

Prolactin₁₇₇, prolactin₁₈₈ and prolactin receptor 2 in the pituitary of the euryhaline tilapia, *Oreochromis mossambicus*, are differentially osmosensitive

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Abstract

Two forms of prolactin (Prl), prolactin 177 (Prl₁₇₇) and prolactin 188 (Prl₁₈₈), are produced in the *rostral pars distalis* (RPD) of the pituitary gland of euryhaline Mozambique tilapia, *Oreochromis mossambicus*. Consistent with their roles in fresh water (FW) osmoregulation, release of both Prls is rapidly stimulated by hyposmotic stimuli, both *in vivo* and *in vitro*. We examined the concurrent dynamics of Prl₁₇₇ and Prl₁₈₈ hormone release and mRNA expression from Prl cells in response to changes in environmental salinity *in vivo* and to changes in extracellular osmolality *in vitro*. In addition, mRNA levels of Prl receptors 1 and 2 (*prlr1* and *prlr2*) and osmotic stress transcription factor 1 (*ostf1*) were measured. Following transfer from seawater (SW) to FW, plasma osmolality decreased, while plasma levels of Prl₁₇₇ and

Prl₁₈₈ and RPD mRNA levels of *prl177* and *prl188* increased. The opposite pattern was observed when fish were transferred from FW to SW. Moreover, hyposmotically induced release of Prl₁₈₈ was greater in Prl cells isolated from FW-acclimated fish after 6 h of incubation, while the hyposmotically induced increase in *prl188* mRNA levels was only observed in SW-acclimated fish. In addition, *prlr2* and *ostf1* mRNA levels in Prl cells from both FW- and SW-acclimated fish increased in direct proportion to increases in extracellular osmolality both *in vivo* and *in vitro*. Taken together, these results indicate that the osmosensitivity of the tilapia RPD is modulated by environmental salinity with respect to hormone release and gene expression.

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Introduction

A necessary component of the systems that direct osmoregulatory processes are osmoreceptors that detect deviations from salt and water balance and initiate adaptive responses to restore equilibrium. The prolactin (Prl)-secreting cells of the euryhaline Mozambique tilapia (*Oreochromis mossambicus*) comprise a nearly homogeneous portion of the *rostral pars distalis* (RPD) of the pituitary (conservatively >95%) and are directly stimulated by physiologically relevant decreases in extracellular osmolality *in vitro* (Grau *et al.* 1994, Seale *et al.* 2006b). Consistent with its role as an essential hormone for fresh water (FW) acclimation (reviewed by Hirano (1986), Manzon (2002) and Sakamoto & McCormick (2006)) plasma Prl levels increase *in vivo* when tilapia are transferred from seawater (SW) to FW (Yada *et al.* 1994, Seale *et al.* 2002). The operation of tilapia Prl cells as true osmoreceptors and the anatomical organization of these cells in tilapia RPD provide an accessible model to integrate osmosensing

mechanisms with gene transcription, hormone synthesis and hormone secretion (Seale *et al.* 2005).

Hyposmotically induced Prl release from tilapia lactotrophs is dependent on the entry of extracellular Ca²⁺ through stretch-activated ion channels upon cell swelling (Seale *et al.* 2003). There is evidence that the influx of extracellular Ca²⁺ may have distinct intracellular effects depending on the acclimation salinity of the animal from which pituitary cells are collected. For example, hyposmotically induced Prl synthesis (Yoshikawa-Ebesu *et al.* 1995) and release (Seale *et al.* 2006a) is greater in RPDs collected from fish acclimated to FW than in the RPDs collected from SW fish. One likely explanation for this pattern is the fact that Prl cells from FW tilapia contain more stored Prl than those from SW fish (Nagahama *et al.* 1975, Specker *et al.* 1993) and, hence, exhibit a greater capacity to respond rapidly with hormone secretion when faced with a hyposmotic stimulus. In addition, recent evidence suggests that this difference in responsiveness may result from the diminished capability of

Prl cells from SW-acclimated tilapia to initiate osmotic signal transduction. The relative rate of change in cell volume and water influx into Prl cells following an osmotic challenge was found to be significantly lower in cells from SW tilapia compared to those in FW (Watanabe *et al.* 2009). The question remains, however, as to whether the responsiveness of *prl* gene expression differs between FW- and SW-acclimated fish.

The tilapia pituitary releases two Prl molecules that differ in length and amino acid composition, and are encoded by separate genes (Specker *et al.* 1985, Yamaguchi *et al.* 1988, Rentier-Delrue *et al.* 1989). While the existence of two distinct Prl molecules, designated Prl₁₇₇ and Prl₁₈₈, suggests an evolution of differing actions (Chen *et al.* 1994), a bioassay failed to identify any clear differences in their osmoregulatory activities (Specker *et al.* 1985). On the other hand, it has been reported that Prl₁₇₇ exhibits somatotrophic activity while Prl₁₈₈ does not (Shepherd *et al.* 1997). The tilapia Prls have also been found to respond differently to acclimation salinity. Borski *et al.* (1992) reported significant differences in the relative amounts of Prl₁₈₈ and Prl₁₇₇ in the RPDs of tilapia acclimated to FW, SW, or during transition from one salinity to another. Specifically, the ratio of Prl₁₈₈ to Prl₁₇₇ is >1 in FW and <1 in SW (Borski *et al.* 1992). This disparate control suggests that the two Prls are potentially regulated through discrete mechanisms. To date, however, it is not known whether acclimation salinity affects only the synthesis and release of these two hormones, or whether it also exercises differential effects on their gene expression. In a broader context, understanding the regulatory mechanisms governing the release and expression of both Prls *in vivo* and *in vitro* is essential for understanding the intricacies of the tilapia Prl cell as a model to better understand vertebrate osmoreceptors.

The main objectives of this study were to determine whether there are differential effects of acclimation salinity *in vivo* and extracellular osmolality *in vitro* on the release and expression of Prl₁₇₇, and Prl₁₈₈. We also explored the effects of acclimation salinity and extracellular osmolality on the mRNA expression of two Prl receptors (*prlr*) in the RPD to explore possible feedback mechanisms on Prl expression/release. Originally cloned by Sandra *et al.* (1995) in Nile tilapia (*Oreochromis niloticus*), the tilapia Prl receptor (PrLR1) is expressed in an array of tissues, including gill, intestine and kidney (Pierce *et al.* 2007). *prlr1* mRNA expression is sensitive to salinity, with enhanced expression occurring in the gill when Mozambique tilapia are transferred from SW to FW (Breves *et al.* 2011) and down-regulated expression after transfer from FW to SW (Breves *et al.* 2010a). Recently, a second Prl receptor, PrLR2, was identified in tilapia gill on the basis of its induced expression following a hyperosmotic challenge (Fiol *et al.* 2009). It is unknown whether either of these PrLRs exhibit changes in expression in the pituitary following salinity challenges. Lastly, we explored the effects of acclimation salinity and extracellular osmolality on the gene expression of osmotic stress transcription factor 1 (*osf1*) in the RPD. Fiol *et al.* (2006) characterised *osf1* as an immediate

response gene in tilapia gill following exposure to hyperosmotic conditions and suggested that it may participate in conferring osmotic tolerance (Fiol & Kultz 2005, Fiol *et al.* 2006). To our knowledge, environmental factors that modulate *osf1* expression have not been reported in extra-branchial tissues. In the current study, we examined the effects of salinity transfers on plasma osmolality, plasma Prl₁₇₇ and Prl₁₈₈ levels, and mRNA levels of *prl177*, *prl188*, *prlr1*, *prlr2* and *osf1* from RPDs, *in vivo*. We also investigated *in vitro* effects of various incubation osmolalities on the release of Prl₁₇₇, Prl₁₈₈ and mRNA levels of *prl177*, *prl188*, *prlr1*, *prlr2* and *osf1* of dispersed Prl cells isolated from fish acclimated to either FW or SW.

Materials and Methods

Fish

Mature Mozambique tilapia (*O. mossambicus*) of both sexes (50–300 g), were obtained from a population maintained at the Hawaii Institute of Marine Biology, University of Hawaii. Fish were reared in outdoor tanks (700 l) with a continuous flow of FW (14 mOsmolal) or SW (1042 mOsmolal) under natural photoperiod. SW-acclimated tilapia employed in this experiment were spawned and reared in SW, having never been previously exposed to FW. FW-acclimated tilapia, on the other hand, were spawned and reared in FW, having never been previously exposed to SW. Water temperature was maintained at 24–26 °C. Animals were fed ~5% of their body weight per day with Silver Cup Trout Chow (Nelson and Sons Inc., Murray, UT, USA). All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

In vivo experiments

Four tanks were employed for salinity transfer experiments. Each tank contained 50 fish of both sexes ranging from 50 to 100 g. At the time of transfer, one tank was transitioned from SW to FW with minimal disturbance by opening an incoming FW valve and closing a SW valve, while the other tank was transitioned from FW to brackish water (BW; 27‰) by reducing the FW valve flow while opening the SW valve. FW and BW conditions were reached after 60 min. After 48 h, the tank that was transitioned to BW was exposed to full strength SW. Two control tanks, which contained fish from the original pool raised in either FW or SW, were held at constant salinity throughout the duration of the experiment. Fish were fed for the duration of the experiment and water temperature was maintained between 24 and 26 °C. Fish were sampled ($n=6-10$) at 0 h (immediately before opening the FW or SW valves), 6 h, 48 h and 10 days after transfer. At the time of sampling, fish were netted and anaesthetised with a lethal dose of 2-phenoxyethanol (2 ml/l). Blood was collected

from the caudal vasculature by a needle and syringe coated with ammonium heparin (200 U/ml, Sigma–Aldrich). Plasma was separated by centrifugation and stored at -80°C until later analyses. Fish were rapidly decapitated and the pituitary extracted. The RPD was dissected under a microscope and placed in separate tubes containing 750 μl TRI Reagent (MRC, Cincinnati, OH, USA). Samples were stored at -80°C before RNA extraction and analyses of gene expression.

Cell cultures

Incubations of dispersed Prl cells were carried out as previously described (Seale *et al.* 2006a) with minor modifications. After removal of pituitaries from tilapia (60–300 g) of both sexes, RPDs were placed in isosmotic (330 mOsmolal) modified Ringer's culture medium (Seale *et al.* 2011), diced into fragments and treated with 0.125% trypsin in PBS. After termination of trypsin treatment by adding trypsin inhibitor (0.125% w/v), cells were harvested, and viability determined using the trypan blue exclusion test. Cell yield was estimated using a haemocytometer. Cells were re-suspended in isosmotic medium, plated ($4\text{--}6 \times 10^4$ cells/well on 96 well plates) and incubated at 26°C under saturated humidity. Cells were pre-incubated for 1 h in isosmotic medium before exposure to a series of osmolalities (280, 300, 330, 355 and 420 mOsmolal). Medium osmolality was adjusted by varying the concentration of NaCl and verified using a vapor pressure osmometer (Wescor 5100C; Wescor, Inc., Logan, UT, USA). Culture medium was collected after 6 h of incubation and stored at -80°C before RIA. At the end of the incubation period, 200 μl TRI Reagent was added to each well. The mixture of TRI Reagent and cells was added to a 1.5 ml tube containing an additional 500 μl TRI Reagent and stored at -80°C before RNA extraction and gene expression analyses.

RIAs

Prl₁₇₇ and Prl₁₈₈ levels in plasma and culture medium were measured by homologous RIA (Ayson *et al.* 1993, Yada *et al.* 1994). Assay parameters, including inter-assay coefficients of variations, cross reactivity between Prl₁₇₇ and Prl₁₈₈, and competitive binding curves, are reported in a previous study (Ayson *et al.* 1993). For *in vivo* experiments, plasma values are reported as nanograms per milliliter. For dispersed cell cultures, values obtained as nanograms per milliliter were first converted to micrograms per 10^4 cells and then calculated as relative percent change from isosmotic (330 mOsmolal) SW treatments.

Quantitative real-time PCR

Total RNA was extracted from intact RPDs and dissociated Prl cells using TRI Reagent according to the manufacturer's protocols and then reverse transcribed using a cDNA reverse

transcription kit (Applied Biosystems, Carlsbad, CA, USA). All primer pairs are previously described: *prlr1* (Pierce *et al.* 2007), *prlr2* (long isoform; Breves *et al.* 2010b), *ostf1* (Breves *et al.* 2010a), *prl177* and *prl188* (Magdeldin *et al.* 2007) and *ef1- α* (Breves *et al.* 2010b). The quantitative real-time PCRs were set up as previously described (Pierce *et al.* 2007). Briefly, 200 nM of each primer, 3 μl cDNA and 12 μl SYBR Green PCR Master Mix (Applied Biosystems) were added to 15 μl final reaction volume. The following cycling parameters were employed: 2 min at 50°C , 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min using the StepOnePlus real-time PCR system (Applied Biosystems). After verification that mRNA levels did not vary across treatments and time, *ef1- α* levels were used to normalise target genes. Reference and target genes were calculated by the absolute quantification method, in which relative expression levels are obtained based on a standard curve produced by the amplification of target tissue at known concentrations. For *in vivo* experiments, expression of target genes is reported as fold-change from 0 h SW controls. For *in vitro* experiments, gene expression is reported as fold-change from the 330 mOsmolal SW group.

Statistical analysis

Comparisons among different treatments were analysed by two-way ANOVA, with treatment and time as main effects. Significant ($P < 0.05$) main effects of treatment were followed up by a Student's *t*-test at all time points. Comparisons among slopes were carried out by linear regression and analysis of covariance (ANCOVA). Statistical calculations were performed using a statistical software program, Prism 5.0 (GraphPad, La Jolla, CA, USA).

Results

Effects of salinity transfers on plasma Prl₁₇₇ and Prl₁₈₈ in vivo

Plasma osmolality decreased when fish were transferred from SW to FW, with the greatest reduction (to ~ 305 mOsmolal) occurring 48 h after transfer. Conversely, plasma osmolality markedly increased when fish were transferred from FW to SW, reaching ~ 390 mOsmolal by 6 h after transfer (Fig. 1A). Plasma Prl₁₈₈ increased by 6 h after transfer from SW to FW, and reached the highest level at 48 h (Fig. 1B). After 10 days, plasma Prl₁₈₈ levels of fish transferred from SW to FW remained significantly elevated from plasma levels in SW controls, but at levels lower than fish maintained in FW. This difference disappeared by 72 days after transfer; Prl₁₈₈ levels of SW–FW and FW–FW groups were statistically similar at 6–7 ng/ml (data not shown). This pattern changed when fish were transferred from FW to SW. Six hours after transfer from FW to BW, plasma Prl₁₈₈ dropped to levels observed in fish maintained in SW and remained low throughout the duration of the experiment (Fig. 1B). Although less drastic, a similar

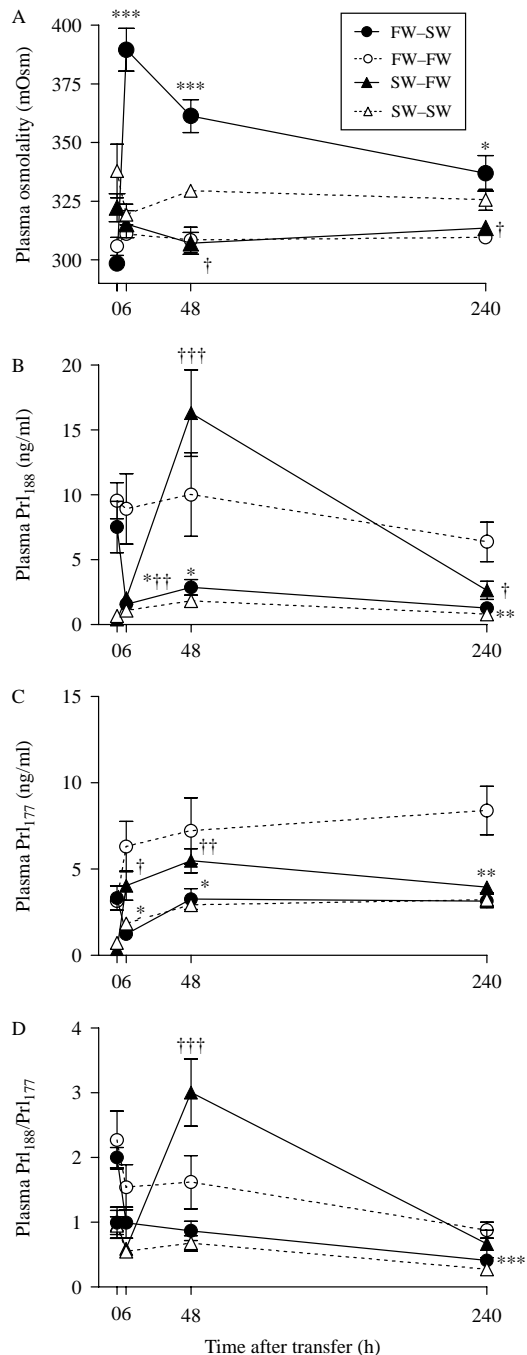


Figure 1 Changes in (A) plasma osmolality, (B) Prl₁₈₈, (C) Prl₁₇₇ and (D) Prl₁₈₈/Prl₁₇₇ after transfer of Mozambique tilapia from FW to SW (circles) and SW to FW (triangles). Symbols represent \pm S.E.M. ($n=6-10$). Control fish were maintained in FW or SW and sampled on the same time course as fish subjected to salinity change. Differences among groups were evaluated by two-way ANOVA. *, **, ***Significantly different from the corresponding FW-FW controls (open circles) at $P<0.05$, 0.01 and 0.001 respectively by Student's *t*-test. \dagger , \ddagger , $\ddagger\ddagger$ Significantly different from corresponding SW-SW controls (open triangles) at $P<0.05$, 0.01 and 0.001 respectively by Student's *t*-test.

trend was observed with respect to plasma Prl₁₇₇. Prl₁₇₇ was increased at all time points after transfer from SW to FW when compared with levels in SW-SW controls; Prl₁₇₇ decreased at all time points upon transfer from FW to SW when compared with levels in FW-FW controls (Fig. 1C). The ratio of Prl₁₈₈/Prl₁₇₇ in plasma following salinity challenges confirmed differential responsiveness to changes in environmental salinity. While Prl₁₈₈/Prl₁₇₇ was increased 48 h after transfer from SW to FW, Prl₁₈₈/Prl₁₇₇ decreased 10 days after transfer from FW to SW (Fig. 1D).

Effects of salinity transfers on prl₁₇₇ and prl₁₈₈ gene expression in vivo

Expression of *prl₁₈₈* in RPD increased significantly by 6 h after transfer from SW to FW, and reached the highest value, tenfold higher than SW controls, after 48 h. These elevated levels were maintained at 10 days (Fig. 2A). An opposite pattern was observed when fish were transferred from FW to SW, where *prl₁₈₈* mRNA levels dropped significantly by 48 h and remained low throughout the duration of the experiment.

Expression of *prl₁₇₇* in response to salinity transfers followed overall patterns that were similar to, though less drastic than, those observed with *prl₁₈₈* (Fig. 2B). Transfer from SW to FW elicited a ~ 2.5 -fold increase by 48 h, and a threefold increase by 10 days. Transfer from FW to SW, on the other hand, induced significant decreases in *prl₁₇₇* mRNA by 48 h, but levels in these fish did not reach the levels of SW-SW controls until 10 days. The ratio of mRNA for *prl₁₈₈* to *prl₁₇₇* increased significantly after 6 h, 48 h and 10 days of transfer from SW to FW. Transfer from FW to SW, by contrast, resulted in reduced *prl₁₈₈/prl₁₇₇* mRNA ratios that were significantly different from FW-FW controls at all time points (Fig. 2C). This trend was similar to that observed with plasma levels of both Prls (Fig. 1D).

Effects of extracellular osmolality on Prl₁₇₇ and Prl₁₈₈ release and gene expression in vitro

Initially, *prl₁₈₈* and *prl₁₇₇* mRNA expression in dispersed Prl cells of FW and SW fish incubated in isosmotic conditions (330 mOsmolal) was compared between time 0 and 6 h. While there was no effect of incubation time on *prl₁₈₈* and *prl₁₇₇* mRNA expression in dispersed cells of SW fish, there was a twofold increase in mRNA expression of *prl₁₈₈* in Prl cells of FW fish incubated for 6 h when compared with cells of FW fish at time 0 (data not shown). There was no effect of incubation time on mRNA expression of *prl₁₇₇* in dispersed cells of FW fish.

Dispersed Prl cells from fish acclimated to either FW or SW were then incubated under a range of medium osmolalities. The effect of reducing medium osmolality on Prl₁₈₈ release was more robust, i.e. a significantly steeper slope, in incubations of Prl cells from FW fish (slope = -0.809 ± 0.161 ; $R^2=0.407$) than that observed with Prl cells from SW fish (slope = -0.404 ± 0.103 ; $R^2=0.288$; Fig. 3A).

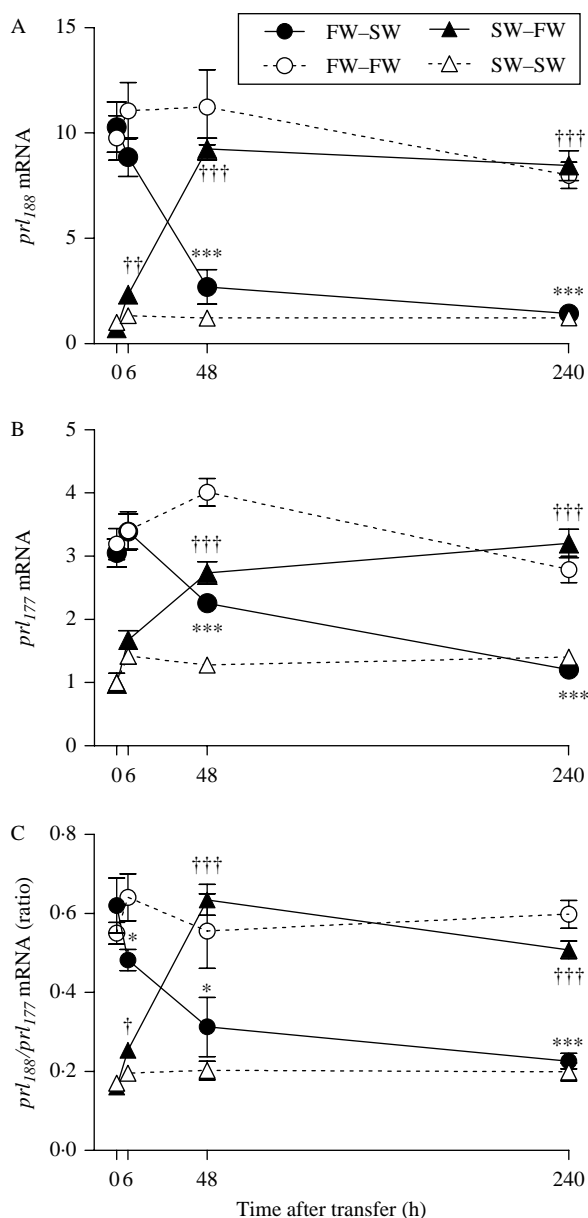


Figure 2 Changes in mRNA levels of (A) *prl*₁₈₈ (B) *prl*₁₇₇ and (C) *prl*₁₈₈/*prl*₁₇₇ after transfer of Mozambique tilapia from FW to SW (circles) and SW to FW (triangles). Symbols represent mRNA levels from individual RPDs \pm S.E.M. ($n=8-10$). Control fish were maintained in FW (FW-FW) or SW (SW-SW) and sampled on the same time course as fish subjected to salinity change. Expression levels are presented relative to the time 0 SW-SW group. Differences among groups were evaluated by two-way ANOVA. *, ***Significantly different from the corresponding FW-FW controls (open circles) at $P<0.05$, and 0.001 respectively by Student's *t*-test. †, ††, †††Significantly different from the corresponding SW-SW controls (open triangles) at $P<0.05$ and 0.001 respectively by Student's *t*-test.

The effect of medium osmolality on *prl*₁₈₈ gene expression differed between the two acclimation salinities. In Prl cells from SW fish, *prl*₁₈₈ mRNA levels were inversely related to medium osmolality (slope = $-0.468 \pm 7.43 \times 10^{-2}$; $R^2=0.511$). With Prl cells from FW fish, on the other hand, already high *prl*₁₈₈ mRNA levels were unresponsive to changes in medium osmolality (slope = $-4.09 \times 10^{-2} \pm 6.19 \times 10^{-2}$; $R^2=0.006$; Fig. 3B).

*Prl*₁₇₇ showed a different pattern of response to medium osmolality. *Prl*₁₇₇ release from dispersed Prl cells was significantly and equally responsive to extracellular osmolality regardless of the salinity in which the tilapia were reared (SW slope = $-0.291 \pm 7.78 \times 10^{-2}$; SW $R^2=0.28$; FW slope = -0.432 ± 0.158 ; FW $R^2=0.172$; Fig. 3C). The mRNA levels of *prl*₁₇₇ in Prl cells from SW-acclimated fish increased as medium osmolality was reduced, while in Prl cells from FW fish, *prl*₁₇₇ mRNA levels were unresponsive to the changes in medium osmolality (SW slope = -0.182 ± 0.083 ; SW $R^2=0.111$; FW slope = $-7.81 \times 10^{-2} \pm 0.121$; FW $R^2=0.012$; Fig. 3D). The ratio of *Prl*₁₈₈/*Prl*₁₇₇ release was inversely related to changes in medium osmolality in FW fish with more *Prl*₁₈₈ being released relative to *Prl*₁₇₇ as osmolality was reduced (SW slope = $-2.72 \times 10^{-4} \pm 1.66 \times 10^{-4}$; SW $R^2=0.065$; FW slope = $-1.61 \times 10^{-3} \pm 7.03 \times 10^{-4}$; FW $R^2=0.124$; Fig. 3E). This pattern was not observed when Prl cells from SW fish were exposed to various osmolalities. The mRNA ratio of *prl*₁₈₈ to *prl*₁₇₇ showed a response that was opposite to that for release, rising significantly when Prl cells from SW fish were exposed to lower medium osmolalities (SW slope = $-3.07 \times 10^{-3} \pm 7.79 \times 10^{-4}$; SW $R^2=0.289$; FW slope = $-2.09 \times 10^{-4} \pm 1.16 \times 10^{-3}$; FW $R^2=0.001$; Fig. 3F). This pattern was not observed with Prl cells from FW fish in which medium osmolality had no effect on the *prl*₁₈₈/*prl*₁₇₇ mRNA ratio.

Effects of salinity transfers and extracellular osmolality on *ostf*₁, *prlr*₁ and *prlr*₂ gene expression in RPD

The expression of *prlr*₁, *prlr*₂ and *ostf*₁ in RPD following salinity challenges was measured *in vivo*, and to changes in medium osmolality *in vitro*. Comparisons of the expression levels of the three genes at time 0 and 6 h of incubation were carried out. The levels of *prlr*₁ mRNA increased by twofold and decreased by twofold, in Prl cells of FW and SW fish respectively by 6 h of incubation when compared with time 0 (data not shown). The levels of *prlr*₂ mRNA decreased by 1.5-fold and by twofold in Prl cells of FW and SW fish respectively by 6 h of incubation when compared with time 0 (data not shown). Finally, the levels of *ostf*₁ mRNA decreased by twofold and by threefold in Prl cells of FW and SW fish respectively by 6 h of incubation when compared with time 0 (data not shown). The levels of *prlr*₁ mRNA in RPDs did not change in response to either FW or SW transfer (Fig. 4A), nor in response to changes in medium osmolality *in vitro* (SW slope = $4.48 \times 10^{-3} \pm 0.11$; SW $R^2<0.001$; FW slope = $5.29 \times 10^{-2} \pm 0.104$; FW $R^2=0.007$; Fig. 4B).

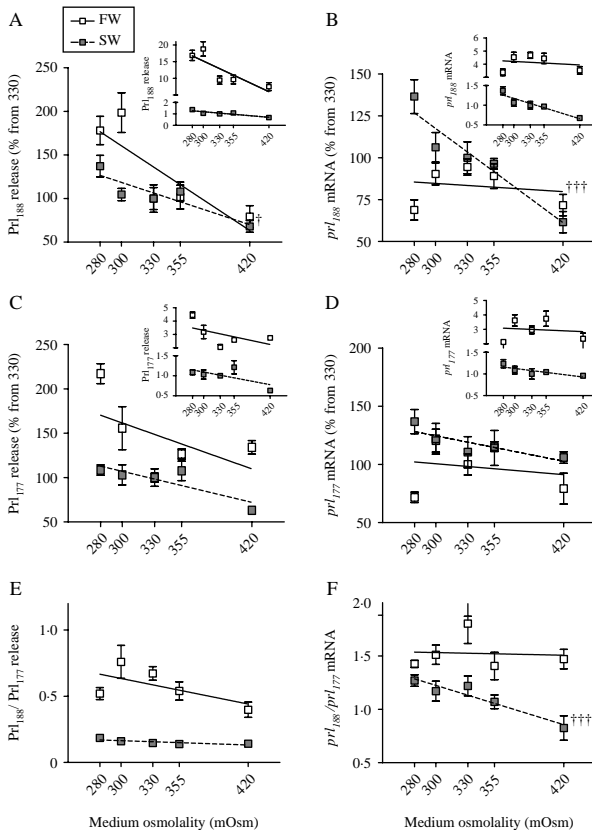


Figure 3 Linear regression of (A) Prl₁₈₈ release and (B) mRNA levels, (C) Prl₁₇₇ release and (D) mRNA levels and (E) Prl₁₈₈/Prl₁₇₇ release and (F) mRNA levels from dispersed Prl cells exposed to different medium osmolalities during a 6-h incubation. Open squares and a solid line represent Prl release or mRNA levels and linear regression respectively from fish acclimated to FW, whereas shaded squares and a dashed line represent these same parameters from fish acclimated to SW. Values represent percent Prl release (A and C) or percent mRNA levels (B and D) relative to the 330 mOsmolal incubation group of each respective salinity \pm S.E.M. ($n=8$, SW; $n=12-16$, FW). Values presented in panels E and F represent ratios of Prl release and expression respectively. Insets represent the values relative to the SW 330 mOsmolal incubation groups \pm S.E.M. †, †††Regression slopes significantly different from each other at $P<0.05$ and 0.001 respectively.

The levels of *prlr2* mRNA, on the other hand, increased in RPDs by 6 h after transfer from FW to SW compared to levels in FW-FW controls (Fig. 4C). The levels of *prlr2* mRNA from Prl cells isolated from both FW- and SW-acclimated fish increased in direct proportion to extracellular osmolality (SW slope = 1.24 ± 0.178 ; SW $R^2=0.571$; FW slope = 0.955 ± 0.094 ; FW $R^2=0.733$; Fig. 4D). Similarly, *ostf1* mRNA increased by 6 h after transfer from FW to SW compared to FW-FW controls (Fig. 4E). While the Prl cells isolated from both FW- and SW-acclimated fish showed increased *ostf1* gene expression in response to increased medium osmolality, this response was more robust in Prl cells isolated from FW-acclimated fish (SW slope = 0.399 ± 0.133 ; SW $R^2=0.204$; FW slope = 2.148 ± 0.377 ; FW $R^2=0.488$; Fig. 4F).

Discussion

The present study examined the modulation of osmotic responsiveness in the tilapia Prl cell by environmental salinity. Our findings indicate that: 1) Prl cells from fish acclimated to FW exhibited a greater capacity for hormone release in response to hyposmotic stimulation than those from SW fish; 2) *prl* gene expression in Prl cells from fish acclimated to SW was increased as medium osmolality was reduced *in vitro*, this did not occur in Prl cells from FW-acclimated fish; 3) release and gene expression of Prl₁₈₈ is more responsive to changes in medium osmolality than release and gene expression of Prl₁₇₇; and 4) gene expression of *prlr2* and *ostf1* in Prl cells is induced in direct proportion to increases in plasma osmolality *in vivo* and culture medium osmolality *in vitro* respectively. The differences in osmosensitivity between Prl₁₈₈ and Prl₁₇₇ we document suggest that different osmotic signal transduction mechanisms regulate their release and gene transcription. Moreover, the osmosensitivity of *prlr2* and *ostf1* expression provides evidence that these two factors contribute to osmotic control of tilapia Prl cell function.

Prl-secreting cells of the RPD are true osmoreceptors in the Mozambique tilapia (Seale *et al.* 2006b). In tilapia, Prl₁₇₇ and Prl₁₈₈ are encoded by separate genes and differ in both amino acid number and composition, and consequently exhibit only 69% homology (Specker *et al.* 1985, Yamaguchi *et al.* 1988, Rentier-Delrue *et al.* 1989). While the discovery of two distinct Prl molecules in tilapia suggested the evolution of distinct actions, no clear differences in osmoregulatory action have been demonstrated (Specker *et al.* 1985). This issue has been recently revisited by Fiol *et al.* (2009), who reported that Prl₁₇₇ and Prl₁₈₈ activate distinct downstream signalling pathways in transfected HEK293 cells expressing tilapia PrlRs.

Early studies have shown greater RPD and Prl cell size (Dharmamba & Nishioka 1968), greater Prl cell activity (Nagahama *et al.* 1975), and greater hypophyseal Prl content (Nicoll *et al.* 1981) in tilapia maintained in FW compared to tilapia maintained in SW. Later, Yoshikawa-Ebesu *et al.* (1995) reported that hyposmotically induced Prl₁₇₇ and Prl₁₈₈ synthesis is more robust in RPDs of fish acclimated to FW than in RPDs of fish acclimated to SW. Likewise, mRNA levels for both *prl* genes were higher in pituitaries of tilapia acclimated to FW than in pituitaries of fish acclimated to $\frac{1}{4}$ SW or SW (Shepherd *et al.* 1999). More recently, we showed with dispersed Prl cells that Prl₁₈₈ release in response to *in vitro* reductions in osmolality is more sensitive in Prl cells of FW fish than in Prl cells of fish acclimated to SW (Seale *et al.* 2006a). To specifically address the differential osmosensitivity of Prl₁₇₇ and Prl₁₈₈ in the current study, we examined the effects of salinity transfers and a range of physiologically relevant extracellular osmolalities on the release and gene expression of these hormones. Consistent with previous reports (Seale *et al.* 2002, Breves *et al.* 2011), plasma osmolality decreased, and plasma Prl₁₇₇ and Prl₁₈₈ levels increased, following transfer from SW to FW.

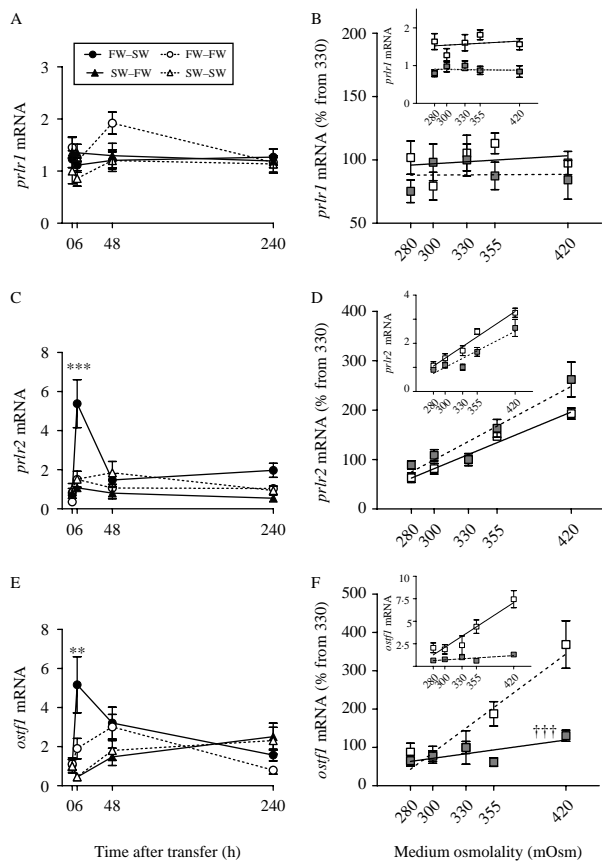


Figure 4 Changes in mRNA levels of (A) *prlr1* (C) *prlr2* and (E) *ostf1* after transfer of Mozambique tilapia from FW to SW (circles) and SW to FW (triangles). Symbols represent mRNA levels from individual RPDs (\pm S.E.M.; $n=8-10$). Control fish were maintained in FW (FW-FW) or SW (SW-SW) and sampled on the same time course as fish subjected to salinity change. Expression levels are presented relative to the time 0 SW-SW group. Differences among groups were evaluated by two-way ANOVA. ** ***Significantly different from the corresponding FW-FW controls (open circles) at $P<0.01$, and 0.001 respectively by Student's *t*-test. Linear regression of (B) *prlr1* mRNA, (D) *prlr2* mRNA and (F) *ostf1* mRNA from dispersed Prl cells exposed to different medium osmolalities during a 6-h incubation. Open squares and a solid line represent mRNA levels and linear regression respectively from fish acclimated to FW, whereas shaded squares and a dashed line represent these same parameters from fish acclimated to SW. Expression levels are presented relative to the 330 mOsmol group of each respective salinity (\pm S.E.M.; $n=8$, SW-acclimated groups; $n=12-16$, FW-acclimated groups). Insets represent the values relative to the SW 330 mOsmol incubation groups \pm S.E.M. +++Regression slopes significantly different from each other at $P<0.001$.

Gene expression of both *prl* genes followed a similar pattern. On the other hand, when fish were transferred from FW to SW, increased plasma osmolality was associated with decreases in plasma Prl₁₇₇ and Prl₁₈₈, as well as in *prlr177* and *prlr188* mRNA levels in RPDs; this finding is in agreement with a previous SW acclimation study (Breves *et al.* 2010a). These patterns are consistent with the hyperosmoregulatory actions of Prls. Nevertheless, there are changes in the ratio

of Prl₁₈₈/Prl₁₇₇ that indicate that Prl₁₈₈ is more sensitive to changes in environmental salinity than Prl₁₇₇. Previously, Borski *et al.* (1992) reported relative RPD Prl₁₈₈/Prl₁₇₇ ratios close to 1.5:1 in FW fish and 0.5:1 in SW and in fish that had been transferred from SW to FW or from FW to SW after 49 days. In the present study, plasma Prl₁₈₈/Prl₁₇₇ ratio increased to 3:1 by 48 h after transfer from SW to FW, and declined to 0.5:1 by 10 days after transfer from FW to SW. Changes in the relative expression of both *prl* genes followed a pattern that is similar to that seen with plasma Prls. When faced with a decrease in environmental salinity, plasma Prl₁₈₈ and *prlr188* mRNA rose to a greater extent than plasma Prl₁₇₇ and *prlr177* mRNA, whereas after transfer of tilapia from FW to SW, both plasma levels and mRNA for Prl₁₈₈ declined to a greater extent than for Prl₁₇₇. These results indicate that, *in vivo*, the mechanisms responsible for both transcription and release of Prl₁₈₈ are more responsive to changes in environmental salinity than those of Prl₁₇₇. The greater responsiveness of Prl₁₈₈ to changes in environmental salinity may therefore be a reflection of greater osmosensitivity of the signal transduction mechanisms controlling release/transcription of this hormone.

We compared the relative osmosensitivity of the two Prls by exposing dispersed Prl cells from FW- and SW-acclimated fish to alterations in extracellular osmolality. Despite the fact that Prl cells from FW-acclimated fish released tenfold more Prl₁₈₈ than did those of SW fish after 6 h, changes in extracellular osmolality elicited responses in cells of fish from both acclimation salinities. The sensitivity of Prl₁₈₈ release in Prl cells from FW fish, however, was far stronger than that in Prl cells from SW fish, as indicated by the significant difference in the slopes of osmolality vs Prl₁₈₈ (Fig. 3A). The greater responsiveness of Prl cells from FW fish from that of SW fish to hyposmotic stimulation *in vitro* has been previously observed in dispersed Prl cells (Seale *et al.* 2006a), but was not observed under the conditions provided by Borski *et al.* (1992), who employed intact RPDs in 18–20 h incubations. In that study, Prl release *in vitro* was clearly discernible between RPDs of FW and SW fish but not between RPDs incubated with hypo- and hyperosmotic media. Differences in experimental design and in the method of Prl assay—electrophoresis in the previous case and RIA in this study—may have ultimately prevented the detection of differences in FW and SW Prl cell osmosensitivity in the earlier study. In the current study, different osmosensitivity regarding the release of the two Prls was observed in FW fish. As medium osmolality decreased, the Prl₁₈₈/Prl₁₇₇ release ratio increased. Overall, hyposmotically induced release of both Prls from SW-acclimated tilapia showed a lower sensitivity that may be derived from the lower quantity of Prl stored in the pituitaries of these fish (Nagahama *et al.* 1975). Recent evidence also suggests that this difference may result from the diminished ability of Prl cells from SW-acclimated tilapia to initiate osmotic signal transduction. The relative rates of cell volume increase and water influx into Prl cells following an osmotic challenge—essential steps of the osmotic signal transduction

mechanism (Seale *et al.* 2005)—were found to be significantly lower in cells from SW tilapia compared to those in FW (Watanabe *et al.* 2009). These disparities were associated with lower gene expression, protein content and immunoreactivity for the water channel aquaporin 3 (Aqp3) in the Prl cells of SW fish as compared to those of FW fish (Watanabe *et al.* 2009).

Contrary to release patterns, the hyposmotically induced rise in *prl*₁₈₈ mRNA only reached statistical significance in Prl cells of SW fish. Absolute levels of *prl*₁₈₈ mRNA, however, were 4.5-fold higher in Prl cells of FW compared to those of SW fish after 6 h incubation in isosmotic conditions (330 mOsmolal). The fact that mRNA levels of the two Prls are much higher in the RPD of FW fish, and perhaps maximal, may render them unable to respond further to osmotic stimulation *in vitro*. The difference observed between *prl*₁₈₈ and *prl*₁₇₇ mRNA in response to salinity transfer *in vivo* was also observed *in vitro*. The ratio of the gene expression of *prl*₁₈₈ to that of *prl*₁₇₇, in Prl cells of SW fish, but not of FW fish, rose as medium osmolality was reduced. In earlier studies, employing ³H-leucine to label newly synthesised Prls, it was shown that the ratio of the appearance of newly synthesised ³H-Prl₁₈₈ to that of ³H-Prl₁₇₇ was 1.3 in FW tilapia RPD and 0.5 in SW RPD after exposure to a hyposmotic stimulus (Yoshikawa-Ebesu *et al.* 1995). This nearly threefold difference between FW and SW ratios is similar to the difference in ratios of plasma Prls between FW and SW fish *in vivo*, as well as the ratios of release and mRNA levels between Prl cells of FW and SW fish observed *in vitro*.

The current study provides the most in-depth analysis of differential Prl₁₈₈ and Prl₁₇₇ regulation by environmental salinity and extracellular osmolality, and is the first to identify the differences in Prl₁₈₈ and Prl₁₇₇ osmosensitivity *in vitro*. Both Prls have been shown to co-localise in the same secretory granules (Specker *et al.* 1993), indicating that differences in the release of both isoforms are unlikely to result from different Prl cell types or stored hormone pools within the same cell type. It is possible, therefore, that both hormones may be regulated by separate transduction pathways, or are differentially sensitive to intracellular spikes in Ca²⁺ and cAMP—second messengers that are known to be activated during osmotic signal transduction (Seale *et al.* 2005, 2011).

In the present study, we also measured the expression of two *prlr*s in the RPDs of fish transferred from SW to FW and vice versa, and compared these responses with those exhibited by Prl cells *in vitro*. Branchial expression of *prlr*₂ has been shown to be higher in the gill of tilapia abruptly transferred to SW than in those maintained in FW (Fiol *et al.* 2009). HEK293 cells transfected with *prlr*₂ exhibited greater tolerance to hyperosmotic stress when compared with empty vector controls or those transfected with *prlr*₁ (Fiol *et al.* 2009). Expression of branchial *prlr*₁ decreases with transfer from FW to SW (Breves *et al.* 2010a) and increases with transfer from SW to FW (Breves *et al.* 2011). Furthermore, branchial *prlr*₁ levels decrease after hypophysectomy in SW but not in FW, suggesting that *prlr*₁ expression is regulated, at least in part, by the pituitary (Breves *et al.* 2010b). We found *prlr*₁ in

the RPD to be unresponsive either to salinity transfers *in vivo* or to changes in extracellular osmolality *in vitro*. Nagahama *et al.* (1975) suggested that Prl secretion may be subject to negative feedback from Prl itself; this finds support in the basic observation that *prlr* genes are expressed in Prl cells. In mammalian lactotrophs, autocrine or paracrine inhibitory responses to local Prl are reported under physiological (lactation) and pathological (prolactinoma) conditions (Freeman *et al.* 2000). Autoregulation of Prl cell activity by Prl, through PrlRs, may play a similar role in tilapia to fine-tune hormone synthesis and secretion. This mode of autocrine or paracrine regulation is supported by a recent study in zebrafish embryos reporting that PrlR knockdown by morpholino induced drastic increases in Prl expression in lactotrophs (Liu *et al.* 2006). We therefore propose that increased *prlr*₂ expression in tilapia RPDs in response to hyperosmotic conditions (both *in vivo* and *in vitro*) represents a mechanism to maintain low plasma Prl levels in hyperosmotic environments.

Levels of *ostf*₁ mRNA in RPDs increased by 6 h after transfer from FW to SW and in response to an increase in medium osmolality *in vitro*. After 48 h and 10 days of transfer from FW to SW, however, *ostf*₁ and *prlr*₂ levels subsided to pre-transfer levels, possibly indicating that changes in *ostf*₁ and *prlr*₂ may be part of a transient response targeted to mitigate the marked increase in plasma osmolality (~390 mOsmolal) observed at 6 h. The time course of *ostf*₁ induction in the RPD following transfer from FW to SW is similar to that reported in the gills of tilapia exposed to the same hyperosmotic challenge (Fiol & Kultz 2005). Rapid induction of *ostf*₁ in response to hyperosmotic stress has also been shown to occur in branchial cell cultures (Fiol *et al.* 2006). This response was shown to occur independently from systemic factors, which instead appear to prevent sustained transcriptional induction of *ostf*₁ (Fiol *et al.* 2006). While induction of *ostf*₁ in the RPDs of fish transferred from FW to SW *in vivo* is restricted to the first 6 h, further studies are required to determine whether hyperosmotic induction of *ostf*₁ in Prl cells *in vitro* is also transient in nature. The greater hyperosmotically induced increase of *ostf*₁ observed in Prl cells from FW fish suggests that these cells are more sensitive to hyperosmotic stimuli than those from SW fish.

Studies executing *in vitro* knockdowns of *ostf*₁ and *prlr*s will be useful to reveal whether these genes support the adaptive osmotic responses of Prl cells. In order to explore the potential adaptive roles that *prlr*₂ plays in tilapia Prl cells, further studies must identify the effects of the two Prls on both *prlr*₂ expression and the osmosensitivity of hormone release and gene expression in Prl cells isolated from fish acclimated to various salinities.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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