Hormonal regulation of aquaporin 3: opposing actions of prolactin and cortisol in tilapia gill

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Abstract

Aquaporins (Aqps) are expressed within key osmoregulatory tissues where they mediate the movement of water and selected solutes across cell membranes. We leveraged the functional plasticity of Mozambique tilapia (*Oreochromis mossambicus*) gill epithelium to examine how Aqp3, an aquaglyceroporin, is regulated in response to osmoregulatory demands. Particular attention was paid to the actions of critical osmoregulatory hormones, namely, prolactin (Prl), growth hormone and cortisol. Branchial *aqp3* mRNA levels were modulated following changes in environmental salinity, with enhanced *aqp3* mRNA expression upon transfer from seawater to freshwater (FW). Accordingly, extensive Aqp3 immunoreactivity was localized to cell membranes of branchial epithelium in FW-acclimated animals. Upon transferring hypophysectomized tilapia to FW, we identified that a pituitary factor(s) is required for Aqp3 expression in FW. Replacement with ovine Prl (oPrl) was sufficient to stimulate Aqp3 expression in hypophysectomized animals held in FW, an effect blocked by coinjection with cortisol. Both oPrl and native tilapia Prls (tPrl₁₇₇ and tPrl₁₈₈) stimulated *aqp3* in incubated gill filaments in a concentration-related manner. Consistent with *in vivo* responses, coincubation with cortisol blocked oPrl-stimulated *aqp3* expression *in vitro*. Our data indicate that Prl and cortisol act directly upon branchial epithelium to regulate Aqp3 in tilapia. Thus, within the context of the diverse actions of Prl on hydromineral balance in vertebrates, we define a new role for Prl as a regulator of Aqp expression.

Key Words

- ▶ aquaporin
- ▶ prolactin
- ▶ cortisol
- ▶ gill
- ▶ osmoregulation

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Introduction

Aquaporin (Aqp) proteins are important mediators of transepithelial fluid transport and cell volume regulation in vertebrate tissues (Boury-Jamot *et al.* 2006, Verkman 2012). Spanning the plasma membrane, Aqps facilitate passive movements of water and, in some cases, small

nonionic compounds. Diverse activities, such as cell migration, neural signaling, fat metabolism and osmotic balance, rely upon the regulated movement of water and solutes via Aqps. Accordingly, misregulated or genetic interruption of Aqp function underlies a host of

disease states such as polyuria, edema, cataracts, diabetes insipidus, and obesity (Verkman 2012, Papadopoulos & Verkman 2013). A nuanced understanding of how Aqps underlie both physiological and disease states requires varied experimental approaches and model systems that reflect the diverse roles of Aqps in vertebrate systems.

Owing to the osmoregulatory demands associated with living in aqueous environments, teleost fishes are intensely studied to build the mechanistic models of how vertebrate ion-transporting epithelia are regulated. A limited subset (<10%) of teleosts (Schultz & McCormick 2013) has evolved the capacity to thrive in environments ranging from freshwater (FW) to seawater (SW) by efficiently modulating effectors of ion and water transport in the gill, kidney, gut, and urinary bladder (Marshall & Grossell 2006). The evolved capacities of these 'euryhaline' fishes to tolerate disparate salinities necessitate that epithelial surfaces in direct contact with external conditions possess sensory systems that detect changes in ambient osmolality (Fiol & Kültz 2007). These osmosensory systems couple with regulatory systems that both protect cellular homeostasis and modulate the expression of ion transport effectors responsible for maintaining homeostasis of the organism (Fiol & Kültz 2007, Tse *et al.* 2007).

The vertebrate Aqp superfamily consists of 17 classes of Aqps. Teleosts (e.g., Atlantic salmon (*Salmo salar*)) may express up to 42 paralogs (Finn *et al.* 2014). Multiple teleost Aqps are expressed in osmoregulatory epithelia; for example, Aqp3, an aquaglyceroporin, which transports water, glycerol, and urea, has been studied in a suite of euryhaline teleosts with particular attention to its expression following changes in salinity. In surveyed species, branchial *aqp3* gene expression is markedly elevated in FW- versus SW-acclimated animals (Cutler & Cramb 2002, Lignot *et al.* 2002, Tse *et al.* 2006, Tipsmark *et al.* 2010, Whitehead *et al.* 2010, Jung *et al.* 2012, Madsen *et al.* 2014, Moorman *et al.* 2015). Although the functional significance of plastic *aqp3* expression in the gill stands unresolved, regulated transepithelial water movement, via Aqp3, appears unlikely on the basis that branchial water exchange is disadvantageous for fish in both FW and SW environments (Cerdà & Finn 2010). Alternatively, the roles for Aqp3 in mediating regulatory cell volume and/or autonomous osmosensing capacities of the gill have been proposed (Watanabe *et al.* 2005, Cutler *et al.* 2007, Madsen *et al.* 2015). With salinity-dependent patterns of *aqp3* expression clearly evident from studying fishes in a comparative fashion, there remains little knowledge of how Aqp3 is actually regulated. In mammalian epidermis,

Aqp3 is expressed extensively in the plasma membrane of keratinocytes where it mediates water and glycerol transport supporting skin hydration/elasticity (Boury-Jamot *et al.* 2006). Although the extent of hormonal control of Aqp3 in mammalian epidermis is similarly unresolved, it is noteworthy that both tissues operate as barriers between the organism and the environment.

The endocrine system coordinates the processes supporting hydromineral balance by orchestrating the expression, localization, and activities of subcellular effectors of solute and water transport. These effectors include ion channels, cotransporters, exchangers, Na⁺/K⁺-ATPases, and Aqps (Kaneko *et al.* 2008, Konno *et al.* 2010). Decades of comparative study have revealed that a conserved function for prolactin (Prl) across vertebrates is the regulation of ion and water transport (Bole-Feysot *et al.* 1998). In teleosts, Prl promotes phenotypes associated with FW acclimation, whereas growth hormone (Gh) supports phenotypes associated with SW acclimation (Hirano 1986, McCormick 2001). Cortisol, under the control of adrenocorticotrophic hormone, can seemingly support both FW and SW acclimation (McCormick 2001). The dynamics of plasma Prl, Gh, and cortisol in response to changes in salinity are in strong agreement with their specific actions in coordinating physiological responses to FW and/or SW (Yada *et al.* 1994, Seale *et al.* 2002, Kajimura *et al.* 2004). In light of the scant knowledge of how Aqp3 is regulated by endocrines, Prl, Gh, and cortisol represent plausible regulators of branchial Aqp3 in fishes.

Here, we employed hypophysectomy (Hx), and subsequent hormone replacement, to identify whether systemic hormones underlie the contrasting *aqp3* gene and Aqp3 protein expression patterns observed between SW- and FW-acclimated Mozambique tilapia, *Oreochromis mossambicus* (Peters 1852). Upon finding the opposing effects of Prl and cortisol on Aqp3 *in vivo*, we incubated gill filaments to test whether Prl and cortisol have the capacity to regulate *aqp3* in a gill-autonomous fashion. Our collective findings provide the first evidence that Prl directs the expression of an Aqp in a vertebrate, and moreover, that cortisol has the capacity to block this action.

Materials and methods

Experimental animals

Male tilapia (*O. mossambicus*; 50–125 g) were selected from stocks maintained at the Hawai'i Institute of Marine Biology. Fish were maintained outdoors with a continuous

flow of FW (municipal water; 1.05 mM Na⁺, 0.55 mM Ca²⁺, 0.03 mM K⁺, 0.60 mM Mg²⁺, conductivity=322 µS/cm) under natural photoperiod and fed a commercial diet (Classic Trout, Skretting, Tooele, UT, USA; 40% crude protein, 12% crude fat, 3% crude fiber, ≤12% ash, ≤2% sodium) twice a day. Water temperatures were maintained between 24 and 26°C. The Institutional Animal Care and Use Committee of the University of Hawai'i approved all housing, surgical, and experimental protocols.

Experiment 1: effect of salinity on *aqp3* gene expression and immunoreactivity

To assess the expression of *aqp3* mRNA in FW- vs SW-acclimated animals, we sampled gill filaments from animals acclimated to either FW or SW (34‰, 482 mM Na⁺, 545 mM Cl⁻, 10.7 mM Ca²⁺, 7.46 mM K⁺, 52.6 mM Mg²⁺, conductivity=51 mS/cm, Kane'ohe Bay, Hawai'i, USA) for over 6 months. At sampling, all fish were lethally anesthetized in 2-phenoxyethanol (2-PE; 0.3 mL/L, Sigma), and 15–20 filaments were excised from the first gill arch, frozen in liquid nitrogen, and stored at –80°C until RNA isolation. For whole-mount immunohistochemistry (IHC), the second gill arch was fixed for 24 h in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) at 4°C and subsequently stored in 70% EtOH. Four tanks (500 L; two SW and two FW) were employed for a pair of salinity transfer experiments. One SW tank was transitioned to FW by opening a FW valve and closing the SW valve; one FW tank was transitioned to SW by opening a SW valve and closing the FW valve. Transitions between salinities were completed within 1 h. At 6 and 24 h after the initial valve switching, fish ($n=6-10$) were netted, lethally anesthetized, and sampled for gill filaments. Nontransferred controls were also sampled from the other two tanks at 6 and 24 h.

Experiment 2: effect of Hx on *Aqp3* expression

Hx was performed by the transorbital technique developed by Nishioka (1994). First, tilapia were anesthetized by immersion in 2-PE (0.3 mL/L) in FW. Following removal of the right eye and underlying tissue, a hole was drilled through the neurocranium, and the pituitary was aspirated with a modified Pasteur pipette. The orbit was then packed with a microfibrillar collagen hemostat (Ethicon, Somerville, NJ, USA), and fish were allowed to recover in brackish water (BW; 12‰). During the recovery period, the aspirated tissue was inspected under a stereo microscope. Hypophysectomized animals

were not included in the experiment if a fully intact pituitary was not overtly evident. The success rate for these surgeries (removal of fully intact pituitaries) was >95%. Following recovery, fish were transferred to recirculating experimental aquaria containing aerated BW and treated with kanamycin sulfate (National Fish Pharmaceuticals, Tucson, AZ, USA). Sham operations were carried out in the same manner, but without aspiration of the pituitary.

In a pair of experiments, intact, sham-operated, and Hx tilapia ($n=5-11$) were acclimated to SW or FW for 3 days. Following a 5–7-day postoperative period in BW (12‰), salinity transfers were conducted by adding SW or FW directly to recirculating aquaria. In the first group, SW was gradually added to the aquaria to raise the salinity to 23‰ after 90 min. After 24 h, SW was added again to raise the salinity to full-strength SW (34‰) for the remainder of the experiment. In the second group, FW conditions were reached after 90 min and maintained for the duration of the experiment. Fish were not fed for the duration of the recovery and transfer periods. At sampling, all fish were lethally anesthetized, rapidly decapitated, and checked for completeness of Hx by postmortem inspection of the hypothalamic region under a stereo microscope. Gill filaments were collected as described previously for gene expression analyses and whole-mount IHC.

Experiment 3: effect of hormones on *Aqp3* expression in hypophysectomized tilapia

In the next set of experiments, we investigated the effects of Prl, Gh, and cortisol on *Aqp3* in Hx animals in FW and SW. Following a 3-day postoperative period in BW (12‰), fish ($n=5-11$) were administered ovine Prl (oPrl; 5 µg/g body weight), ovine Gh (oGh; 5 µg/g) or cortisol (1 µg/g), alone or in combination, by intraperitoneal injection concurrently with FW or SW acclimation. Hormone doses were selected based on previous experiments employing intraperitoneal injection in teleosts (Herndon *et al.* 1991, Eckert *et al.* 2001, Jackson *et al.* 2005, Breves *et al.* 2010c, 2014b). oPrl and cortisol (hydrocortisone) were purchased from Sigma; oGh was obtained from the National Hormone and Peptide Program (NIDDK-oGh-15, Torrance, CA, USA). The salinity transfers (FW and SW acclimation) were conducted in the same manner as in the preceding Hx experiment. Hormones were delivered in saline vehicle (0.9% NaCl; 1.0 µL/g body weight). Cortisol was initially dissolved in ethanol and then added to

appropriate injection mixtures to achieve the desired concentrations. Ethanol (without cortisol) was added to all other injection mixtures at an equal dilution. The first injection occurred immediately before transfer to either FW or BW. Twenty-four hours later, fish were netted and given a second injection. Fish were then returned to aquaria and left undisturbed for 48 h, after which time they were sampled as described previously. Sham-operated fish were injected with saline vehicle only. Intact fish were not included in the hormone replacement experiments because there were no significant differences between intact and sham-operated fish in the preceding experiment.

Experiment 4: effects of oPrl, tPrl₁₇₇, tPrl₁₈₈ and cortisol on *aqp3* expression in incubated gill filaments

To identify the direct effects of Prl and cortisol on branchial *aqp3* levels, we incubated filaments from the second and third gill arches following [Watanabe and coworkers \(2016\)](#). Filaments were collected from FW-acclimated tilapia. Briefly, individual filaments were cut from branchial arches, cut sagittally under a dissecting microscope, and then placed in 24-well plates (Becton Dickinson, Franklin Lake, NJ, USA) containing Leibovitz's L-15 medium (Life Technologies) supplemented with 5.99 mg/L penicillin and 100 mg/L streptomycin (Sigma). The medium was adjusted to 330 mOsm/kg by adding 5 mol/L NaCl solution. Two filaments were placed in each well, which contained 500 µL incubation medium supplemented with 0, 0.1, 1, 5, and 10 µg/mL oPrl or 0, 0.01, 0.1, 1.0 and 10 µg/mL cortisol (Sigma) ($n=6-8$). In a subsequent incubation, filaments were treated with oPrl (5 µg/mL), cortisol (1 µg/mL), or the combination of both hormones ($n=6-8$). Finally, we incubated filaments in medium supplemented with tPrl₁₇₇ or tPrl₁₈₈ at 0, 0.01, 0.1, 0.5, and 1.0 µg/mL ($n=8$). oPrl concentrations were selected following [Inokuchi and coworkers \(2015\)](#); tPrl concentrations were selected on the basis that tPrl₁₇₇ and tPrl₁₈₈ levels range between 0 and 90 and 0 and 35 ng/mL, respectively ([Breves et al. 2011](#)). Cortisol concentrations were selected based upon previous experiments employing gill block incubation ([Kiilerich et al. 2007](#)). tPrIs were purified from media following pituitary tissue incubation ([Breves et al. 2014b](#)). All incubations were maintained for 8 h at 26°C; incubations were terminated by collecting the filaments in TRI Reagent (MRC, Cincinnati, OH, USA) for RNA isolation.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated from gill filaments with TRI Reagent according to the manufacturer's protocol. RNA concentration and purity were assessed by spectrophotometric absorbance (Nanodrop 1000, Thermo Scientific). The first-strand cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Relative levels of mRNA were determined by quantitative real-time PCR (qPCR) using the StepOnePlus Real-Time PCR System (Life Technologies). *aqp3*, *ef1α* and *gapdh* primer sequences and cycling parameters are described by [Watanabe and coworkers \(2005\)](#), [Breves and coworkers \(2010b\)](#), and [Tang and Lee \(2010\)](#), respectively. The mRNA levels of normalization (*ef1α* or *gapdh*) and target (*aqp3*) genes were determined by the relative quantification method as specified by StepOne Software v2.0 (Life Technologies) after verification that normalization genes did not vary across treatments. Standard curves were prepared from serial dilutions of untreated gill cDNA and included on each plate to calculate PCR efficiencies. Relative gene expression ratios between treated and control groups are reported as a fold change from controls. Reverse transcription negative control reactions confirmed the absence of contaminating genomic DNA.

Whole-mount immunofluorescence staining

A polyclonal antibody was used for the detection of tilapia Aqp3 that recognizes amino acids 290–303; the production of anti-tilapia Aqp3 has been described previously ([Watanabe et al. 2009](#)). Fixed samples were rehydrated in PB with 0.2% Triton-X 100 (PBST) and immersed in blocking buffer (PBST containing 10% normal goat serum, 0.1% bovine serum albumin, 0.02% keyhole limpet hemocyanin, and 0.01% sodium azide). After blocking, each specimen was incubated at 4°C overnight with anti-Aqp3 (1:1000) diluted with blocking buffer. After several washes with PBST, samples were incubated overnight at 4°C with goat anti-rabbit IgG labeled with Alexa Fluor 488 (Invitrogen) diluted 1:500. After rinsing with PBST, the specimens were observed under a confocal laser scanning microscope (LSM 710, Zeiss). The afferent-vascular edges of gill filaments were examined for a minimum of four individuals per treatment. For localization of Na⁺/Cl⁻ cotransporter (Ncc) in incubated filaments, a T4 monoclonal antibody (developed by Christian Lytle and Bliss Forbush III, and obtained from the Developmental Studies Hybridoma

Bank, Iowa City, IA, USA) was used as described previously (Lytle *et al.* 1995, Inokuchi *et al.* 2008).

Western blotting analysis

Gill filaments collected from FW-acclimated tilapia were lysed in 0.5% SDS and 50mM sodium phosphate (pH 7.5) by sonication. After centrifugation at 10,000g and 4°C for 10min, the supernatant was collected. About 20µL of

this sample was used for BCA assay (Thermo Scientific) to determine protein concentration. Dithiothreitol was added to the sample (final concentration: 40mM) and proteins were denatured at 100°C for 10min in a water bath and then chilled on ice for 5min. The resulting sample was separated by SDS-PAGE at 200V for 50min. After separation, the proteins were transferred to a PVDF membrane with CAPS transfer buffer (10mM CAPS, pH 11). The PVDF membrane was immersed in blocking buffer

Figure 1

aqp3 expression in SW- vs FW-acclimated animals (A) and following the transfer from SW to FW (B), or FW to SW (C). Data are presented as mean ± s.e.m. Sample size is indicated within parentheses. **Significant difference from time-matched controls at $P < 0.01$ by Mann-Whitney *U* test. Immunofluorescent staining for Aqp3 in the afferent-vascular edges of gill filaments (D, arrowhead) of tilapia acclimated to SW (E) or FW (F). Scale bar = 50µm. Cross-sectional view of FW filament with Aqp3 staining (G) and by DIC (H). Arrowhead = ionocyte, arrow = mucous cell, asterisk = basement membrane. Scale bar = 10µm.

(5% skimmed milk in 20mM Tris-buffered saline with 0.1% Tween-20 (TBST)) for 30min at room temperature, and then incubated overnight at 4°C with anti-tilapia Aqp3 diluted 1:4000 with blocking buffer. After several washes with TBST, the membrane was incubated with HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) diluted 1:5000 with blocking buffer for 3h at room temperature. The immunopositive band was detected using a chemiluminescence method with EzWestLumi One (Atto, Tokyo, Japan).

Statistical analyses

For experiment 1, *aqp3* expression in SW- versus FW-acclimated animals was analyzed by Mann–Whitney *U* test. Transfer experiments were analyzed by two-way analysis of variance (ANOVA) with treatment and time as main effects. When a significant effect of treatment or an interaction between treatment and time was detected, a Mann–Whitney *U* test was employed at each time point. For experiments 2–4, group comparisons were performed by one-way ANOVA, followed by Tukey's HSD. When necessary (Shapiro–Wilk test), data were log transformed to meet assumptions of normality. When assumptions of homogeneity were not met (Bartlett's test), group comparisons were conducted by a Kruskal–Wallis test, followed by Dunn's test. All analyses were conducted using GraphPad Prism 5.0. The significance for all tests was set at $P < 0.05$.

Results

Experiment 1: effect of salinity on *aqp3* gene expression and immunoreactivity

aqp3 levels were roughly 20-fold higher in long-term FW- versus SW-acclimated tilapia (Fig. 1A). Following the transfer of tilapia from SW to FW or vice versa, *aqp3* levels were modulated in a fashion to reflect 'steady-state' expression patterns by 24h (Fig. 1B and C). In both transfer paradigms, there were significant treatment, time, and interaction effects. In the afferent-vascular edges of gill filaments (Fig. 1D), we observed a marked difference in the level of Aqp3 immunoreactivity in SW- versus FW-acclimated tilapia (Fig. 1E and F); Aqp3 immunoreactivity was localized to cell membranes of branchial epithelium in filaments collected from FW-acclimated animals (Fig. 1F). Cross-sectional views revealed Aqp3 immunoreactivity in various epithelial cells, including ionocytes, mucous (goblet) cells and pavement

Figure 2

Western blotting analyses with an antibody raised against Aqp3 amino acids 290–303. Gill filaments were collected from FW-acclimated tilapia and protein samples were separated by SDS-PAGE. Positions of molecular mass markers (kDa) are shown on the left. A single protein band with a molecular mass of ~30 kDa was detected. This ~30 kDa band corresponds to the predicted molecular mass of Aqp3 (33 kDa).

cells (Fig. 1G and H). Apical crypts characterize ionocytes, while mucous cells are larger and ovoid in shape (Wilson & Laurent 2002, Kaneko *et al.* 2008). Pavement cells are the most abundant cells in branchial epithelium, a pattern consistent with the extensive Aqp3 immunoreactivity observed in filaments collected from FW-acclimated fish (Fig. 1F) (Wilson & Laurent 2002). As specified previously, the first and second gill arches were sampled for gene and protein expression, respectively. Although we did not directly assess whether Aqp3 expression varied across arches, the strong agreement between Aqp3 gene and protein expression within a given salinity suggests that there were no overt differences in how Aqp3 was regulated across the sampled arches.

By Western blotting analysis, the Aqp3 antibody employed in this study recognized a single protein with a molecular mass of ~30 kDa (Fig. 2). As calculated from the translated amino acid composition of the *aqp3* open reading frame (ExPASy), the molecular mass of Aqp3 is 33 kDa.

Experiment 2: effect of Hx on Aqp3 expression

To identify whether the pituitary mediates salinity-dependent Aqp3 expression, we transferred Hx fish from

BW (12‰) to either SW or FW. In tilapia transferred to FW, Hx fish showed markedly reduced *aqp3* expression compared with intact and sham-operated controls (Fig. 3A). Expression of *aqp3* was considerably lower in all SW-treatment groups compared with intact and sham-operated fish in FW; there was no effect of Hx on *aqp3* expression in SW. In parallel with diminished *aqp3* levels in Hx animals in FW, we observed a clear reduction in Aqp3 immunoreactivity in branchial epithelium from Hx fish (Fig. 3D) compared with intact and sham-operated controls (Fig. 3B and C).

Experiment 3: effect of hormones on Aqp3 expression in hypophysectomized tilapia

Inasmuch as an intact pituitary was required for salinity-dependent Aqp3 expression, we tested whether hormones (Prl, Gh and cortisol) either secreted by or under the control of the pituitary regulate Aqp3. In SW, there were no effects of any hormone, administered either alone or in combination, on *aqp3* mRNA levels (Fig. 4A). In Hx animals in FW, oPrl stimulated *aqp3* levels by 8.6-fold from vehicle-injected controls; oGh did not affect *aqp3* levels. Although there was no effect of cortisol alone on *aqp3* levels, cortisol was sufficient to block the effect of oPrl on *aqp3* (Fig. 4B). Accordingly, Aqp3 immunoreactivity in the branchial epithelium of oPrl-injected animals was more intense than in vehicle-injected controls (Fig. 4C and D). In contrast with *aqp3* mRNA patterns, there was

only a modest reduction in Aqp3 immunoreactivity in animals injected with oPrl and cortisol compared with vehicle-injected controls (Fig. 4C and E).

Experiment 4: effects of oPrl, tPrl₁₇₇, tPrl₁₈₈, and cortisol on *aqp3* expression in incubated gill filaments

To assess whether Prl and cortisol regulate *aqp3* expression in a gill-autonomous fashion, we incubated gill filaments in the presence or absence of Prl and/or cortisol for 8 h. oPrl at 5 and 10 µg/mL stimulated the expression of *aqp3* by ~40-fold from controls (Fig. 5A); cortisol did not impact *aqp3* expression at any of the tested concentrations (Fig. 5B). In a subsequent incubation, we administered oPrl (5 µg/mL) alone or in combination with cortisol (1 µg/mL). Although the magnitude of response to oPrl was less robust in this subsequent experiment (~4-fold vs 40-fold), cotreatment with cortisol blocked the ability of oPrl to stimulate *aqp3* expression (Fig. 5C); this result was consistent with *in vivo* patterns (Fig. 4B). Mozambique tilapia secrete two Prl peptides from the pituitary, denoted tPrl₁₇₇ and tPrl₁₈₈ (Specker *et al.* 1985). We found that purified tPrl₁₇₇ and tPrl₁₈₈, compared with oPrl, stimulated *aqp3* at a lower concentration (0.5 and 1 vs 5 µg/mL) (Fig. 6).

In a separate experiment, we confirmed that our incubation techniques accurately model *in vivo* responses to oPrl by demonstrating an induction of Ncc immunoreactivity following 24 h of oPrl treatment

Figure 3

Effects of Hx on *aqp3* expression following transfer to SW or FW (A). Data are presented as mean $\pm$ s.e.m. Sample size is indicated within parentheses. Five days after Hx, intact (open bars), sham-operated (shaded bars), and Hx (solid bars) tilapia were transferred from BW (12‰) to FW or SW and sampled after 3 days. Within a given environmental salinity, means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD, $P < 0.05$). Immunofluorescent staining for Aqp3 in gill filaments of intact (B), sham-operated (C), and Hx tilapia (D) transferred to FW for 3 days. Scale bar = 50 µm.

Figure 4

Effects of oPrI, oGh, and cortisol on branchial *aqp3* expression in SW (A) and FW (B). Data are presented as mean $\pm$ S.E.M. Sample size is indicated within parentheses. Fish received two intraperitoneal injections of oPrI (5 µg/g body weight) or oGh (5 µg/g) alone or in combination with cortisol (1 µg/g). The first injection occurred immediately prior to transfer from BW (12‰) to SW or FW; the second occurred at 24 h. Fish were sampled 3 days after initial transfer to SW or FW. Hx fish receiving saline injections (open bars) served as controls. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD, $P < 0.05$). Immunofluorescent staining for Aqp3 in gill filaments of tilapia transferred to FW injected with saline vehicle (C), oPrI (D) or oPrI + cortisol (E). Scale bar = 50 µm.

(Fig. 7); Ncc is an established target of Prl (Breves *et al.* 2010c, 2013, Inokuchi *et al.* 2015).

Discussion

We hypothesized that Prl, Gh, and/or cortisol participate in the regulation of salinity-dependent Aqp3 expression in euryhaline tilapia. Through a combination of experimental approaches, including salinity transfer, Hx, hormone replacement and gill filament incubation, we provide evidence that Prl is both necessary and sufficient to promote Aqp3 expression within branchial epithelium. Moreover, we found that cortisol antagonizes the stimulatory effect of Prl on Aqp3 expression. We first discuss these regulatory patterns within the context of how plasma levels of Prl and cortisol vary during salinity acclimation of tilapia. We then consider the adaptive significance of these regulatory connections between Prl, cortisol and Aqp3.

We and others have shown that branchial *aqp3* expression in teleosts is rapidly modulated in response to changes in salinity, with elevated expression in FW versus SW (Cutler & Cramb 2002, Lignot *et al.* 2002, Tse *et al.* 2006, Tipsmark *et al.* 2010, Whitehead *et al.* 2010, Jung *et al.* 2012, Lam *et al.* 2014, Madsen *et al.* 2014, Moorman *et al.* 2015). Prl has long been recognized as “the FW-adapting hormone” in fishes based upon its capacity to support branchial processes essential to life in FW (Hirano 1986, McCormick 2001). Accordingly, euryhaline fishes exhibit

dramatic changes in circulating Prl in parallel with salinity acclimation, with an inverse relationship between plasma Prl levels and ambient salinity. In Mozambique tilapia, plasma Prl rose by 4–6 h after transfer from SW to FW (Yada *et al.* 1994, Seale *et al.* 2002, Kajimura *et al.* 2004, Breves *et al.* 2011). The latency of this Prl response to FW is consistent with the activation of *aqp3* sometimes between 6 and 24 h following transfer (Fig. 1B). Our Hx and hormone replacement data, however, provide the strongest evidence that circulating Prl directs Aqp3 expression in FW (Figs 3 and 4). Both here and previously (Breves *et al.* 2010a), we observed a sharp attenuation of *aqp3* expression following transfer from FW to SW. Recall that cortisol injected into oPrI-treated animals held in FW also attenuated *aqp3* expression (Fig. 4B). As plasma cortisol responds more rapidly than Prl to SW transfer (Kajimura *et al.* 2004), cortisol released during the acute phase of SW acclimation is likely overriding the effect of Prl levels that have yet to fall. Cutler and coworkers (2002) reported that gill *aqp3* was diminished in FW-acclimated eel infused with cortisol. Thus, inhibition of *aqp3* by cortisol may constitute an important, and conserved, facet of SW acclimation by preventing excessive water loss across branchial epithelium, which would further threaten hydromineral balance. Interestingly, there was no effect of oPrI on *aqp3a* in incubated zebrafish (*Danio rerio*) gill filaments (J P Breves, unpublished observations). As zebrafish are stenohaline, a Prl–*aqp3* regulatory connection may be restricted to euryhaline species routinely exposed to varying salinities. Clearly, a wider

Figure 5

Effects of oPrl (A) and cortisol (B) concentration on *aqp3* expression in gill filaments incubated for 8 h. Effect of cortisol on basal and oPrl-stimulated *aqp3* expression (C). Data are presented as mean $\pm$ s.e.m. Sample size is indicated within parentheses. *aqp3* expression is presented as a fold change from the zero concentration groups. Means not sharing the same letter are significantly different (one-way ANOVA or Kruskal-Wallis, Tukey's HSD or Dunn's test, $P < 0.05$).

Figure 6

Effects of native tPrl₁₇₇ (A) and tPrl₁₈₈ (B) concentration on *aqp3* expression in gill filaments incubated for 8 h. Data are presented as mean $\pm$ s.e.m. Sample size is indicated within parentheses. *aqp3* expression is presented as a fold change from the zero concentration groups. Means not sharing the same letter are significantly different (one-way ANOVA or Kruskal-Wallis, Tukey's HSD or Dunn's test, $P < 0.05$).

suite of teleosts must be surveyed to resolve the degree to which Prl and Aqp3 are linked, and how it relates to salinity tolerance.

The rapid attenuation of *aqp3* by SW, cortisol, and tidally driven changes in salinity suggests that Aqp3 function in the gill is maladaptive to life in SW (Cutler *et al.* 2007, Breves *et al.* 2010a, Moorman *et al.* 2015). Accordingly, we observed that ambient salinity had an 'overriding' effect on whether Prl stimulated *aqp3* (Fig. 4A and B). In other words, salinity-dependent systemic and/or

Figure 7

Immunofluorescent staining for Aqp3 (green) and Ncc (red) in gill filaments incubated for 24 h with control (A) or oPrl-supplemented (B) medium. Scale bar = 50 µm.

local factors seemingly modulate branchial responsiveness to Prl. Osmotic/ionic conditions (external and/or internal) are plausible modulators of Prl responsiveness. Thus, our future work will combine tPrl treatments with varying osmotic conditions *in vitro* to dissect the nature of these interactions. Moreover, we tested *in vitro* effects of Prl on filaments collected from FW-acclimated animals. In Atlantic salmon, *in vitro* hormone responsiveness (cortisol) was impacted by the salinity in which donor animals were maintained (Kiilerich *et al.* 2007). It will be interesting to learn whether filaments isolated from FW-acclimated tilapia are more responsive to Prl given their higher expression levels of Prl receptors compared with SW-acclimated tilapia (Breves *et al.* 2011).

The question of how Aqp3 underlies distinct branchial function(s) remains unanswered. Multiple investigators have approached this question by localizing

Aqp3 protein in gill tissue. In eel (*Anguilla anguilla*), killifish (*Fundulus heteroclitus*), and rainbow wrasse (*Coris julis*), Aqp3 immunoreactivity was observed in basolateral membrane of ionocytes (Lignot *et al.* 2002, Brunelli *et al.* 2010, Jung *et al.* 2012). Here, we observed extensive Aqp3 immunoreactivity in ionocytes, mucous cells, and pavement cells of the afferent-vascular edges of FW filaments (Fig. 1E, G and H). We propose that Aqp3 confers osmoreceptivity in these branchial cell types. Aqp3 plays a well-characterized, and essential, role in mediating osmoreception in Prl cells of the tilapia pituitary (Watanabe *et al.* 2009). For example, the inhibition of Aqp3-mediated water movement by mercury treatment abolished the osmotically driven cell volume increases that follow reductions in extracellular osmolality (Watanabe *et al.* 2009). These cell volume changes are essential to coupling extracellular conditions with appropriate Prl release via the stretch-activated Ca^{2+} channel, transient receptor potential vanilloid 4 (Seale *et al.* 2003, Watanabe *et al.* 2012). Therefore, if the presumed role of branchial Aqp3 is to facilitate changes in cell volume, as in the pituitary, then Prl may play a key role in supporting osmosensitivity in the gill via Aqp3. For example, we have recently characterized the *in vitro* capacity of tilapia ionocytes to modulate ion transporter expression in response to changes in medium osmolality. These modulations of gene expression occurred in response to plasma osmotic conditions routinely experienced during the acute phases of FW (~280 mOsm/kg) and SW (~450 mOsm/kg) acclimation (Inokuchi *et al.* 2015). Hyperosmotic conditions stimulate the gene expression of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter 1a (*nkcc1a*), a key cotransporter underlying ion extrusion by teleost ionocytes (Kaneko *et al.* 2008, Inokuchi *et al.* 2015), as well as volume regulation by pavement cells (Tse *et al.* 2007). Thus, enhanced *aqp3*/Aqp3 expression in the basolateral region of branchial cells in FW-acclimated tilapia (Fig. 1A and F) may render epithelial cells competent to rapidly activate *nkcc1a* expression in response to increases in plasma osmolality along the basolateral membrane. Our current observations do not exclude the possibility that Prl and cortisol regulate glycerol permeability in the gill via Aqp3, operating as an aquaglyceroporin. Unlike in murine skin where glycerol permeability (via Aqp3) was shown to play an important role in maintaining skin elasticity (Ma *et al.* 2002), the consequence(s) of glycerol accumulation/transport in the branchial epithelium of tropical fishes, such as tilapia, is unresolved.

Our *in vitro* results demonstrate that Prl and cortisol have the capacity to directly regulate *aqp3*

expression independent from additional systemic factors. Gill filament incubation is a practical approach to characterize branchial responses to hormones that do not require additional input from the intact animal. Depending upon how filaments are incubated, gill-autonomous functions are maintained for 1–4 days (Kiilerich *et al.* 2007, Breves *et al.* 2013, Watanabe *et al.* 2016). In tilapia, Ncc-expressing ionocytes (type II ionocytes) are key effectors of Cl⁻ uptake (Hiroi *et al.* 2008, Horng *et al.* 2009), and we have shown that Prl stimulates *ncc* expression and the recruitment of Ncc-expressing ionocytes *in vivo* (Breves *et al.* 2010c). Employing the presence of Ncc-expressing ionocytes as readout of Prl responsiveness (Breves *et al.* 2010c, Inokuchi *et al.* 2015), we confirmed the utility of the filament incubation system to model physiological responses to Prl (Fig. 7). In agreement with the direct effects that we observed, receptors for Prl and cortisol are highly expressed in tilapia gill (Dean *et al.* 2003, Fiol *et al.* 2009). We are now poised to leverage *in vitro* approaches to resolve how cortisol antagonizes Prl in the context of Aqp3 expression. We hypothesize that cortisol inhibits signal transduction pathways (JAK2/STAT, PI3K, MEK) demonstrated to underlie Prl-stimulated osmoregulatory pathways (Ferlazzo *et al.* 2012).

In summary, our current data establish Aqp3 as a new target of Prl in a vertebrate. Although a demonstrable function for Aqp3 in teleost gill remains elusive, this apparent link between Prl and Aqp3 may provide a means to modulate the osmosensitivity of multiple branchial cell types. We also revealed a novel capacity for cortisol to block Prl-stimulated *aqp3* transcription. This instance of Prl–cortisol antagonism, at play in the gill, operates in concert with the modulation of Prl cell function by cortisol. In the pituitary, cortisol inhibits Prl release within minutes via nongenomic mechanisms (Borski *et al.* 1991). Recent advances in our understanding of how the teleost gill operates at the molecular level have provided the fodder for identifying novel, and conserved, hormone targets (Breves *et al.* 2014a, Guh *et al.* 2015). For example, the connection between Prl and Ncc first identified in the tilapia gill has been recently found to operate in mouse and rat kidney. This Prl–Ncc link may contribute to hypertension in pregnant women (Rojas-Vega *et al.* 2015). Moreover, Aqp3 was localized to secretory alveolar cells of the rat mammary gland, but functional regulation of Aqp3 in this context remains unresolved (Matsuzaki *et al.* 2005). Future work in euryhaline fishes will thus continue to

provide insight into how highly plastic osmoregulatory systems are regulated while simultaneously revealing conserved modes of hormone action in vertebrates.

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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