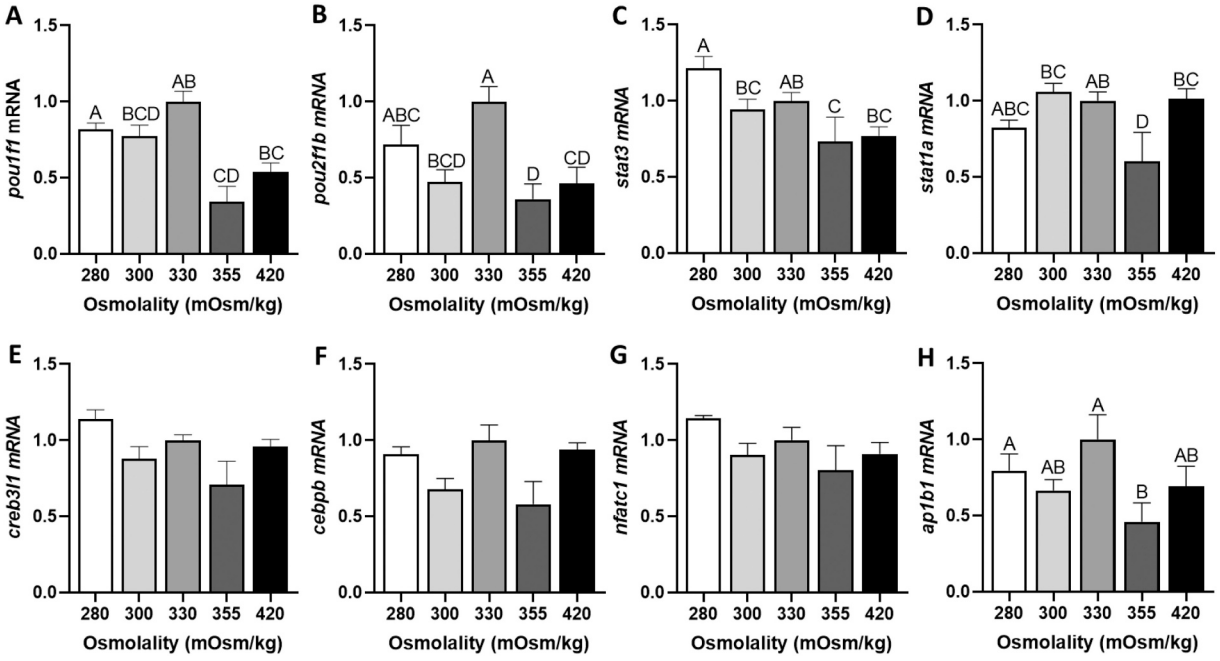

Fig. 6. Changes in mRNA expression of (A) *pou1f1*, (B) *pou2f1b*, (C) *stat3*, (D) *stat1a* (E) *creb3l1*, (F) *cebpb*, (G) *nfatc1* and (H) *ap1b1* in Prl cells from FW-acclimated tilapia incubated in a range of osmolalities for 6 h. mRNA levels are presented as mean fold-change from the 330 mOsm/kg (control) group ± SEM. Differences among groups were analyzed by one-way ANOVA ($n = 8$). When there was a significant effect of media osmolality, *post hoc* group comparisons were followed up with protected Fisher's LSD test. Groups not sharing the same uppercase letter are significantly different.

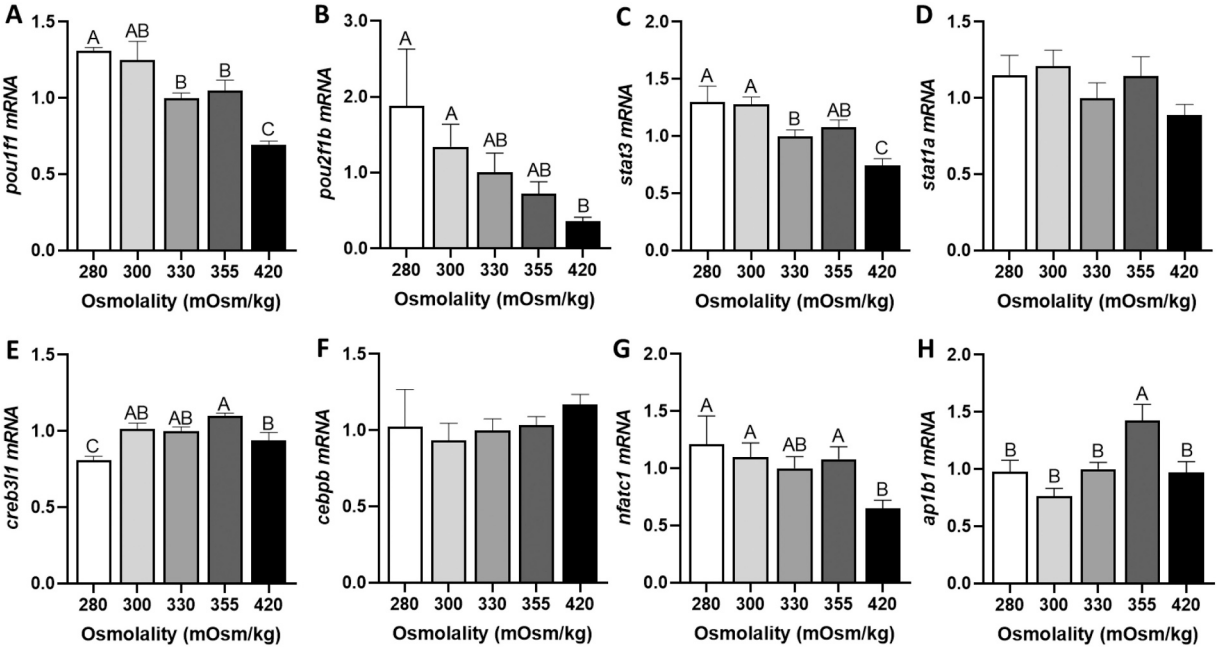

Fig. 7. Changes in mRNA expression of (A) *pou1f1*, (B) *pou2f1b*, (C) *stat3*, (D) *stat1a* (E) *creb3l1*, (F) *cebpb*, (G) *nfatc1* and (H) *ap1b1* in Prl cells of SW-acclimated tilapia incubated in a range of osmolalities for 6 h. mRNA levels are presented as mean fold-change from the 330 mOsm/kg (control) group ± SEM. Differences among groups were analyzed by one-way ANOVA (n = 8). When there was a significant effect of media osmolality, *post hoc* group comparisons were followed up with protected Fisher's LSD test. Groups not sharing the same uppercase letter are significantly different.

binding sites for SORY_PAX3 02 in the promoter regions of both prl_{177} and prl_{188} (Seale et al., 2020; Fig. 2). Inasmuch as this TFM may exert inhibitory effects on transcription, the increase in SORY_PAX3 02 activation observed in SW- acclimated tilapia may underlie the lowering of prl_{177} and prl_{188} mRNA levels and Prl_{177} and Prl_{188} release observed in hyperosmotic environments. SP1F_SP1F 06, SP1F_SP1F 09, which binds to the distal promoter region of prl_{177} is also activated in SW- acclimated tilapia. In mammalian cell lines, SP1 has been reported to be activated in hyperosmotic environments and involved the inhibition of transcription (Tajitsu et al., 2013). Taken together, the actions of SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 may at least in part contribute to the suppression of *prls* and Prl release in SW- acclimated tilapia.

Next, we characterized the activation of TFMs in RPDs of SW- and FW- acclimated tilapia incubated under hypo- and hyperosmotic

conditions, respectively. When we incubated SW- acclimated tilapia RPDs in hyposmotic media, CAAT_AP1F 01, OCT1_CEBP 01, AP1F_SMAD 01, SORY_PAX6 01 and CREB_EBOX 02, EBOX_AP2F 01, EBOX_MITF 01 were activated at higher levels than they were in isosmotic conditions. In FW- acclimated tilapia, OCT1_PIT1 01 was the highest activated TFM; OCT1 alone, also a POU domain TF, was previously reported to become activated in response to different types of stress such as oxidative stress and genotoxic stress in both mammals and teleost fish (Kang et al., 2009; Lennard Richard et al., 2007). Thus, even though there were no differences observed in OCT1_PIT1 01 activation between iso- and hyposmotic conditions, OCT1 might be involved in short-term hyposmotically-induced prl_{188} transcription through the activation of OCT1_CEBP 01.

Hyposmotically-induced Prl release is dependent on the entry of extracellular Ca^{2+} into Prl cells (Grau et al., 1981; Seale et al., 2003a; Watanabe et al., 2012). Moreover, cAMP accumulates in Prl cells in response to a fall in extracellular osmolality and influx of extracellular Ca^{2+} (Helms et al., 1991; Seale et al., 2011). The role of cAMP and Ca^{2+} second messenger systems in cellular signalling has been extensively studied, including downstream activation of TFs such as cyclic AMP response element binding protein (CREB) and CAAT/enhancer binding protein (CEBP) (Thiel et al., 2005; Wang et al., 2019). CEBP was shown to be sensitive to a hyposmotic stimulus in the intestine of medaka (*Oryzias latipes*) (Wong et al., 2014). Both CREB and CAAT are also known to be responsive to hyposmotic stimuli in mammalian cell models as well as in teleost fish (Gao et al., 2008; Kausel et al., 2006; Thiel et al., 2005). In the present study, CEBP, CAAT and CREB constitute TFMs that were highly responsive to a hyposmotic stimulus *in-vitro*. Importantly, the activation of these TFMs occured in RPDs of SW-acclimated tilapia, where expression of *prls* is constituitevly low (Seale et al., 2012b). The activation of CAAT_AP1F 01, OCT1_CEBP 01, and CREB_EBOX 02, EBOX_AP2F 01, EBOX_MITF 01 *in-vitro*, is therefore consistent with the short-term activation of the cAMP second messenger in Prl cells of tilapia acclimated to a SW environment following exposure to a hyposmotic stimulus.

The TFM SORY_PAX6 01 was also activated in SW- acclimated tilapia RPDs incubated in hyposmotic media. The TFs of the PAX family have been shown to have both activating and inhibitory effects through regions near the C-terminus and N-terminus, respectively (Chalepakis et al., 1994). The combined activation and suppressive effects of PAX3 and PAX6 have been reported as a means of fine tuning transcriptional regulation by the PAX family of TFs (Wakamatsu, 2011). In the current study, SORY_PAX3 02 did not change while SORY_PAX3 06 activation increased in RPDs from SW-acclimated tilapia incubated in hyposmotic media. These divergent actions by TFs of the PAX family may underlie the fine regulation of prl_{188} transcription as both these TFs are predicted to bind the prl_{188} promoter, albeit in different regions.

When we incubated FW- acclimated tilapia RPDs in hyperosmotic media, the activation of SORY_PAX3 01 increased by four-fold compared with its activity in isosmotic media. This result was consistent with those of other TF activation plate arrays, where the activity of this TFM was increased in SW- acclimated fish compared with FW-acclimated fish, and unresponsive in RPDs of SW-acclimated fish incubated in hyposmotic media. Because SORY_PAX3 01, a TFM common to the promoter regions of both prl_{177} and prl_{188}, is stimulated by hyperosmotic conditions and SORY_PAX6 01 is not, PAX3 may play a role in both short- and long-term negative regulation of tilapia Prl cells. In addition, the activation of AP1F_SMAD 01 was stimulated by hyperosmotic media. The result is consistent with the mammalian hypothalamus, where AP1 has been shown to be activated in hyperosmotic conditions (McCabe and Burrell, 2001; Ying et al., 1996). Even though SMAD is not reported to be osmotically sensitive, the hyperosmotically-induced activation of AP1 may be involved in the short-term inhibition of prl_{188} transcription in Prl cells of FW- acclimated tilapia responding to hyperosmotic stress. These findings further underscore the high osmosensitivity of Prl cells from FW- acclimated fish, where transition from FW to a SW environment necessitates rapid inhibition of Prl secretion.

Based on the osmotic sensitivity of TF activation and known signal transduction mediators in tilapia Prl cells, we then examined the transcriptional responses of select TFs in Prl cells of FW- and SW- acclimated tilapia incubated under a range of medium osmolalities. When we incubated Prl cells from FW- acclimated fish, mRNA expression of several transcripts varied in response to extracellular osmolality. Encoding PIT1 and OCT1, which formed the most highly activated TFM in FW- acclimated fish, *pou1f1* and *pou2f1b* showed similar responses to extracellular osmolality, decreasing at 355 and 420 mOsm/kg. This pattern is consistent with the observed role of OCT1_PIT1 01 in FW-acclimation. It is worth noting, however, that OCT1_PIT1 01 activation only exhibited a tendency to lower under hyperosmotic conditions. Because the RPDs from FW- acclimated tilapia already show high expression levels of both *prls*, it is expected that they would have high mRNA levels of the TFs needed to maintain the high baseline of *prl* transcription, which could be up to ~30 fold higher in fish acclimated to FW compared with those in SW (Seale et al., 2012a; Seale et al., 2012b). Consequently, in FW- acclimated fish, those TF transcripts may not be as sensitive to hyposmotic challenges as they would in SW-acclimated fish. In fact, Prl cells from SW- acclimated tilapia responded more consistently to extracellular osmolality. Both *pou1f1* and *pou2f1b* mRNA levels were inversely related to the extracellular osmolality (Fig. 7 A and B). These patterns are consistent with those reported for prl_{177} and prl_{188} (Seale et al., 2012b) and the notion that in SW fish *prl* mRNA levels are low, but are rapidly stimulated through the hyposmotically-induced expression of activating TFs.

Prl activates its target cells by interacting with a pair of single-transmembrane domain receptors that are linked to the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathways (Brooks, 2012). Inasmuch as Prl cells can be regulated in autocrine fashion (Yamaguchi et al., 2016), the STAT family of TFs may also play a role in regulating *prl* transcription in response to osmotic stimuli. In goldfish, the main STAT involved in Prl signalling is STAT3 (Yan et al., 2017). Moreover, we observed *stat3* to be the most abundant *stat* transcript based on an earlier transcriptome analysis of the RPD of Mozambique tilapia (Seale et al., 2020). Here, we observed *stat3* transcription from SW-acclimated fish inversely related to extracellular osmolality, following a similar trend as transcripts of the POU family. Despite the osmotic sensitivity of *stat3* regulation, the activation of IRFF_STAT 01 did not differ between FW- and SW-acclimated fish nor it was activated by hyposmotic conditions. Nonetheless, the observed hyposmotic induction of *stat3* but not *stat1a*, suggests that the former TF plays a role in the osmotic sensitivity of prl_{188} expression.

Prl cells transduce hyposmotic stimuli through the activation of second messenger systems, such as cAMP and Ca^{2+}, which in turn initiate *prl* transcription (Seale et al., 2003a, 2011). Accordingly, OCT1_CEBP1 became activated in hyposmotic conditions (Fig. 4). Moreover, we looked at the expression patterns of two transcripts, *creb* and *cebp,* encoding for TFs known to be activated by cAMP and Ca^{2+} (Thiel et al., 2005). The gene transcripts, *creb3l1* and *cebpb* were the most highly expressed from CREB and CEBP families of TFs in RPDs of Mozambique tilapia based on our previous transcriptomic analysis (Seale et al., 2020). Nonetheless, we did not observe any differences in *cebpb* expression in response to extracellular osmolality and *creb3l1* was suppressed in Prl cells of SW-acclimated fish incubated at 280 mOsm/kg (Fig. 7 E and F). One possible reason for the discrepancy between TF activation and mRNA expression may stem from the lack of osmosensitivity of these transcripts and a possible negative feedback role played by *creb3l1*with respect to the regulation of a Ca^{2+}-dependent hyposmotic response of the Prl cell. Moreover, RPDs of SW- acclimated tilapia had greater mRNA levels of *creb3l1* than those of FW fish (Seale et al., 2020), supporting the notion that in hyperosmotic environments Prl cells are more responsive to Ca^{2+}-dependent signalling, such as that known to occur during hyposmotically-induced Prl release (Seale et al., 2003a). A similar hyperosmotic upregulation of *creb3l1* was observed in

the hypothalamus of rats when they were subjected to hyperosmotic stress by preventing water uptake for 1–3 days or replacing water intake by NaCl solution (Greenwood et al., 2015). While the osmotic response of *creb3l1* and *cebpb* may not necessarily coincide with that of TFMs containing CREB and CEBP, other transcripts involved in Ca^{2+}- and cAMP- dependent downstream signalling may exhibit greater osmosensitivity.

Recent findings indicate that the activation of secondary metabolite pathways, including those involved in the production of myo-inositol and glutamine synthetase, are involved in SW- acclimation of Mozambique tilapia (Kim and Kültz, 2020; Wang and Kültz, 2017). The sensitivity of these pathways to hyperomotic stimuli has been reported to be dependent on the osmolality/salinity- responsive enhancer 1 (OSRE1) located in intron 1, which is activated by the TF, nuclear factor of activated T-cells (NFAT) (Kim and Kültz, 2020). NFAT has three distinctive binding regions in the promoter region of prl_{177} *via* two TFMs, NFAT_GATA 01 and NFAT_AP1F 01. In the proximal region, NFAT_AP1F 01 was highly activated in hyposmotic and hyperosmotic conditions while in the distal region, activation was low. The activation of NFAT_GATA 01 was also low regardless of extracellular osmolality. Even though we did not observe any hyperosmotically-induced activation of NFAT or *nfatc1* mRNA expression, studies in mammalian cell models, common fruit fly (*Drosophila melanogaster*) and Atlantic salmon (*Salmo salar*) have shown that NFAT5 is activated by hyperosmotic stress (Keyser et al., 2007; Kim and Kültz, 2020; López-Rodríguez et al., 1999; Lorgen et al., 2017; Yoshimoto et al., 2021). While different *nfat* transcripts may be differentially osmosensitve, *nfatc1* was the most abundant transcript of the NFAT family in RPDs of Mozambique tilapia based on a previous transcriptome analysis (Seale et al., 2020).

In the prl_{177} proximal promoter region, the highly activated NFAT-containing TFM was combined with AP1. Another TFM including AP1, AP1F_SMAD 01, which binds at 622 bp upstream of the prl_{188} promoter region, was significantly activated by both hyperosmotic and hyposmotic media. Both hyperosmotic and hyposmotic activation of AP1 has also been reported in mammalian cell models (Kim et al., 2001; McCabe and Burrell, 2001; Ying et al., 1996). The mRNA expression of *ap1b1* was similar at all osmolalities, except at 355 mOsm/kg, where a decrease and increase in expression was observed in FW- and SW-acclimated fish, respectively. The high activation of AP1 in both hyposmotic and hyperosmotic conditions underlie the high activation of NFAT_AP1F 01 observed in the proximal promoter region of prl_{177}.

Collectively, our results reveal the complex patterns of activation of different TFMs predicted to bind regulatory elements upstream of prl_{177} and prl_{188} depending on acclimation salinity and short-term osmotic stimulation. The marked activation of OCT1_PIT1 01 and CEBP_CEBP 01 in FW- acclimated fish and OCT1_CEBP 01 in response to hyposmotic stimulation was noteworthy as their TFs have been previously implicated in the basal regulation of *prl* transcription and response to Ca^{2+} and cAMP signalling pathways, two of the hallmarks of hyposmotically-induced Prl synthesis and release. Inasmuch as two of these TFMs are only found upstream of prl_{188}, these findings also support the notion that the enhanced responsiveness of prl_{188} transcription and Prl_{188} release to hyposmotic stimuli compared with prl_{177} and Prl_{177} may be dependent on the activation of TFs of the POU 1 domain. The observed activation of SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 TFMs in RPDs of SW-acclimated fish indicate potential transcription inhibitors of both *prls* when fish face hyperosmotic environments. The mRNA expression data suggests that transcripts such as *pou1f1* and *pou2f1b* respond to incubation osmolality more rapidly, despiteTF activation not decreasesing quickly in response to hyperosmolality. Conversely, activation of CEBP and CREB was observed while *cebpb* mRNA did not vary and *creb3l1* was suppressed at 280 mOsm/kg. Hence, different elements in the cAMP second messenger pathway might be readily available while others would be osmosensitive and act to fine-tune the induction of hyposmotically-driven *prl* transcription. A potentially key transcript in the autocrine regulation of Prl, *stat3* was inversely related to osmolality even though its product did not significantly change in TF activation assays. This might be due to very low STAT protein availability in Prl cell nuclei.

5. Conclusions

The present study reveals that some of the TFs shown and/or predicted to regulate *prl* transcription are themselves osmosensitive, both at the level of their activation as TFMs and the transcriptional regulation of their genes. By employing the osmoreceptive tilapia Prl cell model, linkages between their direct responses to extracellular osmolality and the rise in intracellular secondary messengers leading to Prl synthesis and release can now be supported at the level of osmosensitve TFs, which orchestrate the regulation of these osmoregulatory hormones. These findings provide insights into the osmosensitivty of transcriptional regulators involved in osmoreception, which in turn may explain the observed differences in salinity tolerance and Prl cell osmosensitivity of similarly related species (Yamaguchi et al., 2018) and underlie the adapative responses to extracellular osmolality observed in other organs and study models.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgements

This work was funded in part by grants from the National Science Foundation (IOS-1755016), National Oceanic and Atmospheric Administration (#NA18OAR4170347), National Institute of Health (R21DK111775-01) and National Institute for Food and Agriculture (#HAW02051-H). We are also grateful for Mr. Ryan Chang, Mr. Daniel Woo, Mr. Reilly Merlo and Mr. Tyler Goodearly for their help in fish sampling and laboratory analyses.

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