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Acute salinity tolerance and the control of two prolactins and their receptors in the Nile tilapia (*Oreochromis niloticus*) and Mozambique tilapia (*O. mossambicus*): a comparative study

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Abstract

Osmoregulation in vertebrates is largely controlled by the neuroendocrine system. Prolactin (PRL) is critical for the survival of euryhaline teleosts in fresh water by promoting ion retention. In the euryhaline Mozambique tilapia (*Oreochromis mossambicus*), pituitary PRL cells release two PRL isoforms, PRL₁₈₈ and PRL₁₇₇, in response to a fall in extracellular osmolality. Both PRLs function via two PRL receptors (PRLRs) denoted PRLR1 and PRLR2. We conducted a comparative study using the Nile tilapia (*O. niloticus*), a close relative of Mozambique tilapia that is less tolerant to increases in environmental salinity, to investigate the regulation of PRLs and PRLRs upon acute hyperosmotic challenges *in vivo* and *in vitro*. We hypothesized that differences in the regulation of PRLs and PRLRs underlie the variation in salinity tolerance of tilapias within the genus *Oreochromis*. When transferred from fresh water to brackish water (20‰), Nile tilapia increased plasma osmolality and decreased circulating PRLs, especially PRL₁₇₇, to a greater extent than Mozambique tilapia. In dispersed PRL cell incubations, the release of both PRLs was less sensitive to variations in medium osmolality in Nile tilapia than in Mozambique tilapia. By contrast, increases in pituitary and branchial *prlr2* gene expression in response to a rise in extracellular osmolality were more pronounced in Nile tilapia relative to its congener, both *in vitro* and *in vivo*. Together, these results support the conclusion that inter-specific differences in salinity

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tolerance between the two tilapia congeners are tied, at least in part, to the distinct responses of both PRLs and their receptors to osmotic stimuli.

Keywords

prolactin; tilapia; salinity tolerance; osmosensitivity; osmoregulation

1. Introduction

Euryhaline fishes possess the ability to adapt to a wide range of environmental salinities, typically between fresh water (FW) and seawater (SW). It is not uncommon, however, that fish of the same family or genus exhibit very different salinity tolerances, a phenomenon that is often attributed to their life histories and native distributions. Distinct osmoregulatory capacities are exemplified by killifish of the genus *Fundulus*, which are known for large variation in salinity tolerance among species (Griffith, 1974a). Likewise, tilapias of the genus *Oreochromis* display remarkably different abilities to survive in SW, which are linked closely with their natural distributions (Trewavas, 1983). The Nile tilapia (*Oreochromis niloticus*), for example, lives in FW habitats of northern and western river basins of Africa and can only tolerate salinities up to 25‰ (Trewavas, 1983; Watanabe et al., 1985). Populations of Mozambique tilapia (*Oreochromis mossambicus*), on the other hand, live in a range of habitats that include estuaries, hypersaline ponds and tidal rivers in southeast Africa and can acclimate to salinities up to double-strength SW (Fiess et al., 2007; Trewavas, 1983).

Salinity acclimation requires the successful detection of osmotic changes and subsequent activation of physiological responses that restore osmotic homeostasis. A key mediator of this process in teleosts is the hypophyseal hormone, prolactin (PRL), which promotes ion retention in hyposmotic conditions (Manzon, 2002; McCormick and Bradshaw, 2006). In *O. mossambicus*, PRL cells comprise a nearly homogeneous portion of the *rostral pars distalis* (RPD) of the pituitary and function as osmoreceptors (Grau and Helms, 1989; Nagahama et al., 1981; Seale et al., 2012b, 2006b). Consistent with its essential role in FW-acclimation, plasma PRL levels in *O. mossambicus* increase when fish are transferred from SW to FW and fall upon transfer from FW to SW (Breves et al., 2011, 2010a; Seale et al., 2002, 2012a; Yada et al., 1994). Furthermore, PRL release is strongly and directly regulated by physiologically relevant changes in extracellular osmolality, *in vitro* (Grau et al., 1986; Seale et al., 2002, 2003a, 2006a, 2012a).

The pituitary of both Nile and Mozambique tilapia releases two PRL molecules, PRL₁₈₈ and PRL₁₇₇, which differ in length and amino acid composition; these two PRLs are encoded by separate genes (Rentier-Delrue et al., 1989; Specker et al., 1985; Yamaguchi et al., 1988). The Mozambique and Nile tilapia are quite closely related, and the amino acid and nucleotide sequences of their pituitary hormones and cognate receptors are nearly identical (Rentier-Delrue et al., 1989; Yamaguchi et al., 1988). Increasing evidence indicates that the two PRLs are distinctive in their physiological functions. In *O. niloticus*, PRL₁₈₈ induced FW-type ionocyte morphology, and was found to be more potent than PRL₁₇₇ in increasing

plasma Na⁺ and Cl⁻ levels (Auferin et al., 1994; Pisam et al., 1993). In *O. mossambicus*, PRL₁₈₈ was found to be more sensitive to hyposmotic stimulation than PRL₁₇₇ (Borski et al., 1992; Seale et al., 2012a). Conversely, the suppression of PRL₁₇₇ in response to hyperosmotic stimulation is less accentuated than that of PRL₁₈₈, both *in vivo* and *in vitro* (Borski et al., 1992; Seale et al., 2012a). Furthermore, and following the same trend for PRL peptides, there is a difference in the relative sensitivity of *prl*₁₈₈ and *prl*₁₇₇ mRNA expression in tilapia PRL cells to extracellular osmolality (Seale et al., 2012a).

Both PRLs exert their actions through two PRL receptors, PRLR1 and PRLR2, which are distinct in downstream signaling pathways triggered upon ligand binding and in responses to *in vivo* and *in vitro* osmotic stimuli (Breves et al., 2011; Fiol et al., 2009; Huang et al., 2007; Sandra et al., 1995). Branchial expression of *prlr1* mRNA is inversely related to extracellular osmolality *in vivo* (Breves et al., 2011, 2010a). Moreover, hypophysectomy in SW, but not in FW, suppresses branchial *prlr1* expression, suggesting that *prlr1* is regulated, at least in part, by a pituitary hormone(s) (Breves et al., 2010d). By contrast, branchial and pituitary *prlr2* expression is stimulated by elevated environmental osmolality, both *in vivo* and *in vitro* (Fiol et al., 2009; Inokuchi, et al., 2015; Seale, et al., 2012a). When expressed in human embryonic kidney cells (HEK293), PRLR2, but not PRLR1, improves the osmotic tolerance of transformed cells (Fiol et al., 2009). Together, these studies reveal a dynamic relationship between both PRL isoforms and their receptors in response to osmotic challenges.

Based on this framework, we hypothesized that a critical difference in the ability of Nile and Mozambique tilapia to tolerate increases in salinity is tied, at least partly, to how PRL cells and target tissues, such as the gill, respond to changes in extracellular osmolality. By employing both *in vivo* and *in vitro* approaches, we determined whether the distinct salinity tolerances of Nile and Mozambique tilapia were associated with interspecific differences in the osmotic regulation of PRL release and mRNA expression, as well as pituitary and branchial *prlr* mRNA expression.

2. Materials and methods

2.1. Animals

Mature Nile tilapia (*O. niloticus*) and Mozambique tilapia (*O. mossambicus*) of both sexes were obtained from stocks maintained at the University of Hawai'i Hilo Farm Laboratory (Panaewa, Hawai'i) or at the Hawai'i Institute of Marine Biology (Kaneohe, Hawai'i), respectively. Fish were reared in outdoor tanks with a continuous flow of FW under natural photoperiod and fed Catfish EXTR 350 (*O. niloticus*; Rangen Inc., Buhl, ID) or Silver Cup Trout Chow (*O. mossambicus*; Nelson and Sons Inc., Murray, UT), respectively. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawai'i.

2.2. *In vivo* experiments

O. niloticus and *O. mossambicus* of both sexes, ranging from 31 to 361 g and from 27 to 116 g, respectively, were placed in aquaria containing either FW or brackish water (BW; 20‰); BW was prepared by mixing FW with SW (Hilo Bay or Kaneohe Bay, Hawai'i). A total of 2

FW and 2 BW aquaria, each containing 8 fish, were employed for each species. Fish were not fed for the duration of the experiment and water temperature was maintained at 22 °C. Fish were sampled ($n=8$) at 0 h (from the outdoor FW tank, just before transfer to each aquarium), 6 h, and 24 h after transfer from FW to BW. At the time of sampling, fish were netted and anesthetized with 2-phenoxyethanol (0.2 ml/L; Sigma-Aldrich, St. Louis, MO). Blood was collected from the caudal vasculature by a needle and syringe coated with heparin (200 U/ml, Sigma-Aldrich). Plasma was separated by centrifugation and stored at -20 °C until later analyses. Fish were rapidly decapitated and the pituitary extracted and placed in individual cryogenic vials containing RNAlater (Life Technologies, Carlsbad, CA). Samples were stored at 4 °C prior to RNA extraction.

2.3. Dispersed PRL cell incubations

Incubations of dispersed PRL cells from FW-acclimated *O. niloticus* (23-368 g) and *O. mossambicus* (45-309 g) of both sexes were carried out as previously described (Seale et al., 2006a; Seale et al., 2012a). Briefly, after removal of pituitaries from tilapia, RPDs were dissected and pooled in PBS (330 mOsm/kg), diced into pieces and treated with 0.125% trypsin (Sigma-Aldrich) in PBS. After terminating the trypsin treatment by the addition of trypsin inhibitor (0.125% w/v in PBS; Sigma-Aldrich), cells were harvested, and cell viability was determined using the trypan blue (Sigma-Aldrich) exclusion test. Cell yield was estimated using a hemocytometer. Cells were then re-suspended in 330 mOsm/kg incubation medium (Yamaguchi et al., 2016), plated on 96-well plates at densities of $8.4\text{--}12.9 \times 10^4$ cells (*O. mossambicus*) per well, and incubated at 26 °C under saturated humidity. Cells were pre-incubated for 1 h in 330 mOsm/kg medium prior to exposure to a series of osmolalities (280, 300, 330, 355 and 420 mOsm/kg). Medium osmolality was adjusted by varying the concentration of NaCl and verified using a vapor pressure osmometer (Wescor 5100C; Wescor, Logan, UT). Medium was collected after 6 h of incubation and stored at -20 °C prior to radioimmunoassay. At the end of the incubation period, cells were collected by adding 100 μ l of TRI Reagent (MRC, Cincinnati, OH) to each well, and transferred to 1.5 mL tubes. An additional 300 μ L of TRI Reagent was added to each tube before total RNA was extracted as described below.

2.4. Radioimmunoassays (RIAs)

PRL₁₈₈ and PRL₁₇₇ levels in plasma, culture medium and tissue homogenates were measured by RIAs developed for Mozambique and Nile tilapia (Ayson et al., 1993; Yada et al., 1994), as previously described in detail (Yamaguchi et al., 2016). Both pituitary homogenates and plasma from Nile tilapia have been shown to produce displacement curves parallel to those of Mozambique tilapia PRL₁₈₈ and PRL₁₇₇ RIA standards (Ayson et al., 1993). For *in vivo* experiments, plasma values are reported as ng/mL. For dispersed cell incubations, values are shown as fold-change from 330 mOsm/kg treatments.

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

Total RNA was extracted from intact pituitaries, gills and dispersed PRL cells using TRI Reagent according to the manufacturer's protocols, and 200 ng (pituitaries), 2 μ g (gills) or 50 ng (dispersed PRL cells) of total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). The qRT-PCR reactions

were setup as previously described (Pierce et al., 2007). The reaction mix (15 μ L) contains Power SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of forward and reverse primers, and either sample cDNAs or serially-diluted standard cDNAs; the latter was used to generate standard curves for quantification. 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 sec and 60 °C for 1 min, using a StepOnePlus real-time PCR system (Applied Biosystems). All primer pairs used in the present study were previously described and are listed in Table 1 (Breves et al., 2010c, 2010d; Magdeldin et al., 2007; Pierce et al., 2007). After verification that mRNA levels did not vary across treatments, *efla* levels were used to normalize target genes. For *in vivo* experiments, mRNA levels are shown as fold-changes from 0 h FW controls. For dispersed cell incubations, values are shown as fold-change from 330 mOsm/kg treatments.

2.6. Statistical analysis

Comparisons among different treatments were analyzed by three-way (salinity, time and species) analysis of variance (ANOVA). Significant ($P < 0.05$) main effects of treatment were followed up by Fisher's least significant difference (LSD) test. Comparisons among regression lines and curves were carried out by analysis of covariance (ANCOVA) and extra sum-of-squares F test (F test), respectively. When necessary, data were log transformed to satisfy homogeneity of variance requirements. Three-way ANOVA was performed by EZR (Kanda, 2013) and all other statistical analyses were completed using Prism 6.0 (GraphPad, La Jolla, CA).

3. Results

3.1. Effects of salinity transfer on plasma osmolality

A three-way ANOVA detected significant effects of salinity, time and species on plasma osmolality, as well as two-way interactions between these effects in all possible combinations. Plasma osmolality before the transfer from FW to either FW or BW (0 h) was 304.5 ± 5.6 and 302.8 ± 5.5 mOsm/kg in *O. niloticus*, respectively; these values are significantly lower than those in *O. mossambicus* (328.0 ± 1.7 and 325.1 ± 4.3 mOsm/kg for FW and BW groups, respectively) at 0 h. In both species, plasma osmolalities were stable in FW and remained higher in *O. mossambicus* than in *O. niloticus* during the experimental period. By 6 h, transfer of fish to BW produced significant elevations in plasma osmolality in both species. Plasma osmolality in *O. niloticus* reached 604.9 ± 11.0 mOsm/kg by 24 h following transfer from FW to BW; this is comparable to the osmolality of the surrounding environment (603 and 598.5 mOsm/kg in the experiments using *O. niloticus* and *O. mossambicus*, respectively). These BW-transferred Nile tilapia exhibited reduced opercular movement and were lying on their side by 24 h. By contrast, while plasma osmolalities of *O. mossambicus* transferred from FW to BW also increased by 6 and 24 h, they were lower than that of *O. niloticus*, reaching a maximum of 382.5 ± 4.8 mOsm/kg by 24 h (Fig. 1).

3.2. Effects of salinity transfer on plasma PRL₁₈₈ and PRL₁₇₇

For plasma PRL₁₈₈ (Fig. 2A), an interaction effect was found between salinity and time. In both species, plasma PRL₁₈₈ fell by 6 h following salinity transfer, and was maintained at

low levels until 24 h. For plasma PRL₁₇₇ (Fig. 2B), a three-way ANOVA detected significant effects of salinity and species, and an interaction effect between these factors. Consistent with PRL₁₈₈, plasma PRL₁₇₇ in *O. niloticus* fell drastically by 6 h following transfer from FW to BW. On the other hand, plasma PRL₁₇₇ in *O. mossambicus* was not significantly altered by salinity transfer. Although *O. niloticus* showed considerable individual variation in plasma PRL₁₇₇ in FW, it maintained higher levels than *O. mossambicus* at all time points. The drop in PRL₁₇₇ by 6 h following salinity transfer (19.9- and 7-fold lower than the initial levels, in *O. niloticus* and *O. mossambicus*, respectively) was more pronounced than that of PRL₁₈₈ (8.5- and 2.4-fold lower than the initial levels, in *O. niloticus* and *O. mossambicus*, respectively); plasma levels of both PRL isoforms decreased to a greater extent in *O. niloticus* than in *O. mossambicus* (Figs. 2A and 2B).

3.3. Effects of salinity transfer on pituitary *prl*₁₈₈ and *prl*₁₇₇ mRNA levels

Expression of *prl*₁₈₈ and *prl*₁₇₇ mRNAs in the pituitary was downregulated by transfer of both tilapia species from FW to BW (Fig. 3). A three-way ANOVA detected significant effects of salinity and time, as well as an interaction effect between salinity and either time or species, on *prl*₁₈₈ gene expression. In both species, pituitary *prl*₁₈₈ mRNA levels declined by 24 h after transfer from FW to BW relative to initial levels; the suppression of *prl*₁₈₈ mRNA at 24 h was more prominent in *O. niloticus* than in *O. mossambicus*. Significant suppression of *prl*₁₈₈ mRNA in BW fish relative to FW fish was detected by 6 h and 24 h in *O. niloticus* and by 24 h in *O. mossambicus*. In both species, FW control groups maintained rather stable *prl*₁₈₈ mRNA levels, although *prl*₁₈₈ expression in *O. niloticus* was elevated at 24 h and a significant interspecies difference was detected by 6 h.

For pituitary *prl*₁₇₇ mRNA, a three-way ANOVA detected significant effects of salinity, time and species, as well as an interaction effect between salinity and time. Gene expression of *prl*₁₇₇ was significantly lower in BW groups relative to those in FW groups by 6 and 24 h in *O. niloticus*, but only by 24 h in *O. mossambicus*; this reduction in *O. mossambicus* was significant from its initial level and from that of *O. niloticus*. Similar to *prl*₁₈₈, pituitary expression of *prl*₁₇₇ was stable in FW control groups, while an interspecies difference was detected at 6 h.

3.4. Effects of salinity transfer on *prlr1* and *prlr2* mRNA levels in the pituitary and gill

A three-way ANOVA detected a significant effect of time, and an interaction effect between time and species on pituitary *prlr1* mRNA expression (Fig. 4A). On the other hand, in the gill, an effect of salinity and interaction effects among salinity, time and species were detected (Fig. 4C). Significant changes in gene expression following salinity transfer were found only in *O. mossambicus*, where *prlr1* mRNA levels declined by 6 and 24 h in the pituitary (Fig. 4A) and by 24 h in the gill (Fig. 4C), relative to initial levels; differences between BW and parallel FW control groups were significant only in the gill by 6 and 24 h (Fig. 4C). A significant reduction in pituitary *prlr1* mRNA was also detected in FW control *O. mossambicus* at 6 h, relative to time 0 and to *O. niloticus* (Fig. 4A).

For both pituitary and branchial expression of *prlr2*, a three-way ANOVA detected significant effects of salinity, time and species, as well as interaction effects of these factors

in all possible combinations (Figs. 4B and 4D). Similar to *prlr1*, *prlr2* mRNA was relatively stable in control fish maintained in FW. Transfer from FW to BW produced a robust stimulation of pituitary and branchial *prlr2* mRNA by 6 and 24 h, regardless of species (Figs. 4B and 4D). In both tissues, increases in *prlr2* in response to transfer from FW to BW were more prominent in *O. niloticus* than in *O. mossambicus* (Figs. 4B and 4D).

3.5. Effects of extracellular osmolality on PRL₁₈₈ and PRL₁₇₇ release from dispersed PRL cells

The release of PRL₁₈₈ from dispersed PRL cells of both *O. niloticus* and *O. mossambicus* was negatively correlated with medium osmolality; the slope of the regression line was significantly steeper in *O. mossambicus* (slope = -0.005748 ± 0.0007911 , $R^2 = 0.5880$) versus *O. niloticus* (slope = -0.001463 ± 0.0004404 , $R^2 = 0.2396$) (Fig. 5A). A similar negative correlation was also detected between extracellular osmolality and PRL₁₇₇ release from the dispersed PRL cells of *O. mossambicus* (slope = -0.006009 ± 0.0008236 , $R^2 = 0.5899$), but not from PRL cells of *O. niloticus* (Fig. 5B).

3.6. Effects of extracellular osmolality on *prlr1* and *prlr2* mRNA expression in dispersed PRL cells

The expression of *prlr1* mRNA in dispersed PRL cells was positively correlated with medium osmolality in both *O. niloticus* and *O. mossambicus* (Fig. 5C). *prlr2* mRNA levels in both species were also positively correlated with extracellular osmolality (Fig. 5D), but were better fitted by exponential curves ($y = y_0 e^{kx}$) than by a linear function. In both cases, hyperosmotically-induced stimulations of *prlr* genes were more prominent in *O. niloticus*, especially at the highest osmolality tested (420 mOsm/kg), yielding a significantly steeper regression line or curve (slope = 0.007438 ± 0.0008627 , $R^2 = 0.6676$ for *prlr1*; $k = 0.01524$, $R^2 = 0.8576$ for *prlr2*) than those in *O. mossambicus* (slope = 0.003042 ± 0.0002771 , $R^2 = 0.7699$ for *prlr1*; $k = 0.009072$, $R^2 = 0.9701$ for *prlr2*) (Figs. 5C and 5D).

4. Discussion

In the present study, we compared Nile and Mozambique tilapia with respect to their salinity tolerances and associated endocrine responses. Specifically, we investigated: 1) changes in plasma osmolality, circulating PRLs, and pituitary *prl* mRNA expression following transfer from FW to BW; 2) changes in PRL release from dispersed PRL cells in response to a range of extracellular osmolalities *in vitro*; and 3) changes in the mRNA levels of *prlrs* from pituitary and gill, *in vivo*, and from dispersed PRL cells, *in vitro*. By characterizing these parameters, it is apparent that these two species, which differ markedly in their salinity tolerance, respond differently with respect to the regulation of PRL isoforms and their receptors when exposed to comparable changes in extracellular osmolality *in vitro* and *in vivo*.

Under steady-state FW conditions, plasma osmolality of *O. mossambicus* (306-348 mOsm/kg) is significantly higher than that of *O. niloticus* (279-326 mOsm/kg), which is consistent with a previous report (Velan et al., 2011). Upon transfer to BW (20‰), plasma osmolality of *O. mossambicus* did not exceed 400 mOsm/kg. In previous reports, where *O.*

mossambicus were transferred to even higher salinities than the present study, plasma osmolality transiently rose to ~450 mOsm/kg and returned to below 400 mOsm/kg by 24 h and thereafter (Breves et al., 2010a; Seale et al., 2012a; Yada et al., 1994). For *O. niloticus*, on the other hand, plasma equilibrated with external osmolality (600 mOsm/kg) by 24 h (Fig. 1). In another study, plasma osmolalities of both *O. mossambicus* and *O. niloticus* were similar following transfer from FW to BW, peaking at 400 mOsm/kg by 24 h and stabilizing at 350 mOsm/kg over the course of 1 week (Velan et al., 2011). The BW salinity employed in that study, however, was 15‰, lower than that employed in the present study (20‰) or in most acclimation studies involving *O. mossambicus*. The BW salinity we employed in the current study is closer to the threshold of salinity tolerance of Nile tilapia, and is therefore better suited for comparing their osmoregulatory physiologies.

Since the landmark demonstration that PRL replacement enables hypophysectomized killfish to survive in FW (Pickford and Phillips, 1959), PRL has been shown to be critical in supporting hydromineral balance in various teleosts inhabiting FW. Interestingly, Griffith reported differences in the survival of 23 species of the genus *Fundulus* in FW following hypophysectomy (Griffith, 1974b). The survival time seemed to be related to their native environmental salinity, suggesting that, upon hyposmotic challenge, the reliance on pituitary control of osmoregulation was less critical in FW species relative to their congeners that were naturally subject to variations in salinity. In anadromous fishes, such as salmonids and ayu (*Plecoglossus altivelis*), PRL seems to be involved in their migration from FW to SW and vice versa (Onuma et al., 2003; Taniyama et al., 1999; Yada et al., 2010; Yada et al., 2014), but of limited importance in FW osmoregulation (Makino et al., 2007; Manzon, 2002). Two PRL isoforms have been reported in tilapias (Specker et al., 1985), chum salmon (*Oncorhynchus keta*; Yasuda et al., 1986), common carp (*Cyprinus carpio*; Yasuda et al., 1987) and Japanese eel (*Anguilla japonica*; Suzuki et al., 1991). While the two PRLs in non-tilapiine species are highly homologous, tilapia PRL₁₈₈ and PRL₁₇₇ differ significantly in molecular weight and amino acid sequence (Yamaguchi et al., 1988). Hence, differences in the osmoregulatory roles of PRLs in different species may be tied to the variations in PRL structures. When transferred from FW to hyperosmotic environments, such as BW and SW, Mozambique tilapia suppress circulating PRLs, as well as *prl*_{188/177} mRNA expression in the pituitary (Breves et al., 2010a, Seale et al., 2012a, 2002; Yada et al., 1994). Nile tilapia similarly exhibit lower circulating PRL₁₈₈ and PRL₁₇₇ levels after long term acclimation in BW (3-4 weeks in 50‰ SW; Ayson et al., 1993). Velan and colleagues reported (2011) that *prl*₁₈₈ mRNA expression is significantly suppressed in the pituitary of both *O. niloticus* and *O. mossambicus*, by 6 and 24 h after transfer from FW to BW (15‰), respectively. Our current *in vivo* results are in strong agreement with these previous studies, confirming that PRL signaling in *O. niloticus* and *O. mossambicus* is suppressed by increases in salinity via transcriptional and post-transcriptional processes. It is important to note, however, that by 6 h of transfer from FW to BW, the magnitude of plasma PRL₁₇₇ suppression in *O. niloticus* was ~5 times greater than that of *O. mossambicus* (Fig. 2B). This difference may play a role in the ability of Nile tilapia to tolerate increases in salinity, inasmuch as an increase in PRL₁₇₇ levels relative to those of PRL₁₈₈ are associated with SW acclimation, as previously suggested in Mozambique tilapia (Borski et al., 1992; Seale et al., 2012a).

Our *in vitro* experiments indicate that pituitary PRL cells of *O. niloticus* are less responsive to changes in extracellular osmolality than those of *O. mossambicus*. In PRL cells of *O. mossambicus*, a decrease in extracellular osmolality triggers an increase in cell volume which is followed by the activation of the stretch-gated Ca^{2+} channel, transient receptor potential vanilloid 4, resulting in the elevation of intracellular Ca^{2+} concentration and ultimately the release of PRLs (Seale et al., 2005, 2003a, 2003b; Watanabe et al., 2012; Weber et al., 2004). While the PRL cells of *O. niloticus* may be equipped with similar osmosensing machinery, the effects of osmotic stimuli on PRL₁₈₈ release were rather muted in *O. niloticus* compared with *O. mossambicus* (Fig. 5A). Furthermore, the release of PRL₁₇₇ from dispersed PRL cells of *O. niloticus* was unaffected by medium osmolality, further indicating a difference in osmosensitivity between the two species (Fig. 5B). Considering that the plasma osmolality of FW-acclimated *O. niloticus* is lower than that of *O. mossambicus* reared in the same environment (Fig. 1), the differential osmosensitivity of PRL cells observed *in vitro* suggests that the physiologically responsive range of *O. niloticus* PRL cells is shifted to lower osmolalities relative to that of *O. mossambicus* cells. These findings may be reflective of salinity variations in the native environments of both species.

Besides the regulation of transcription and release of PRLs, the dynamic expression of PRLRs is also an important aspect of PRL signaling. Following the molecular characterization of two PRLRs in tilapia (Sandra et al., 1995; Huang et al., 2007; Fiol et al., 2009), these receptors have been suggested to be distinct in their function. When over-expressed in HEK293 cells, tilapia PRLR1 and PRLR2 trigger distinct signaling pathways in response to PRL₁₈₈ and PRL₁₇₇. Moreover, PRLR2 significantly increased tolerance to hyperosmotic stress of HEK293 cells (Fiol et al., 2009). With a focus on discerning whether PRLR isoforms play distinct physiological roles in the gill and pituitary, we have investigated how each receptor isoform responds to osmotic challenges in *O. mossambicus* in both *in vivo* and *in vitro* settings (Breves et al., 2011, 2010a, 2010b; Inokuchi et al., 2015; Seale et al., 2012a). The mRNA levels of *prlr2*, but not *prlr1*, are upregulated in the RPD upon transfer of intact animals from FW to SW (Seale et al., 2012a). Following a similar trend, *in vitro* incubations of dispersed PRL cells (Seale et al., 2012a) and gill filaments (Inokuchi et al., 2015) revealed that *prlr2* expression positively correlates with medium osmolality. Despite the established roles of PRL in FW acclimation of teleosts, these observations suggest that PRLR2 facilitates acclimation responses to increased extracellular osmolality. Here, we demonstrated that hyperosmotically-induced gene expression of *prlr2* is not only present in *O. niloticus*, but is more pronounced than in *O. mossambicus*. While the greater stimulation of branchial and pituitary *prlr2* expression in *O. niloticus in vivo* is likely attributable to a higher plasma osmolality in BW (Figs. 4B and D), our *in vitro* experiments revealed that hyperosmotically-induced expression of *prlr2* in dispersed PRL cells is fitted by a steeper regression curve in *O. niloticus* than in *O. mossambicus*, with the most prominent difference at the highest osmolality tested (420 mOsm/kg; Fig. 5D). Thus, it is possible that differences in osmosensitivity contribute to the observed interspecific variation in the expression of *prlr2*. By contrast, branchial *prlr1* expression is generally inversely correlated with environmental osmolality (Breves et al., 2011, 2010a, 2010b; Pierce et al., 2007; Sandra et al., 1995). In dispersed PRL cells and gill filaments *in vitro*, *prlr1* expression was not affected by extracellular osmolality (Inokuchi et al., 2015; Seale et al.,

2012a). In the present study, osmotic stimulation elicited limited responses in *prlr1* expression in both species, especially when compared with the responses of *prlr2*. Hence, these results suggest that the regulation of *prlr2* is more dependent on extracellular osmolality than *prlr1*.

It has been postulated that the wide salinity tolerance of Mozambique tilapia is attributable, at least in part, to the ability of its PRL cells to directly sense and respond to physiologically relevant changes in extracellular osmolality (cf. Seale et al., 2012b, 2006b). Our comparative approach suggests that, relative to Mozambique tilapia, the reduced ability of Nile tilapia to survive in hyperosmotic environments could be tied to: (1) diminished PRL cell osmosensitivity; (2) greater suppression of circulating PRL₁₇₇ upon a rise in environmental salinity; and concomitantly, (3) more robust upregulation of *prlr2*. Further studies are required to elucidate the underlying molecular mechanisms associated with the observed differences in PRL signaling (at both the pituitary and gill) between congeners, and how such differences contribute to the variation in osmoregulatory ability of tilapias. Furthermore, in addition to the osmotic regulation of PRL cells, the modulatory effects of various hypothalamic and extra-hypothalamic endocrine factors may differ between congeners and form the basis of future lines of investigation (see Seale et al., 2013). In addition, it is noteworthy that hybrids of *O. mossambicus* and *O. niloticus* demonstrate intermediate tolerances to hyperosmotic salinities compared with their parental lineages (Mateo et al., 2004). This implies that the salinity tolerance of tilapia is dictated, at least in part, by heritable genetic factors. Future studies employing comparative genomic approaches will provide insights into physiological mechanisms that mediate a variety of salinity acclimation strategies in teleosts.

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Nile tilapia is less tolerant to hyperosmotic stress relative to Mozambique tilapia.
Prolactin (PRL) cells of the two tilapia species are differently osmosensitive.
The two tilapia species are distinct in the regulation of circulating PRL₁₇₇.
Gene expression of PRL receptor 2 (PRLR2) was also distinct between species.
Distinct salinity tolerance of tilapias may be tied to regulation of PRLs and PRLRs.

Figure 1.

Plasma osmolalities at 0, 6 and 24 h after transfer from FW to FW (open symbols) or BW (20‰; filled symbols). Circles connected with a solid line depict *O. niloticus*, and triangles connected with a broken line depict *O. mossambicus*. Values represent means $\pm$ S.E.M ($n=8$). *** Significantly different from 0 h at $P < 0.001$. ††† Significantly different from FW at $P < 0.001$, within each species. §§§ Significantly different from time-matched *O. niloticus* counterparts at $P < 0.05$, 0.01 and 0.001, respectively. Three-way ANOVA followed by Fisher's LSD test.

Figure 2.

Plasma PRL₁₈₈ (A) and PRL₁₇₇ (B) at 0, 6 and 24 h after transfer from FW to FW (open symbols) or BW (20‰; filled symbols). Circles connected with a solid line depict *O. niloticus*, and triangles connected with a broken line depict *O. mossambicus*. Values represent means $\pm$ S.E.M ($n=8$). *Significantly different from 0 h at $P < 0.05$. $\dagger$ Significantly different from FW at $P < 0.05$, within each species. $\S\S\S$ Significantly different from time-matched *O. niloticus* counterparts at $P < 0.05$ and 0.01 , respectively. Three-way ANOVA followed by Fisher's LSD test.

Figure 3.

Pituitary gene expression of *prl188* (A) and *prl177* (B) at 0, 6 and 24 h after transfer from FW to FW (solid bars) or BW (20%; hatched bars). Open-solid and open-hatched bars depict *O. niloticus*, while gray-solid and gray-hatched bars depict *O. mossambicus*. Data are normalized by *ef1a* and shown as fold-changes from FW groups at 0 h (solid bars at 0 h), within each species. Values represent means $\pm$ S.E.M ($n=8$). *, **, ***Significantly different from 0 h at $P < 0.05$, 0.01 and 0.001, respectively. †, ††, †††Significantly different from FW at $P < 0.05$, 0.01 and 0.001, respectively, within each species. §, §§, §§§Significantly different from time-matched *O. niloticus* counterparts at $P < 0.05$ and 0.01, respectively. Three-way ANOVA followed by Fisher's LSD test.

Figure 4.

Pituitary and branchial gene expression of *prlr1* and *prlr2* at 0, 6 and 24 h after transfer from FW to FW (solid bars) or BW (20%; hatched bars). Pituitary *prlr1* (A) and *prlr2* (B) mRNA levels. Branchial *prlr1* (C) and *prlr2* (D) mRNA levels. Open-solid and open-hatched bars depict *O. niloticus*, while gray-solid and gray-hatched bars depict *O. mossambicus*. Data are normalized by *ef1a* and shown as fold-changes from FW groups at 0 h (solid bars at 0 h), within each species. *, **, *** Significantly different from 0 h at $P < 0.05$, 0.01 and 0.001, respectively. †, ††, ††† Significantly different from FW at $P < 0.05$, 0.01 and 0.001, respectively, within each species. §, §§, §§§ Significantly different from time-matched *O. niloticus* counterparts at $P < 0.05$ and 0.01, respectively. Three-way ANOVA followed by Fisher's LSD test.

Fig. 5.

PRL₁₈₈ (A) and PRL₁₇₇ (B) release and *prlr1* (C) and *prlr2* (D) gene expression in dispersed PRL cells exposed to different medium osmolalities for 6 h. Data are expressed as fold-changes from 330 mOsm/kg (symbols for *O. niloticus* and *O. mossambicus* at 330 mOsm/kg overlap each other) and fitted by either linear (A-C) or exponential (D) regressions. Each data point represents mean $\pm$ S.E.M ($n=8$). Open circles and solid lines depict *O. niloticus*, and open triangles and broken lines depict *O. mossambicus*. **,*** Significant regression at $P < 0.01$ and 0.001 , respectively. ††† Significant difference in regression slopes/curves from those of *O. niloticus* at $P < 0.001$, respectively. (A-C, ANCOVA; D, F test)

Table 1

List of qRT-PCR primers.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
EF1 α	AGCAAGTACTACGTGACCATCATTG	AGTCAGCCTGGGAGGTACCA	Breves et al., 2010c
PRL ₁₈₈	GGCCACTCCCCATGTTTAAA	GGCATAATCCCAGGAGGAGAC	Magdeldin et al., 2007
PRL ₁₇₇	TGGTTTGGCTCTTTTAACACAGTG	AGACAATGAGGAGTCACAGAGATTTTAC	Magdeldin et al., 2007
PRLR1	TGGGTCAGCTACAACATCACTGT	GGATGGGGCTTGACAATGTAGA	Pierce et al., 2007
PRLR2	GCCCTTGGGAATACATCTTCAG	GTGCATAGGGCTTCACAATGTC	Breves et al., 2010d