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1 Osmosensitive transcription factors in the prolactin cell of a euryhaline teleost

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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.

53 **Introduction**

54 The maintenance of osmotic homeostasis for life in complex organisms necessitates
55 appropriate mechanisms to detect and direct hydromineral balance. A first step in detecting
56 changes in extracellular osmolality involves the activation of osmosensitive and osmoreceptive
57 cells and tissues (Bourque & Oliet, 1997; Kültz, 2012; Seale et al., 2006a; Seale et al., 2012a;
58 Wells, 1998). In euryhaline fish, release of the pituitary hormone prolactin (Prl) increases
59 following a fall in extracellular osmolality, a response required for freshwater (FW) acclimation
60 (Ball & Ingleton, 1973; Dharmamba & Nishioka, 1968; Grau et al., 1981; Pickford & Phillips,
61 1959; Seale et al., 2002). The osmoregulatory actions of Prl are largely driven by the stimulation
62 of ion uptake in epithelial tissues such as gill, kidney and intestine (Breves et al., 2011, 2014;
63 Hirano, 1986; Inokuchi et al., 2015; Seale et al., 2014). The euryhaline Mozambique tilapia,
64 (*Oreochromis mossambicus*) is a tractable model to study osmoreception, largely due to the
65 morphological arrangement of Prl cells, which comprise >99% of the *rostral pars distalis* (RPD)
66 of the pituitary (Nishioka et al., 1988.; Seale et al., 2006a; 2005). Tilapia Prl cells secrete two
67 isoforms of Prl, Prl_{177} and Prl_{188}, which are encoded by separate genes (Rentier-Delrue et al.,
68 1989; Specker et al., 1985; Yamaguchi et al., 1988). Both Prls exert hyperosmoregulatory
69 functions by binding their cognate receptors, Prlr1 and Prlr2 in target tissues (Fiol et al., 2009).
70 Despite similar ion-retaining actions (Specker et al., 1985), Prl_{188} release and prl_{188} mRNA
71 expression respond more robustly to hyposmotic stimulation than Prl_{177} and prl_{177} (Inokuchi et
72 al., 2015; Seale, et al., 2012b).

73 When exposed to a fall in extracellular osmolality, tilapia Prl cells respond through an
74 aquaporin-3 (Aqp3)-dependent increase in cell volume followed by rapid uptake of extracellular
75 Ca^{2+} through transient receptor potential vanilloid (Trpv4) mechanosensitive channels, thereby

76 triggering the release of stored Prl (Seale et al., 2003a, 2003b; Watanabe et al., 2009, 2012,;
77 Weber et al., 2004). The acclimation salinity history of the fish plays a role in modulating the
78 responsiveness of Prl cells to changes in extracellular osmolality. For example, Prl cells of
79 Mozambique tilapia acclimated to FW are larger, and contain more secretory granules and stored
80 Prl than those of fish acclimated to SW (Borski et al., 1992; Dharmamba & Nishioka, 1968).
81 Likewise, mRNA levels of both *prl*s are ~30-fold higher in fish acclimated to FW than those in
82 SW (Seale et al., 2012b). The implications for environmental regulation are distinct: in Prl cells
83 of FW-acclimated fish, *prl* mRNA levels are less sensitive to further osmotic stimulation than in
84 Prl cells of SW-acclimated fish, presumably because those levels are already very high in FW
85 fish (Seale et al., 2012a). By contrast, hyposmotically-induced Prl release from Prl cells of FW-
86 acclimated fish *in vitro* is more robust than that of SW fish (Seale et al., 2006a; 2012a; 2002).

87 While previous studies employing the tilapia Prl cell model to investigate osmoreception
88 have focused on hyposmotically-induced Prl release, less is known about the underlying
89 molecular mechanisms that regulate prl_{177} and prl_{188} genes following osmotic stimulation.
90 Studies on the transcriptional regulation of the *prl* gene have shown similarities between tilapia
91 and other vertebrates in promoter regions that bind transcription factors (TFs), such as Pit 1
92 (Nelson et al., 1988; Poncelet et al., 1996; Sohm et al., 1998). Pit 1 is mainly encoded by the
93 *pou1f1* gene and has shown to be a central regulator of cellular differentiation and function in the
94 pituitary, including Prl cells of both mammals and in fish (Howard et al., 2009; Sobrier et al.,
95 2016; Wang et al., 2017). Through *in silico* analysis of the promoter regions of tilapia *prl* genes,
96 we recently identified a number of putative TFs, in addition to Pit1, and TF modules (TFMs)
97 predicted to play a role in the regulation of prl_{177} and prl_{188} expression (Seale et al., 2020).
98 Whether these TFs and TFMs are activated during osmotic stimulation, however, remained to be

99 determined. Based on the predicted *prl*$_{177}$ and *prl*$_{188}$ promoter binding sequences, we designed a
100 customized TF activation assay to identify the osmotic sensitivity of the TFs and TFMs up to
101 ~1600 bp upstream of *prl*$_{177}$ and *prl*$_{188}$ genes. First, we compared the activation of TFMs from
102 RPDs of FW- and SW- acclimated Mozambique tilapia. Then, we compared the activation of
103 TFMs from RPDs of FW- and SW- acclimated fish exposed to hyperosmotic and hyposmotic
104 stimuli in-vitro, respectively. Last, we incubated dispersed Prl cells from both FW- and SW-
105 acclimated tilapia in static incubation systems to determine how medium osmolality affects the
106 transcription of TFs shown to be most activated by changes in extracellular osmolality and
107 previously identified as being required for *prl* transcription. We hypothesized that TFs regulating
108 osmotically-induced changes in *prl*$_{177}$ and *prl*$_{188}$ expression in the tilapia Prl cell, were
109 themselves osmosensitive, especially those that are responsive to hyposmotic stimulation, a
110 potent trigger of Prl secretion.

111 **Materials and methods**

112 *Animals*

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

121 *Experiment 1: Comparison between RPDs of FW- and SW-acclimated fish*

122 The effects of environmental salinity on the activation of TFs predicted to bind *prl*$_{177}$ and
123 *prl*$_{188}$ promoter regions were determined by comparing RPDs of fish acclimated to FW and SW.
124 Six FW-acclimated Mozambique tilapia of mixed sex weighing 300-1,200 g and six SW-
125 acclimated tilapia of mixed sex weighing 180-400 g were anaesthetized with a 0.3 mL/L dose of
126 2-phenoxyethanol. Blood was collected from the caudal vasculature by a heparinized needle and
127 syringe (200 U/ml, Sigma–Aldrich). Plasma was separated by centrifugation and stored at -80 ºC
128 until later analyses. Fish were decapitated and their pituitaries were sampled. RPDs were
129 dissected from pituitary glands and stored at -80 ºC prior to nuclear protein extraction and
130 subsequent TF activation profiling.

131 *Experiment 2: Static incubations of RPDs*

132 The effects of short-term changes in extracellular osmolality (12 h) on the activation of
133 TFs predicted to bind *prl*$_{177}$ and *prl*$_{188}$ promoter regions were determined by incubating RPDs
134 of fish acclimated to either FW or SW in hyper- or hyposmotic media, respectively. A total of
135 24 FW- and SW-acclimated Mozambique tilapia (*O. mossambicus*) of mixed sex weighing 200-
136 1200 g were used for static RPD incubations *in vitro*. Following euthanasia, RPDs were
137 dissected from the pituitary glands of each fish and placed individually into a single well of a 48-
138 well plate containing 500 µL of isosmotic medium (330 mOsm/kg) (Yamaguchi et al., 2016).
139 The incubation media contained 120 mM NaCl, 4 mM KCl, 0.81 mM $MgSO_4$, 0.99 mM $MgCl_2$,
140 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
141 mM glucose, 2 mM glutamine, 100 IU/mL penicillin, 76.3 IU/mL streptomycin, and milli-Q
142 water. After preincubation for 2 h at 26 °C, the RPDs were rinsed once with isosmotic medium.
143 The RPDs from FW-acclimated fish were incubated in 500 µL of isosmotic medium (330

144 mOsm/kg) or hyperosmotic medium (420 mOsm/kg), while the RPDs from SW-acclimated fish
145 were incubated in 500 µL of isosmotic medium (330 mOsm/kg) or hyposmotic medium (280
146 mOsm/kg). A total of six RPDs (three from males and three from females) per salinity group
147 were incubated for 12 h at 26 °C under saturated humidity. At the end of the incubation, the
148 RPDs were collected and stored at -80 ºC prior to nuclear protein extraction and subsequent TF
149 activation profiling.

150 *Experiment 3: Static incubations of dispersed Prl cells*

151 To detrmine the effects of extracellular osmolality on the gene expression of TFs that
152 were shown to be highly expressed in Prl cells (Seale et al., 2020) and exhibit salinity-dependent
153 TF activation, Prl cells from fish acclimated to either FW or SW were incubated in media
154 spanning a range of osmolalities. A total of 60 FW- and SW-acclimated Mozambique tilapia (*O.*
155 *mossambicus*) of mixed sex weighing 60-300 g were used in static incubations of dispersed Prl
156 cells as previously described (Seale et al., 2012b). Briefly, RPDs dissected from either FW- or
157 SW-acclimated tilapia were pooled in PBS (0.02 M, 330 mOsm/kg) and treated with 0.125%
158 (wt/vol) trypsin (Sigma-Aldrich) for 30 min on a gyratory platform set at 120 rpm to allow for
159 complete cell dissociation. The cells were centrifuged for 5 min at 1200 rpm and the supernatant
160 decanted and discarded; cells were resuspended and triturated in trypsin inhibitor (0.125%
161 wt/vol; Sigma-Aldrich) to terminate the trypsin treatment. Cells were washed with PBS twice
162 and then re-suspended in isosmotic medium. Cell viability was determined by trypan blue
163 exclusion test and yield was estimated with a hemocytometer. Dispersed Prl cells were plated (4-
164 6 x10^4 cells/well on 96 well plate) and preincubated in isosmotic medium for 1 h. The cells were
165 then incubated in media spanning a range of osmolalities (280, 300, 330, 355 and 420 mOsm/kg)
166 for 6 h under saturated humidity (Seale et al., 2012b). At the end of the 6 h incubation, 200 µL of

167 TRI Reagent (MRC, Cincinnati, OH) was added to each well. The mixture of cells and TRI

168 Reagent was then transferred to 1.5 mL tubes and stored at -80 ºC prior to RNA extraction and

169 gene expression analyses.

170 *Radioimmunoassay*

171 Prl_{177} and Prl_{188} levels in the collected plasma samples were measured by homologous

172 radioimmunoassay (RIA) using the primary antibodies developed in rabbit against Prl_{177} and

173 Prl_{188} (anti- Prl_{177} and anti-Prl_{188}) and secondary antibody raised in goat against rabbit IgG (anti-

174 rabbit IgG) as previously described and validated (Ayson et al., 1994; Yada et al., 1994;

175 Yamaguchi et al., 2016). Dilutions employed for anti-Prl_{177}, anti-Prl_{188} and anti-rabbit IgG were

176 1:8000, 1:35000 and 1:100 respectively.

177 *Nuclear protein extraction and quantification*

178 A commercial nuclear extraction kit (Signosis, Santa Clara, CA) was used for the

179 preparation of nuclear extracts following the manufacturer's protocol with minor modifications.

180 For each treatment, two RPDs were combined (one male and one female) to represent one

181 mixed-sex sample of nuclear extract. Briefly, the manufacturer's protocol for cell culture was

182 followed with an extra step to add 1X Buffer 1 and repeat the incubation on a shaking platform

183 to ensure a better separation between cytoplasm and nuclear proteins. Also, an overnight freeze-

184 thawing step at -80 ºC was included to maximize the release of nuclear proteins. At the end of

185 the procedure, the harvested nuclear protein extract was quantified using a Pierce BCA protein

186 assay kit following the manufacturer's protocol (Thermo scientific, Rockford, IL). The

187 concentrations of nuclear protein ranged between 200-900 µg/mL and were used to normalize

188 each sample. The nuclear protein samples were stored at -80 ºC until further analysis.

189 *Transcription factor activation profiling assay*

190 A custom-made TF activation profiling plate array (Signosis, Santa Clara, CA) was used
191 to determine the activated TFs in the nuclei of tilapia Prl cells. Twenty six predicted TFMs in the
192 1000-1600 bp upstream promoter regions of prl_{177} and prl_{188} were included in this screening
193 (Figure 2). The sequences of the predicted binding sites of each TFM in the prl_{177} and prl_{188}
194 promoter regions were obtained through *in silico* analysis using MatInspector: Matrix family
195 library V.11.0 (Genomatix, Munich, Germany) (Seale et al., 2020) and used to design
196 biotinylated probes in the customized TF activation plate (Table 1). The TF activation assay was
197 conducted using 5 µg of nuclear protein per sample according to the manufacturer's protocol
198 with minor modifications. Briefly, two additional washing steps were carried out to assure the
199 complete removal of free probes from the TF-DNA complex and the incubation period with
200 Streptavidin-HRP was reduced from 40 to 20 min to avoid background noise. Luminescence was
201 read in a luminometer (Synergy Lx, BioTek, Santa Clara, CA) with gain set at 100
202 photomultiplier tube amplification units (PMT) and without the use of filters to avoid emission
203 cutoff. The activity of ETSF_ETSF 06, a TFM that did not vary significantly among plates or
204 treatments (one-way ANOVA, $P>0.05$), was used to normalize TFM activation. Values are
205 expressed as relative activity, defined as the ratio between target TFM and ETSF_ETSF 06.

206 *Quantitative real-time PCR (qRT-PCR)*

207 Total RNA was extracted from Prl cells frozen in TRI Reagent following the
208 manufacturer's protocol and reverse transcribed using a High Capacity cDNA Reverse
209 Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The levels of reference and target
210 genes were determined by the relative quantification method using a StepOnePlus real-time
211 qPCR system (Thermo Fisher Scientific). The qPCR reaction mix (15 µL) contained Power

212 SYBR Green PCR Master Mix (Thermo Fisher Scientific), 200 nmol/L forward and reverse
213 primers, and 1 µL of cDNA. PCR cycling parameters were as follows: 2 min at 50 °C, 10 min at
214 95 °C followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Target gene transcripts
215 were identified using the previously reported TF transcriptome data (Seale et al., 2020) and
216 selected based on abundance and osmotically-induced activation. New primers were designed
217 using the Primer3 software (Kõressaar et al., 2018) and their specificities were confirmed by
218 melt curves. Primer sequences are listed in Table 2. The geometric mean of three reference
219 genes (*ef1-α*, *18S*, and *β-actin*) was used to normalize target genes. Data are expressed as mean
220 fold change ± SEM (*n* = 8) from the isosmotic treatment (330 mOsm/kg).

221 *Statistics*

222 Data representing the activation of TFs from steady-state salinity comparisons and from
223 static incubations of RPDs were analyzed by Student's t-test. Data from static incubations of Prl
224 cells were analyzed by one-way ANOVA. Significant effects of medium osmolality were
225 followed up by protected Fisher's LSD test. When necessary, data were log-transformed to
226 satisfy normality and homogeneity of variance requirements prior to statistical analysis. All
227 statistics were performed using Prism 6 (GraphPad, La Jolla, CA) and data are reported as means
228 ± SEM.

229

230 **Results**

231 *1. Effects of acclimation salinity on plasma Prl*

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

235

236 *2. Effects of acclimation salinity on transcription factor activation*

237 Transcription factor activation assays in FW- and SW- acclimated Mozambique tilapia

238 were developed based on predicted binding sites of TFs and TFMs to cis-regulatory elements of

239 prl_{177} and prl_{188} promoter regions (Figure 2).

240 The activation of TFMs in RPDs of fish acclimated to FW and SW is presented in order,

241 from lowest to highest relative activity in FW (Figure 3). The threshold for activation of a TFM

242 was set at a relative activity of 1.5, based on (Ding et al., 2020) . OCT1_PIT1 01 was the highest

243 activated TFM in FW-acclimated tilapia. Activation of OCT1_PIT1 01, BRNF_RXRF and

244 CEBP_CEBP 01 in FW-acclimated tilapia was higher than that of SW-acclimated fish. By

245 contrast, the activation of SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 was higher in

246 tilapia acclimated to SW compared with those in FW. NFAT_GATA 01, SORY_PAX3 02,

247 SP1F_SP1F 06, SP1F_SP1F 09, PBXC_PDX 01 and GATA_SP1F were not activated in FW-

248 acclimated fish, while NFAT_GATA 01 and NFAT-AP1F 01 in the distal region of prl_{177}

249 promoter were not activated in SW-acclimated fish.

250 *3. Effects of extracellular osmolality on transcription factor activation in vitro*

251 To characterize the activation of TFMs by a hyposmotic stimulus, RPDs from SW-

252 acclimated tilapia were incubated in either isosmotic (330 mOsm/kg) or hyposmotic media (280

253 mOsm/kg) for 12 h prior to nuclear extraction and subsequent TFM profiling (Figure 4). The

254 activation of TFMs is presented in the order of activity in hyposmotic media. The activation of

255 CAAT_AP1F 01, OCT1_CEBP 01, AP1F_SMAD 01, GATA_SP1F 01 and SORY_PAX6 01

256 was higher in SW-acclimated tilapia RPDs incubated in hyposmotic media than the RPDs

257 incubated in isosmotic media. The TFMs NFAT_GATA 01, SORY_PAX3 02, FKHD_NF1F 01,

258 SP1F_SP1F 06, SP1F_SP1F 09, NFAT_AP1F 01, PBXC_PDX 01 and IRFF_STAT 01 were not
259 activated in hyposmotic conditions. Even though the relative activity was below 1.5,
260 CREB_EBOX 02, EBOX_AP2F 01, EBOX_MITF 01 had higher activity in hyposmotic media
261 than in isosmotic media.

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

268 *4. Effects of extracellular osmolality on mRNA expression of TF transcripts*

269 The mRNA levels of the TF transcripts in Prl cells from FW- and SW-acclimated tilapia
270 incubated in a range of osmolalities are shown in Figures 6 and 7. In FW- acclimated tilapia Prl
271 cells, *pou1f1* (Fig. 6A), *pou2f1b* (Fig 6B) and *stat3* (Fig. 6C) levels were suppressed by
272 hyperosmotic media compared to the levels in 280 mOsm/kg. Also, the expressions of *stat1a*
273 (Fig. 6D) and *ap1b1* (Fig. 6H) were suppressed at 355 mOsm/kg. All of the TF mRNA levels
274 were similar in FW-acclimated tilapia Prl cells incubated in 280 mOsm/kg and 330 mOsm/kg
275 media. Incubation osmolalities did not have any effect on the expressions of *creb3l1* (Fig. 6E),
276 *cebpb* (Fig. 6F) or *nfatc1* (Fig. 6G).

277 In SW-acclimated tilapia Prl cells, *pou1f1* (Fig. 7A), *pou2f1b* (Fig. 7B) and *stat3* (Fig.
278 7C) mRNA levels were inversely related to incubation osmolality. Incubation in 420 mOsm/kg
279 media suppressed the expression of *nfatc1* (Fig. 7G) compared with its expression in hyposmotic
280 conditions. The expression of *creb3l1* (Fig. 7E) was suppressed at 280 mOsm/kg media while

281 355 mOsm/kg media upregulated the expression of *ap1b1* (Fig. 7H). Incubation osmolalities did
282 not have any effect on the expressions of *cebpb* (Fig. 7F), or *stat1a* (Fig. 7D).
283
284 **Discussion**
285 The present study examined the osmosensitivity of TFs and TFMs in Prl cells of
286 Mozambique tilapia, an established euryhaline fish model for osmoreception studies. Our
287 findings indicate that a range of TFMs are activated in both hypo- and hyperosmotic conditions
288 and in accordance with acclimation salinity. In this discussion, we will consider each group of
289 TFMs separately and discuss their osmosensitive characteristics. TFMs activated in FW-
290 acclimated fish largely coincided with those activated by a hyposmotic stimulus *in vitro*; the
291 reverse was observed in SW-acclimated fish, where the most highly activated TFMs were similar
292 to those activated by hyperosmotic conditions. Most notably, PIT1_OCT1 was robustly activated
293 in fish in FW compared with those in SW, and their transcripts, *pou1f1* and *pou2f1b*, were
294 inversely related to extracellular osmolality *in vitro*. The osmotic response patterns in the
295 activation of other TFMs were also similar to those observed in the expression of their
296 corresponding transcripts. This study, therefore, lays the foundation for characterizing the
297 salinity dependence and osmotically-induced activation of TFs in teleost fishes.
298 Consistent with their roles in promoting ion absorption and retention across
299 osmoregulatory epithelia, the mRNA expression of prl_{177} and prl_{188} and release of their gene
300 products, Prl_{177} and Prl_{188}, are inversely related to extracellular osmolality (Seale et al., 2003a,
301 2006b; 2012b; Yada et al., 1994). Accordingly, both pituitary mRNA (Seale et al., 2012b) and
302 circulating levels of Prls are higher in tilapia acclimated to FW than those acclimated to SW and
303 our results confirm this (Seale et al., 2006b; Fig. 1). Here, we characterized the osmotic

304 responses of TFs previously predicted to bind promoter regions of both tilapia *prl* genes (Seale et
305 al., 2020). To monitor the simultaneous activation of multiple TFs, specific probes corresponding
306 to 26 consensus sequences of TFM-DNA binding sites in the promoter regions of prl_{177} and
307 prl_{188} were used for designing a customized TF profiling array (Fig. 2, Table 1). First, the
308 activation of TFMs in RPDs of FW- and SW-acclimated fish were compared. The highest
309 activated TFM observed in RPDs of FW-acclimated tilapia was OCT1_PIT1 01 (Fig. 3). Both
310 TFs, OCT1 and PIT1, are members of the POU (Pit-Oct-Unc) family of TFs which are encoded
311 by *pou2f1* and *pou1f1* genes respectively (Malik et al., 2018). OCT1_PIT1 01, CEBP_CEBP 01
312 and BRNF_RXRF 01 were highly activated in FW-acclimated tilapia. Conversely, SORY_PAX3
313 02, a common TFM that can bind to the promoter regions of both *prls* and SP1F_SP1F 06,
314 SP1F_SP1F 09 were most highly activated in SW-acclimated tilapia.

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

327 stimulation in tilapia (Seale et al., 2006b). Thus, the high activation of PIT1 in FW is in
328 agreement with the maintenance of sustained *prl*$_{188}$ transcription and elevated synthesis and
329 secretion of Prl$_{188}$ in FW-acclimated fish.

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

338 The TFMs SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 were predominantly
339 activated in SW-acclimated tilapia. Specifically, the activation of SORY_PAX3 02 was 3-fold
340 higher in SW- than in FW-acclimated fish. PAX3 has been reported to contain regions capable of
341 confering both activation and inhibition of gene transcription (Chalepakis et al., 1994). Our
342 previous analysis indicated the presence of binding sites for SORY_PAX3 02 in the promoter
343 regions of both *prl*$_{177}$ and *prl*$_{188}$ (Seale et al., 2020; Fig. 2). Inasmuch as this TFM may exert
344 inhibitory effects on transcription, the increase in SORY_PAX3 02 activation observed in SW-
345 acclimated tilapia may underlie the lowering of *prl*$_{177}$ and *prl*$_{188}$ mRNA levels and Prl$_{177}$ and
346 Prl$_{188}$ release observed in hyperosmotic environments. SP1F_SP1F 06, SP1F_SP1F 09, which
347 binds to the distal promoter region of *prl*$_{177}$ is also activated in SW-acclimated tilapia. In
348 mammalian cell lines, SP1 has been reported to be activated in hyperosmotic environments and
349 involved the inhibition of transcription (Tajitsu et al., 2013). Taken together, the actions of

350 SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 may at least in part contribute to the
351 suppression of *prl*s and Prl release in SW-acclimated tilapia.

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

363 Hyposmotically-induced Prl release is dependent on the entry of extracellular Ca^{2+} into
364 Prl cells (Grau et al., 1981; Seale et al., 2003a; Watanabe et al., 2012). Moreover, cAMP
365 accumulates in Prl cells in response to a fall in extracellular osmolality and influx of extracellular
366 Ca^{2+} (Helms et al., 1991; Seale et al., 2011). The role of cAMP and Ca^{2+} second messenger
367 systems in cellular signaling has been extensively studied, including downstream activation of
368 TFs such as cyclic AMP response element binding protein (CREB) and CAAT/enhancer binding
369 protein (CEBP) (Thiel et al., 2005; Wang et al., 2019). CEBP was shown to be sensitive to a
370 hypoosmotic stimulus in the intestine of medaka (*Oryzias latipes*) (Wong et al., 2014). Both
371 CREB and CAAT are also known to be responsive to hyposmotic stimuli in mammalian cell
372 models as well as in teleost fish (Gao et al., 2008; Kausel et al., 2006; Thiel et al., 2005). In the

373 present study, CEBP, CAAT and CREB constitute TFMs that were highly responsive to a
374 hyposmotic stimulus *in vitro*. Importantly, the activation of these TFMs occured in RPDs of SW-
375 acclimated tilapia, where expression of *prl*s is constituitevly low (Seale et al., 2012b). The
376 activation of CAAT_AP1F 01, OCT1_CEBP 01, and CREB_EBOX 02, EBOX_AP2F 01,
377 EBOX_MITF 01 *in vitro*, is therefore consistent with the short-term activation of the cAMP
378 second messenger in Prl cells of tilapia acclimated to a SW environment following exposure to a
379 hyposmotic stimulus.

380 The TFM SORY_PAX6 01 was also activated in SW-acclimated tilapia RPDs incubated
381 in hyposmotic media.The TFs of the PAX family have been shown to have both activating and
382 inhibitory effects through regions near the C-terminus and N-terminus, respectively (Chalepakis
383 et al., 1994). The combined activation and suppressive effects of PAX3 and PAX6 have been
384 reported as a means of fine tuning transcriptional regulation by the PAX family of TFs
385 (Wakamatsu, 2011). In the current study, SORY_PAX3 02 did not change while SORY_PAX3
386 06 activation increased in RPDs from SW-acclimated tilapia incubated in hyposmotic media.
387 These divergent actions by TFs of the PAX family may underlie the fine regulation of prl_{188}
388 transcription as both these TFs are predicted to bind the prl_{188} promoter, albeit in different
389 regions.

390 When we incubated FW-acclimated tilapia RPDs in hyperosmotic media, the activation
391 of SORY_PAX3 01 increased by four-fold compared with its activity in isosmotic media. This
392 result was consistent with those of other TF activation plate arrays, where the activity of this
393 TFM was increased in SW-acclimated fish compared with FW-acclimated fish, and unresponsive
394 in SW-acclimated RPDs incubated in hyposmotic media. Because SORY_PAX3 01, a TFM
395 common to the promoter regions of both prl_{177} an prl_{188}, is stimulated by hyperosmotic

396 conditions and SORY_PAX6 01 is not, PAX3 may play a role in both short- and long-term
397 negative regulation of tilapia Prl cells. In addition, the activation of AP1F_SMAD 01 was
398 stimulated by hyperosmotic media. The result is consistent with the mammalian hypothalamus,
399 where AP1 has been shown to be activated in hyperosmotic conditions (McCabe & Burrell,
400 2001; Ying et al., 1996). Even though SMAD is not reported to be osmotically sensitive, the
401 hyperosmotically-induced activation of AP1 may be involved in the short-term inhibition of
402 prl_{188} transcription in Prl cells of FW-acclimated tilapia responding to hyperosmotic stress.
403 These findings further underscore the high osmosensitivity of Prl cells from FW-acclimated fish,
404 where transition from a FW to a SW environment necessitates rapid inhibition of Prl secretion.
405 Based on the osmotic sensitivity of TF activation and known signal transduction
406 mediators in tilapia Prl cells, we then examined the transcriptional responses of select TFs in Prl
407 cells of FW- and SW-acclimated tilapia incubated under a range of medium osmolalities. When
408 we incubated Prl cells from FW-acclimated fish, mRNA expression of several transcripts varied
409 based on the extracellular osmolality. Encoding PIT1 and OCT1, which formed the most highly
410 activated TFM in FW-acclimated fish, *pou1f1* and *pou2f1b* showed similar responses to
411 extracellular osmolality, decreasing at 355 and 420 mOsm/kg. This pattern is consistent with
412 the observed role of OCT1_PIT1 01 in FW-acclimation. It is worth noting, however, that
413 OCT1_PIT1 01 activation only exhibited a tendency to lower under hyperosmotic conditions.
414 Because the RPDs from FW-acclimated tilapia already show high expression levels of both *prl*s,
415 it is expected that they would have high mRNA levels of the TFs needed to maintain the high
416 baseline of *prl* transcription, which could be up to ~30 fold higher in fish acclimated to FW
417 compared with those in SW(Seale et al., 2012a; Seale et al., 2012b). Consequently, in FW-
418 acclimated fish, those TF transcripts may not be as sensitive to hyposmotic challenges as they

419 would in SW-acclimated fish. In fact, Prl cells from SW-acclimated tilapia, responded more
420 consistently to extracellular osmolality. Both *pou1f1* and *pou2f1b* mRNA levels were inversely
421 related to the extracellular osmolality (Fig. 7 A and B). These patterns are consistent with those
422 reported for prl_{177} and prl_{188} (Seale et al., 2012b) and the notion that in SW fish *prl* mRNA levels
423 are low, but are rapidly stimulated through the hyposmotically-induced expression of activating
424 TFs.

425 Prl activates its target cells by interacting with a pair of single-transmembrane domain
426 receptors that are linked to the Janus kinase/Signal transducer and activator of transcription
427 (JAK/STAT) pathways (Brooks, 2012). Inasmuch as Prl cells can be regulated in autocrine
428 fashion (Yamaguchi et al., 2016), the STAT family of TFs may also play a role in regulating *prl*
429 transcription in response to osmotic stimuli. In goldfish, the main STAT involved in Prl signaling
430 is STAT3 (Yan et al, 2017). Moreover, we observed *stat3* to be the most abundant *stat* transcript
431 based on an earlier transcriptome analysis of the RPD of Mozambique tilapia (Seale et al.,
432 2020). Here, we observed *stat3* transcription from SW-acclimated fish inversely related to
433 extracellular osmolality, following a similar trend as transcripts of the POU 1 family. Despite
434 the osmotic sensitivity of *stat3* regulation, the activation of IRFF_STAT 01 did not differ
435 between FW- and SW-acclimated fish nor it was activated by hyposmotic conditions.
436 Nonetheless, the observed hyposmotic induction of *stat3* but not *stat1a*, suggests that the former
437 TF plays a role in the osmotic sensitivity of prl_{188} expression.

438 Prl cells transduce hyposmotic stimuli through the activation of second messenger
439 systems, such as cAMP and Ca^{2+}, which in turn initiate *prl* transcription (Seale et al., 2003a,
440 2011). Accordingly, OCT1_CEBP1 became activated in hyposmotic conditions (Fig. 4).
441 Moreover, we looked at the expression patterns of two transcripts, *creb* and *cebp,* encoding for

442 TFs known to be activated by cAMP and Ca^{2+} (Thiel et al., 2005). The gene transcripts, *creb3l1*
443 and *cebpb* were the most highly expressed from CREB and CEB families of TFs in RPDs of
444 Mozambique tilapia based on our previous transcriptomic analysis (Seale et al., 2020).
445 Nonetheless, we did not observe any differences in *cebpb* expression in response to extracellular
446 osmolality and *creb3l1* was suppressed in Prl cells of SW-acclimated fish incubated at 280
447 mOsm/kg (Fig.7 E and F). One possible reason for the discrepancy between TF activation and
448 mRNA expression may stem from the lack of osmosensitivity of these transcripts and a possible
449 negative feedback role played by *creb3l1*with respect to the regulation of a Ca^{2+} dependent
450 hyposmotic response of the Prl cell. Moreover, RPDs of SW-acclimated tilapia had greater
451 mRNA levels of *creb3l1* than FW fish (Seale et al., 2020), supporting the notion that in
452 hyperosmotic environments Prl cells are more responsive to a Ca^{2+}-dependent signalling, such as
453 that known to occur during hyposmotically-induced Prl release (Seale et al., 2003a). A similar
454 hyperosmotic upregulation of *creb3l1* was observed in the hypothalamus of rats when they were
455 subjected to hyperosmotic stress by preventing water uptake for 1-3 days or replacing water
456 intake by NaCl solution (Greenwood et al., 2015). While the osmotic response of *creb3l1* and
457 *cebpb* may not necessarily coincide with that of TFMs containing CREB and CEBP other
458 transcripts involved in Ca^{2+}- and cAMP- dependent downstream signalling may exhibit greater
459 osmosensitivity .
460 Recent findings indicate that the activation of secondary metabolite pathways, including
461 those involved in the production of myo-inositol and glutamine synthetase, are involved in SW-
462 acclimation of Mozambique tilapia (Kim & Kültz, 2020; Wang & Kültz, 2017). The sensitivity
463 of these pathways to hyperomotic stimuli has been reported to be dependent on the
464 osmolality/salinity- responsive enhancer 1 (OSRE1) located in intron 1 which is activated by the

TF, nuclear factor of activated T-cells (NFAT) (Kim & 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 & 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 622bp upstream of *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 & Burrell, 2001; Ying et al., 1996). The mRNA expression levels 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*$_{177}$.

487 Collectively, our results reveal the complex patterns of activation of different TFMs
488 predicted to bind regulatory elements upstream of prl_{177} and prl_{188} depending on acclimation
489 salinity and short-term osmotic stimulation. The marked activation of OCT1_PIT1 01 and
490 CEBP_CEBP 01 in FW-acclimated fish and OCT1_CEBP 01 in response to hyposmotic
491 stimulation was noteworthy as their TFs have been previously implicated in the basal regulation
492 of *prl* transcription and response to Ca^{2+} and cAMP signalling pathways, two of the hallmarks of
493 hyposmotically-induced Prl synthesis and release. Inasmuch as two of these TFMs are only
494 found upstream of prl_{188}, these findings also support that the enhanced responsiveness of prl_{188}
495 transcription and Prl_{188} release to hyposmotic stimuli compared with prl_{177} and Prl_{177} may be
496 dependent on the activation of TFs of the POU 1 domain. The observed activation of
497 SORY_PAX3 02 and SP1F_SP1F 06, SP1F_SP1F 09 TFMs in RPDs of SW-acclimated fish
498 indicate potential transcription inhibitors of both *prl*s when fish face hyperosmotic environments.
499 The mRNA expression data suggests that transcripts such as *pou1f1* and *pou2f1b* react to
500 incubation osmolality more rapidly even though the TF activation did not decrease quickly in
501 response to hyperosmolality. Conversely, CEBP and CREB activation was observed in TFM
502 activation while *cebpb* mRNA did not vary and *creb3l1* was suppressed at 280 mOsm/kg. Hence,
503 different elements in the cAMP second messenger pathway might be readily available while
504 others would be osmosensitive and act as fine-tuning to induce the hyposmotically driven *prl*
505 transcription. A potential key protein in the autocrine regulation of Prl, *stat3* was inversely
506 related to the osmolality even though it was not significant in TF activation levels. This might be
507 due to very low STAT protein availability in Prl cell nuclei.
508

509 **Conclusions**

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

520 **Conflict of interest**

521 The authors declare that they have no conflicts of interest.

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528 **Figure Captions**

529 **Figure 1:** Effects of acclimation salinity on plasma levels of Prl_{177} (A) and Prl_{188} (B) in
530 Mozambique tilapia. Clear and solid bars represent fish acclimated to FW and SW, respectively.

531 Data are expressed as mean ± SEM (n=9-10). The effect of acclimation salinity was analyzed by

532 Student's t-test; **p<0.01, ***p<0.001.

533

534 **Figure 2:** Transcription factor modules (TFM) predicted in prl_{177} (yellow band) and prl_{188} (green

535 band) promoter regions up to -1.6 kb (adapted from Seale et al., 2020, with author's permission)

536 and used to design specific probes employed in the TF/TFM activation profiling assay . TFMs

537 predicted to bind to the (-) strand are indicated by blue text and the TFMs predicted to bind to the

538 (+) strand are indicated by red text.

539

540 **Figure 3:** Relative activation of predicted TFMs in RPDs of FW- and SW- acclimated tilapia.

541 Clear and solid bars represent fish acclimated to FW and SW, respectively, and expressed as

542 mean ± SEM. Data represents the relative activation of each TFM to the activity of ETSF_ETSF

543 01 and are shown in ascending order of activity in FW. TFMs predicted to bind to prl_{177}

544 promoter region are written in italics and the TFMs predicted to bind to prl_{188} promoter region

545 are written in bold letters. TFMs common to both *prls* are written in bold italics. The grey solid

546 line shows the 1.5X activity compared to ETSF_ETSF 01. * Denotes significant differences in

547 activities between FW and SW acclimated fish at p< 0.05, by Student's t-test (n=3).

548

549 **Figure 4:** Relative activation of predicted TFMs in RPDs of SW- acclimated tilapia incubated in

550 hyposmotic (280 mOsm/kg) and isosmotic (330 mOsm/kg) media for 12 h. Clear and grey bars

551 represent RPDs of SW fish incubated in hyposmotic and isosmotic media, respectively, and

552 expressed as mean ± SEM. Data represents the relative activation of each TFM to the activity of

553 ETSF_ETSF 01 and are shown in ascending order of activity in hyposmotic media. TFMs

554 predicted to bind to the *prl_{177}* promoter region are written in italics and the TFMs predicted to

555 bind to *prl_{188}* promoter region are written in bold letters. TFMs common to both *prls* are written

556 in bold italics. The grey solid line shows the 1.5X activity compared to ETSF_ETSF 01. *

557 Denotes significant differences in activities between RPD's incubated in hyposmotic and

558 isosmotic media at $p < 0.05$, by Student's t-test (n=3).

559

560 **Figure 5:** Relative activation of predicted TFMs in RPDs of FW- acclimated tilapia incubated in

561 hyperosmotic (420 mOsm/kg) and isosmotic (330 mOsm/kg) media for 12 h. Black and grey

562 bars represent RPDs of FW fish incubated in hyperosmotic and isosmotic media, respectively,

563 and expressed as mean ± SEM. Data represents the relative activation of each TFM to the

564 activity of ETSF_ETSF 01 and are shown in ascending order of activity in hyperosmotic media.

565 TFMs predicted to bind to the *prl_{177}* promoter region are written in italics and the TFMs

566 predicted to bind to *prl_{188}* promoter region are written in bold letters. TFMs common to both *prls*

567 are written in bold italics. The grey solid line shows the 1.5X activity compared to ETSF_ETSF

568 01. * Denotes significant differences in activities between RPD's incubated in hyperosmotic and

569 isosmotic media at $p < 0.05$, by Student's t-test (n=6).

570

571 **Figure 6:** Changes in mRNA expression of (A) *pou1f1*, (B) *pou2f1b*, (C) *stat3*, (D) *stat1a* (E)

572 *creb3l1*, (F) *cebpb*, (G) *nfatc1* and (H) *ap1b1* in Prl cells from FW-acclimated tilapia incubated

573 in a range of osmolalities for 6 h. mRNA levels are presented as mean fold-change from the 330

574 mOsm/kg (control) group ± SEM. Differences among groups were analyzed by one-way

575 ANOVA (n=8). When there was a significant effect of media osmolality, *post hoc* group

576 comparisons were followed up with Fisher's protected LSD test. Groups not sharing the same
577 uppercase letter are significantly different.
578
579 **Figure 7:** Changes in mRNA expression of (A) *pou1f1*, (B) *pou2f1b*, (C) *stat3*, (D) *stat1a* (E)
580 *creb3l1*, (F) *cebpb*, (G) *nfatc1* and (H) *ap1b1* in Prl cells of SW-acclimated tilapia incubated in a
581 range of osmolalities for 6 h. mRNA levels are presented as mean fold-change from the 330
582 mOsm/kg (control) group ± SEM. Differences among groups were analyzed by one-way
583 ANOVA (n=8). When there was a significant effect of media osmolality, *post hoc* group
584 comparisons were followed up with Fisher's protected LSD test. Groups not sharing the same
585 uppercase letter are significantly different.
586

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842

843

844 **Table 1**: Predicted putative TFM consensus binding sites on *prl*$_{177}$ and *prl*$_{188}$ promoter regions.

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

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

849

850

851 **Table 2**: Gene specific primers used for qPCR

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

	R: CCTGTGAAGCGTACCGTTTT				
nfatc1	F: GCCGCTGTAGCTTTAAGTGG	0.935	84.1	XM_003447265.5	Newly designed
	R: ACACTGAGGCGAGCTCAAAT				
ap1b1	F: CACTGACAGCCTGGAGTGAA	0.961	100.0	XM_005473361.4	Newly designed
	R: CTCATTGACTTCTGCCACGA				

852

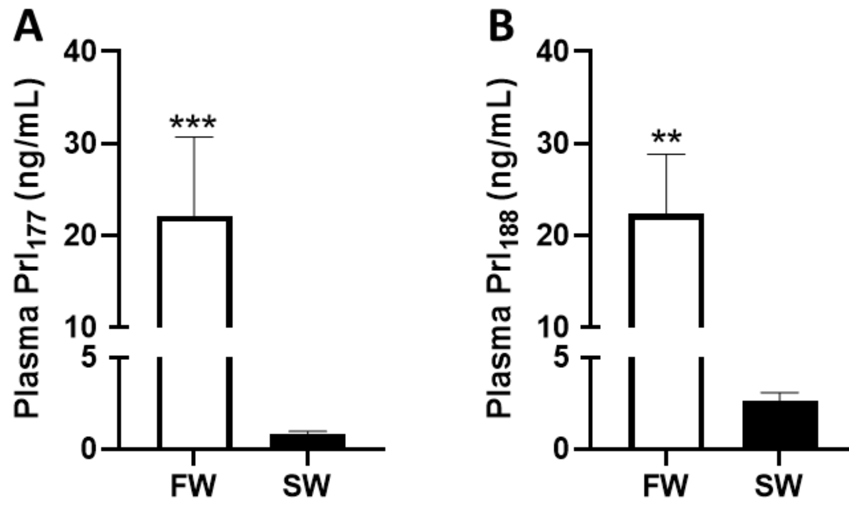


Figure 1

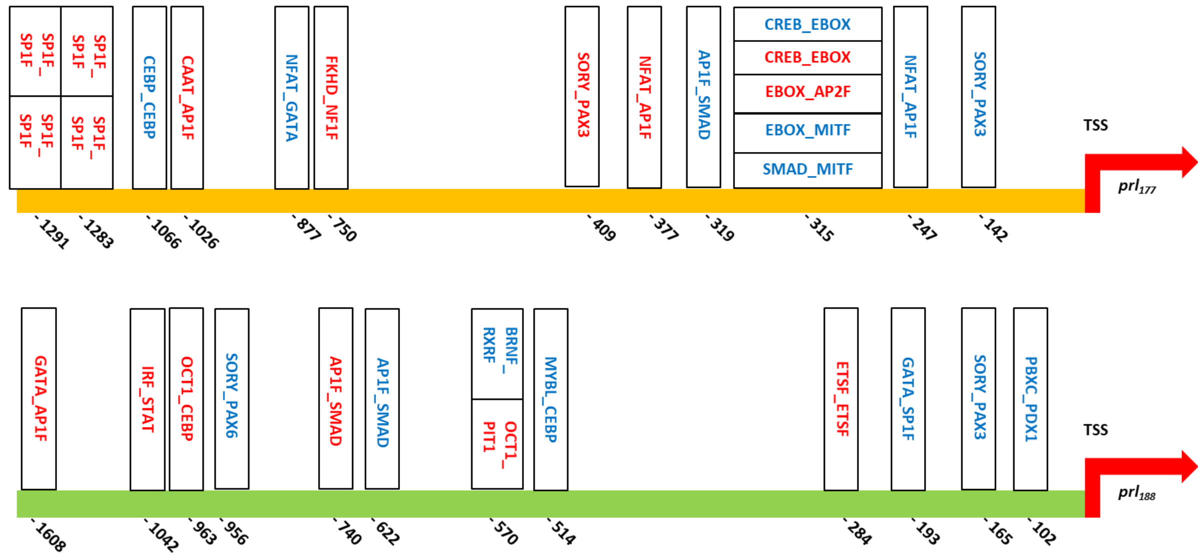


Figure 2

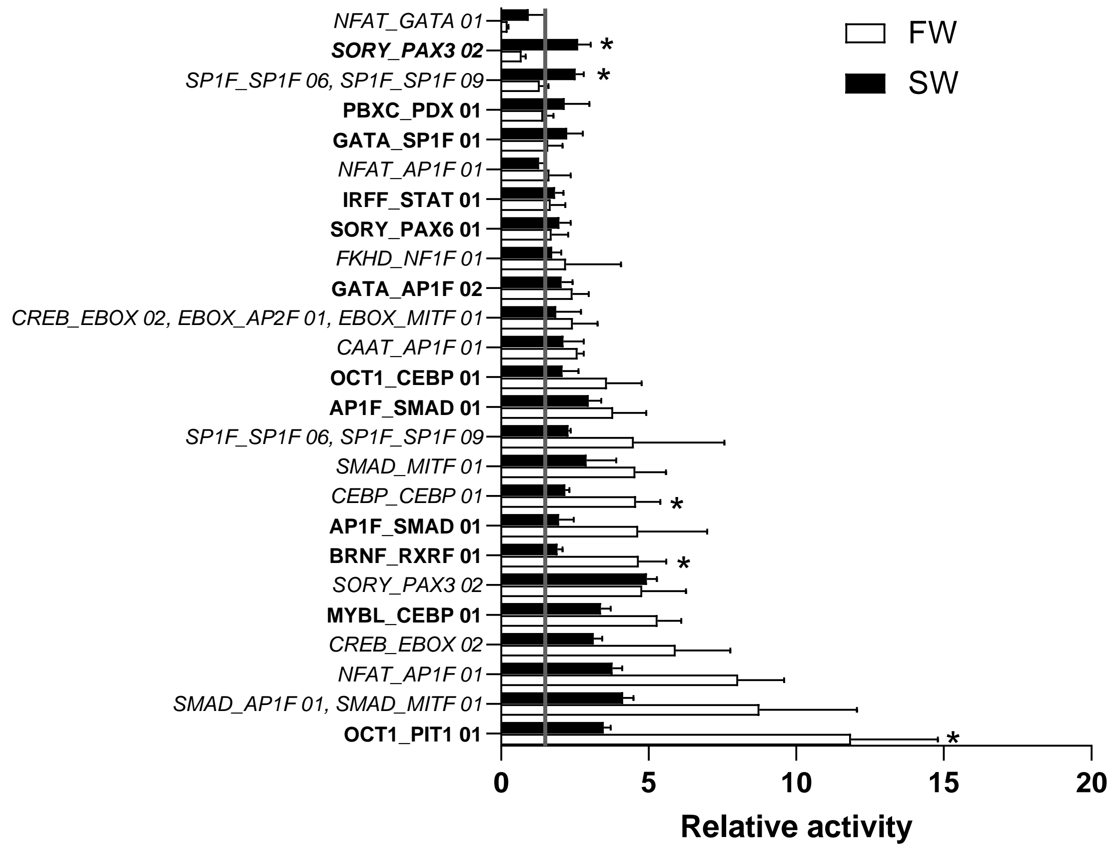


Figure 3

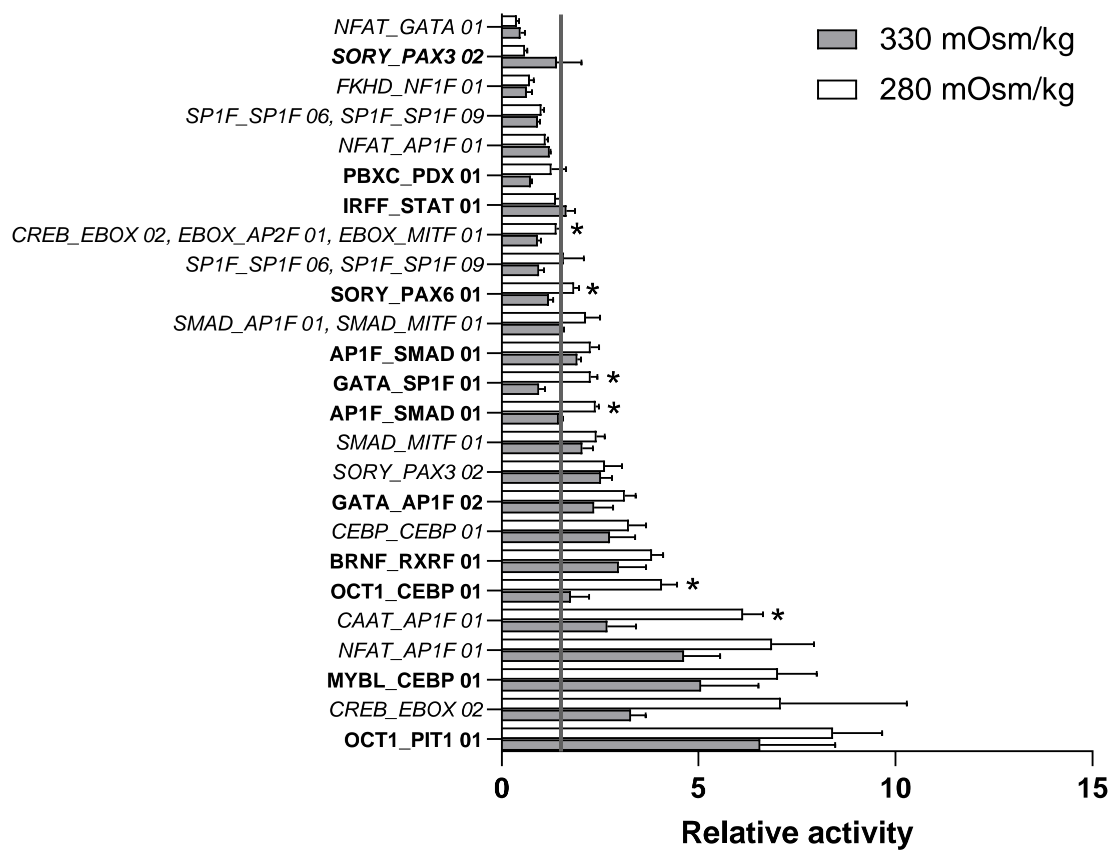


Figure 4

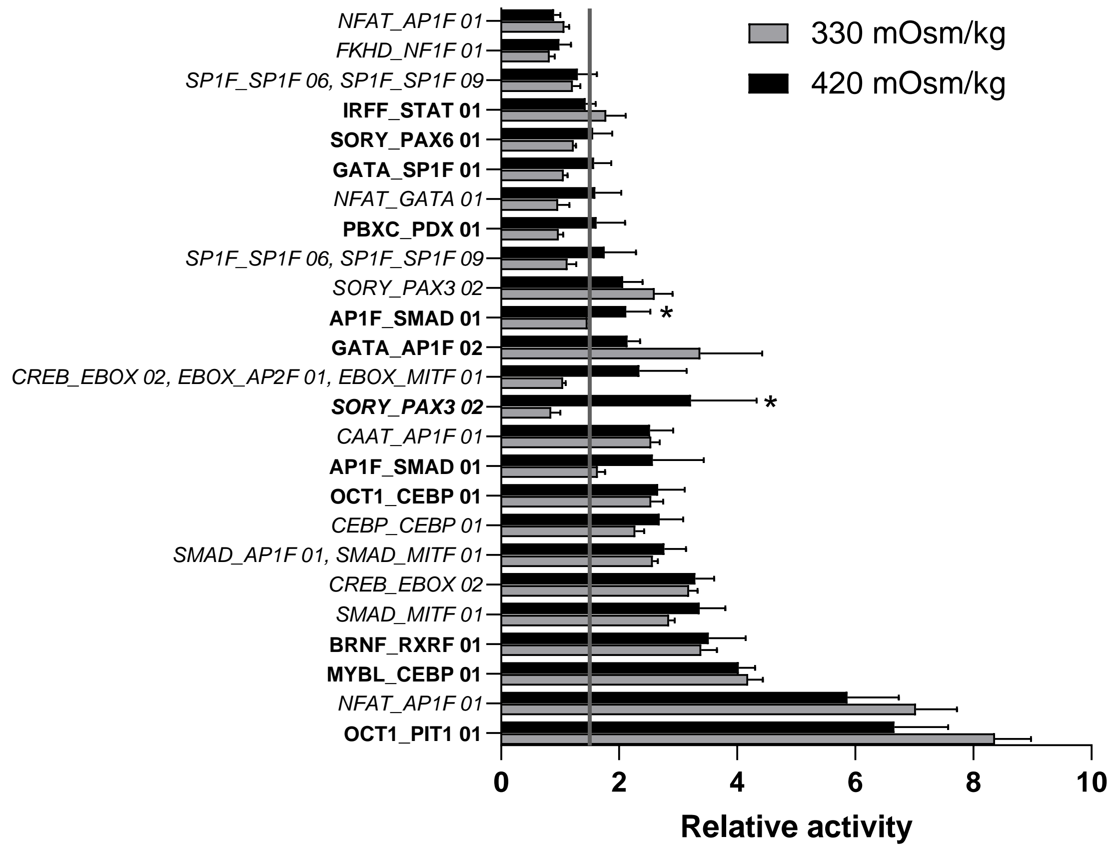


Figure 5

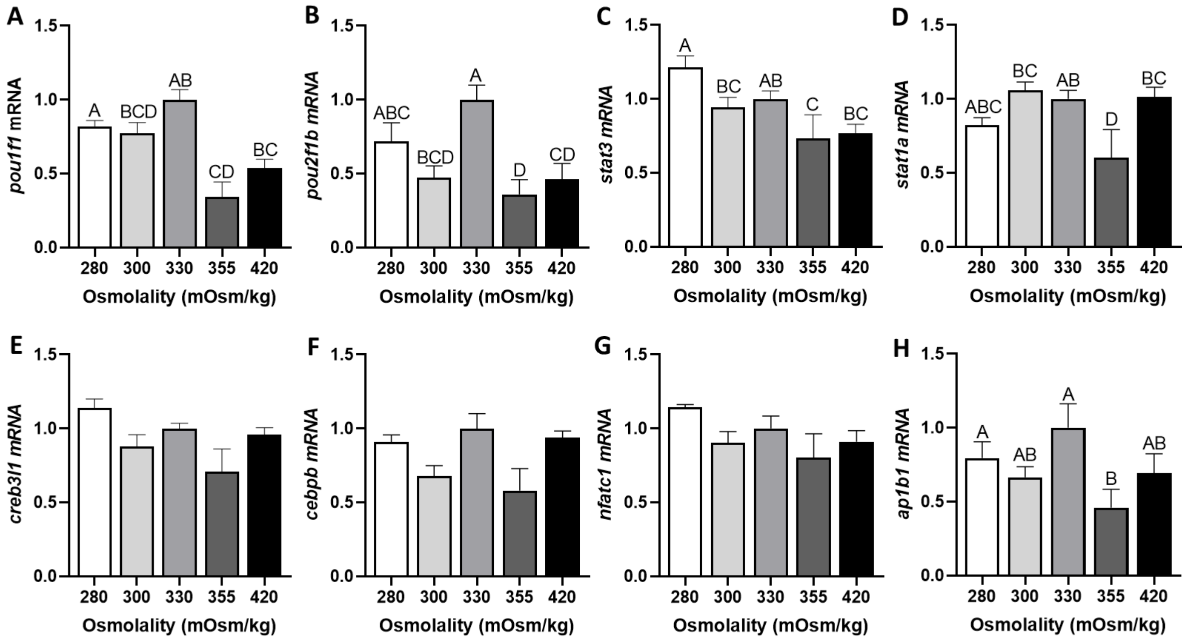


Figure 6

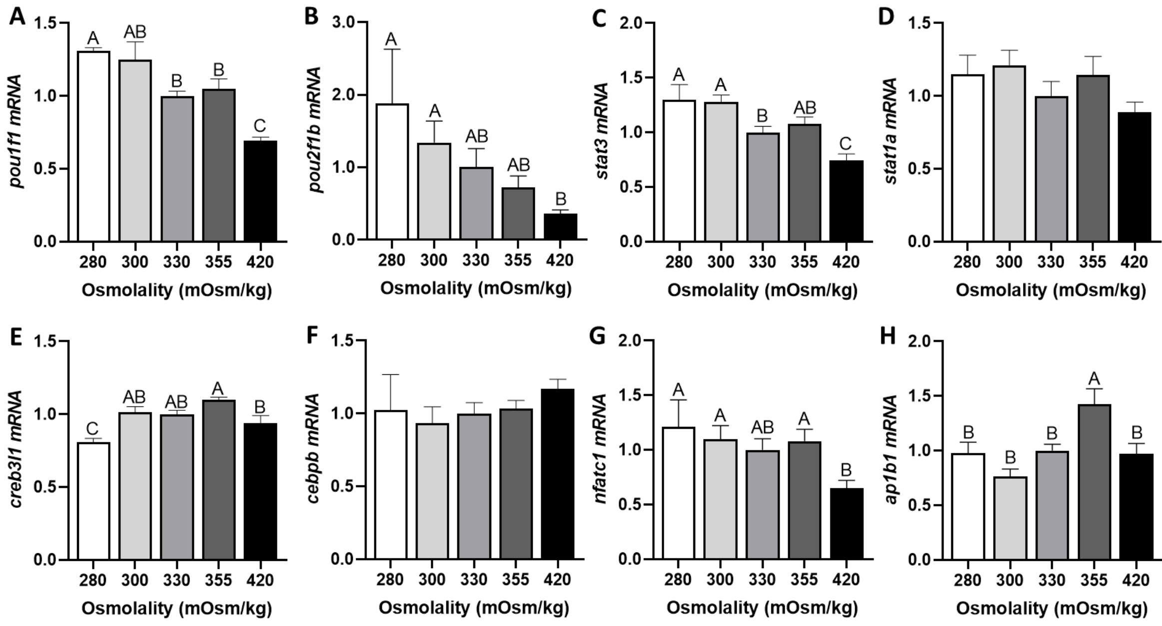
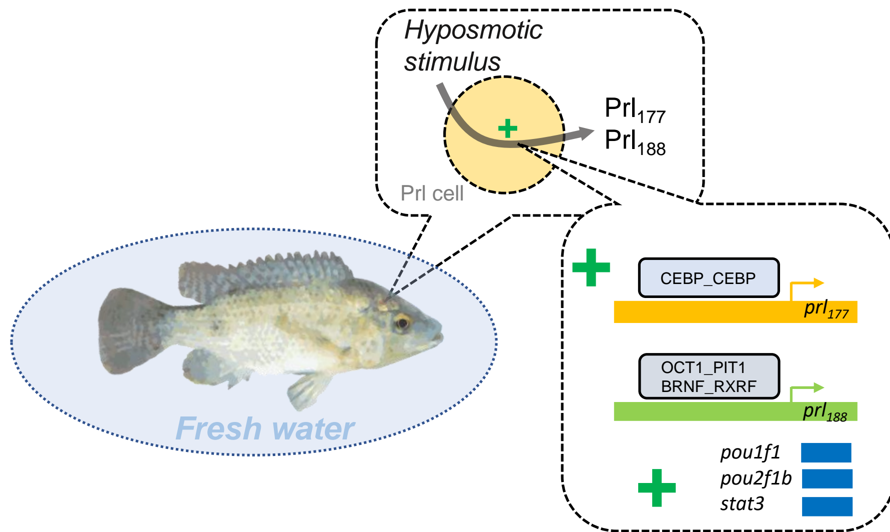
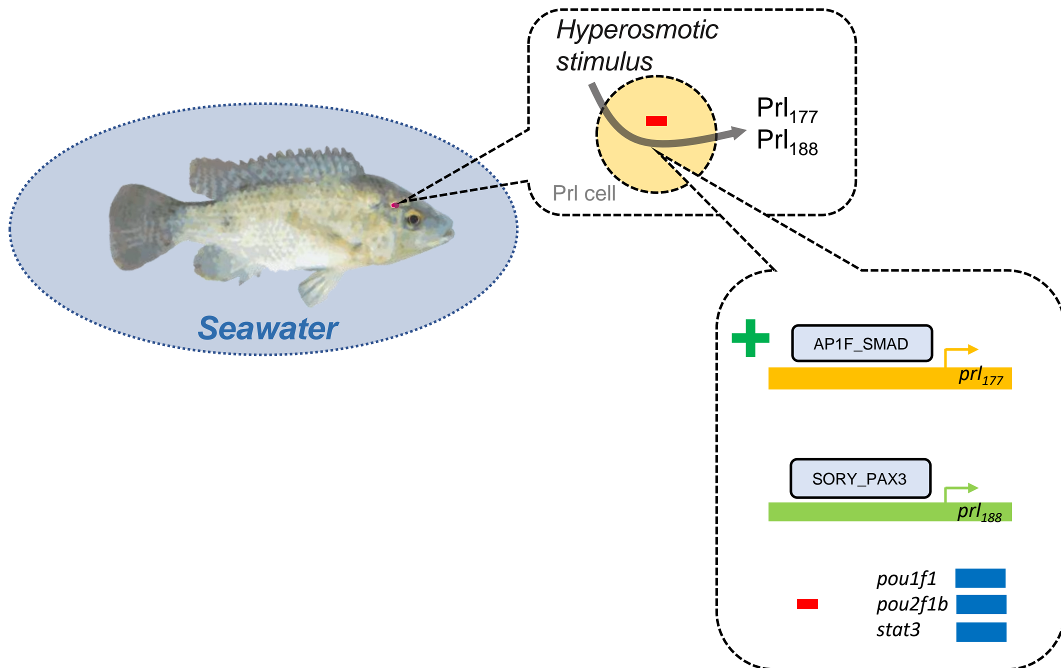


Figure 7


Hyposmotic stimulus
Prl177
Prl188
Prl cell
CEBP_CEBP
prl177
OCT1_PIT1
BRNF_RXRF
prl188
pou1f1
pou2f1b
stat3
Fresh water


Hyperosmotic stimulus
Prl177
Prl188
Prl cell
Seawater
AP1F_SMAD
prl177
SORY_PAX3
prl188
pou1f1
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stat3