clc-2c is regulated by salinity, prolactin and extracellular osmolality in tilapia gill

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Abstract

Teleosts inhabiting fresh water (FW) depend upon ion-absorptive ionocytes to counteract diffusive ion losses to the external environment. A Clc Cl^- channel family member, Clc-2c, was identified as a conduit for basolateral Cl^- transport by Na^+/Cl^- cotransporter 2 (Ncc2)-expressing ionocytes in stenohaline zebrafish (*Danio rerio*). It is unresolved whether Clc-2c/*clc-2c* is expressed in euryhaline species and how extrinsic and/or intrinsic factors modulate branchial *clc-2c* mRNA. Here, we investigated whether environmental salinity, prolactin (Prl) and osmotic conditions modulate *clc-2c* expression in euryhaline Mozambique tilapia (*Oreochromis mossambicus*). Branchial *clc-2c* and *ncc2* mRNAs were enhanced in tilapia transferred from seawater (SW) to FW, whereas both mRNAs were attenuated upon transfer from FW to SW. Next, we injected hypophysectomized tilapia with ovine prolactin (oPrl) and observed a marked increase in *clc-2c* from saline-injected controls. To determine whether Prl regulates *clc-2c* in a gill-autonomous fashion, we incubated gill filaments in the presence of homologous tilapia Prls ($tPrl_{177}$ and $tPrl_{188}$). By 24 h, $tPrl_{188}$ stimulated *clc-2c* expression ~5-fold from controls. Finally, filaments incubated in media ranging from 280 to 450 mosmol/kg for 3 and 6 h revealed that extracellular osmolality exerts a local effect on *clc-2c* expression; *clc-2c* was diminished by hyperosmotic conditions (450 mosmol/kg) compared with isosmotic controls (330 mosmol/kg). Our collective results suggest that hormonal and osmotic control of branchial *clc-2c* contributes to the FW adaptability of Mozambique tilapia. Moreover, we identify for the first time a regulatory link between Prl and a Clc Cl^- channel in a vertebrate.

Key Words
- Clc-2 Cl^- channel
- prolactin
- tilapia
- gill
- ionocyte

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Introduction

Demanded by their close association with aqueous environments, teleost fishes employ complex homeostatic control systems to maintain hydromineral balance. Teleosts generally maintain internal osmotic conditions between 270 and 400 mosmol/kg, with the major dissolved ions, Na^+ and Cl^-, maintained between 130–180 and

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120–150 mmol/L, respectively (Evans *et al.* 2005, Evans & Claiborne 2008). Thus, teleosts inhabiting fresh water (FW) habitats must actively absorb Na^+ and Cl^- from the surrounding environment to counteract diffusive losses. Alternatively, marine teleosts actively secrete Na^+ and Cl^- to mitigate passive influxes. Euryhaline fishes (<10% of teleosts) with the capacity to tolerate salinities ranging from FW to seawater (SW) exhibit remarkable plasticity regarding the direction of active Na^+ and Cl^- transport following changes in ambient salinity (Kaneko *et al.* 2008, Schultz & McCormick 2013).

The maintenance of hydromineral balance in FW environments requires coordination among multiple tissues, including gill, intestine, kidney and urinary bladder. Nonetheless, branchial epithelium is the primary site of Na^+ and Cl^- absorption via specialized FW-type ionocytes (Marshall & Grossell 2006). Various models have been presented to explain how ionocytes absorb ions from dilute environments (Evans 2008). Recent studies on euryhaline Mozambique tilapia (*Oreochromis mossambicus*) and stenohaline zebrafish (*Danio rerio*) have in particular improved our understanding of how ion uptake is accomplished. For example, among the multiple ion-absorptive ionocytes now identified in tilapia and zebrafish (Hiroi & McCormick 2012, Guh *et al.* 2015), cells termed 'Type-II' ionocytes, or 'Ncc cells', express Na^+/Cl^- cotransporter 2 (Ncc2; Slc12a10) in the apical membrane (Hiroi *et al.* 2008, Wang *et al.* 2009). Ncc2 facilitates electroneutral transport of Na^+ and Cl^- from FW into the ionocyte interior. Convincing evidence established that Ncc2-expressing ionocytes are in fact critical to Na^+ and Cl^- homeostasis (Hiroi *et al.* 2008, Inokuchi *et al.* 2008, 2009, Horng *et al.* 2009, Wang *et al.* 2009, Kwong & Perry 2016). A member of the Clc family of Cl^- channels, Clc-2c, was subsequently proposed as a conduit for basolateral transport of Cl^- from the interior of zebrafish Ncc cells into blood plasma (Pérez-Ruis *et al.* 2015, Wang *et al.* 2015). Accordingly, low-Cl^- environments stimulate *clc-2c* mRNA levels and the recruitment of Clc-2c-expressing ionocytes (Wang *et al.* 2015). In tilapia, however, there is no information regarding Clc-2c/*clc-2c* expression in the gill and equivocal data concerning the involvement of Clc-3 in branchial Cl^- uptake (Tang & Lee 2011).

Euryhaline fishes respond to variations in environmental salinity by modulating the levels of gene transcripts that encode effectors of ion transport (Fiol & Kültz 2007). For example, branchial *ncc2* expression is highly labile in tilapia, with a marked increase in expression occurring within hours of transfer from SW to FW, and decreased expression following transfer from FW to SW (Hiroi *et al.* 2008, Breves *et al.* 2011). The dynamics of *ncc2* align closely with the morphological and functional changes occurring within FW-type ionocyte populations during salinity acclimation (Hiroi *et al.* 2008, Inokuchi *et al.* 2008) and following hormone treatments (Breves *et al.* 2010*c*, Inokuchi *et al.* 2015, Watanabe *et al.* 2016). *ncc2* transcript levels thus provide a point of reference from which to characterize parallel transcriptional responses underlying FW acclimation. For example, if Clc-2c/*clc-2c* is coexpressed with Ncc2/*ncc2* in tilapia ionocytes, as in zebrafish (Wang *et al.* 2015), one would predict coordinated increases in *ncc2* and *clc-2c* mRNA levels as adaptive responses to FW. Coordinated responses of this nature could be achieved through common regulators of their expression.

Hormones secreted in response to perturbations of internal osmotic conditions and/or changes in environmental salinity orchestrate the activities of teleost osmoregulatory systems. The so-called 'fast-acting' hormones direct acute responses such as alterations of ion transport, drinking behavior and cardiovascular function, whereas 'slow-acting' hormones modulate osmoregulatory tissues by altering patterns of gene expression and cell proliferation and/or differentiation (McCormick 2001, Takei *et al.* 2014). One 'slow-acting' factor, the hypophyseal hormone prolactin (Prl), is considered the 'FW-adapting hormone' given its actions to stimulate ion-conserving and water-excreting processes (Hirano 1986). Only recently, gene targets of Prl have been identified which underlie discreet solute and water-handling processes. These transcriptional targets encode ion transporters/channels, Na^+/K^+-ATPase subunits, aquaporins and tight-junction proteins (Breves *et al.* 2014*a*).

While Prl and other hormones are certainly important regulators of osmoregulatory processes, there is evidence that local osmotic/ionic control of ion transport occurs in some euryhaline species. Inasmuch as the acute phase of salinity acclimation entails deviations from internal 'set points' before effector mechanisms are fully activated, these deviations themselves may initiate changes in gill function (Marshall *et al.* 2000, Tse *et al.* 2007, Inokuchi *et al.* 2015). In this regard, cells comprising branchial epithelia operate as osmoreceptors in addition to responding to systemic hormones (Zadunaisky *et al.* 1995, Hiroi *et al.* 2005, Marshall *et al.* 2008, Kültz 2012). For instance, tilapia ionocytes respond to varying osmotic conditions *in vitro* by modifying cell morphology and gene transcription (Hiroi *et al.* 2005, Inokuchi *et al.* 2015). Osmotic conditions could be detected by either direct exposure to changes in environmental osmolality

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at the apical surface or through changes in blood plasma conditions along the basolateral surface. In either case, tilapia ionocytes are well suited to uncover how hormones and osmotic conditions work in concert (or antagonistically) to regulate aspects of ionocyte function, such as the expression of mRNAs that encodes solute transporters.

The primary objective of this study was to identify the regulators of branchial *clc-2c* expression in a euryhaline teleost. Given the demonstrated effects of Prl and extracellular osmolality on Ncc2/*ncc2* (Breves *et al.* 2010*c*, 2014*b*, Inokuchi *et al.* 2015), a key effector of ion uptake in tilapia, we hypothesized that Prl and/or local osmotic conditions likewise regulate *clc-2c* expression. The combined results of our *in vivo* (salinity transfers, hypophysectomy and hormone replacement) and *in vitro* (gill filament incubations) experiments suggest that multifactorial control of *clc-2c* underlies the highly plastic ionoregulatory capacities of euryhaline tilapia.

Materials and methods

Experimental animals and rearing conditions

Tilapia (*O. mossambicus*) 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 mmol/L Na^+, 0.55 mmol/L Ca^{2+}, 0.03 mmol/L K^+, 0.60 mmol/L Mg^{2+}, conductivity = 322 µS/cm) or SW (Kāne'ohe Bay, Hawai'i; 34‰, 482 mM Na^+, 545 mM Cl^-, 10.7 mM Ca^{2+}, 7.46 mM K^+, 52.6 mM Mg^{2+}, Conductivity = 51 mS/cm) under natural photoperiod and fed a commercial diet (Silver Cup Trout Chow, Nelson & Sons Inc., Murray, UT, USA). 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 and experimental protocols.

Tissue distribution of *clc-2c* gene expression

Tissues were collected from male tilapia maintained in FW for >1 year ($n=6$). Fish were lethally anesthetized with 2-phenoxyethanol (2-PE; 0.3 mL/L, Sigma-Aldrich) and the following tissues were collected: whole brain, gill, esophagus, stomach, anterior intestine, body kidney, urinary bladder and white muscle. Tissues were stored in TRI Reagent (MRC, Cincinnati, OH, USA) at −80°C until RNA isolation. To compare branchial *clc-2c* mRNA levels among FW-, brackish water (BW; 12‰)- and SW-acclimated animals ($n=6$), gill filaments were collected from animals fully acclimated to various environmental salinities.

Effects of salinity on branchial gene expression (SW and FW transfers)

Sixty SW-acclimated fish and sixty FW-acclimated fish (mixed sex) were allocated randomly across four 700-L tanks supplied with either SW or FW to a final count of thirty fish per tank (two tanks per salinity). Fish were fed once daily to satiation and allowed to acclimate to the experimental tanks for 3 weeks prior to transfers. On day 0, 8 fish from each of the two SW and two FW tanks were sampled. Then, water supplies to one of the SW and one of the FW tanks were changed to FW and SW, respectively. Fish transferred from FW to SW were first exposed to ~85% SW (30‰) over 48 h, and then the water supply was adjusted to full strength SW. One SW tank and one FW tank were maintained as time-matched controls for the duration of the experiment. From each of the four experimental tanks, 8 fish were sampled on day 3 and day 7. Fish sampled over the 7-day period weighed 282.9 ± 10.8 g (mean ± S.E.M.) at the time of sampling.

Effects of oPrl in hypophysectomized (Hx) animals

Hypophysectomy of male tilapia (91.0 ± 2.3 g) was performed by the transorbital technique developed by Nishioka (1994). Tilapia were anesthetized by immersion in buffered tricaine methanesulfonate (100 mg/L, Argent Chemical Laboratories, Redmond, WA, USA) and 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 microfibrillar collagen hemostat (Ethicon, Somerville, NJ, USA) and fish were allowed to recover for 5 days in BW (12‰) composed of SW diluted with FW. Following recovery, fish were transferred to re-circulating 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.

To identify the effects of Prl on branchial gene expression, Hx fish recovered in BW ($n=8$) were administered oPrl (5 µg/g body weight, Sigma-Aldrich) via intraperitoneal (IP) injections over the course of 5 days. Hormone dose was selected following previous experiments employing IP injection of oPrl in teleosts (Herndon *et al.* 1991, Jackson *et al.* 2005, Breves *et al.* 2010*c*,

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2014*b*). Forty-eight hours after an initial injection, second and third injections were administered 48 h apart. Twenty-four hours after the third injection, gill filaments were excised from the first arch (left side) and stored in TRI Reagent. oPrl was delivered in saline vehicle (0.9% NaCl; 1.0 µL/g body weight). Two additional groups, Hx (n=8) and sham-operated (n=9) fish, were injected with saline vehicle only. Fish were not fed for the duration of the recovery and post-injection periods. At sampling, all fish were anesthetized in 2-PE (0.3 mL/L) and blood was collected from the caudal vasculature by a needle and syringe treated with heparin ammonium salt (200 U/mL, Sigma-Aldrich). Plasma was separated by centrifugation for measurement of plasma osmolality using a vapor pressure osmometer (Wescor 5100C, Logan, UT, USA) and plasma Cl^- by the silver titration method using a Buchler–Cotlove digital chloridometer (Labconco, Kansas City, MO, USA). Completeness of Hx was confirmed by post-mortem inspection of the hypothalamic region.

Effects of homologous tilapia Prls (tPrls) and extracellular osmolality

To identify direct effects of two isoforms of tPrl ($tPrl_{177}$ and $tPrl_{188}$) on branchial gene expression, we incubated filaments from the second and third gill arches of FW-acclimated tilapia (males) following Watanabe *et al.* (2016). Excised gill arches were first washed in sterilized balanced salt solution (BSS: NaCl 120 mmol/L, KCl 4 mmol/L, $MgSO_4$ 0.8 mmol/L, $MgCl_2$ 1.0 mmol/L, $NaHCO_3$ 2 mmol/L, $CaCl_2$ 1.5 mmol/L, KH_2PO_4 0.4 mmol/L, Na_2HPO_4 1.3 mmol/L, $CaCl_2$ 2.1 mmol/L, Hepes 10 mmol/L, pH 7.4) and then incubated in 0.025% $KMnO_4$ for 1 min. After a second wash in BSS, individual gill filaments were cut from the arches, cut sagittally under a dissecting microscope and then placed in 24-well plates containing Leibovitz's L-15 culture medium (Life Technologies). Culture medium was supplemented with 5.99 mg/L penicillin and 100 mg/L streptomycin (Sigma-Aldrich), adjusted to 330 mosmol/kg, and sterilized with a 0.2-µm filter. Three gill filaments were placed in each well, which contained 500 µL culture medium supplemented with 0, 0.01, 0.1, 0.5 and 1.0 µg/mL of $tPrl_{177}$ or $tPrl_{188}$ (n=8). tPrls were purified by HPLC from media following pituitary tissue culture (Seale *et al.* 2002) as described previously (Breves *et al.* 2014*b*). After 24 h at 26°C, incubations were terminated by collecting the gill filaments in TRI Reagent.

To test the effect of extracellular osmolality on *clc-2c*, gill filaments were incubated in four osmolalities: 280, 330, 380 and 450 mosmol/kg (n=8). Media were prepared as described by Inokuchi and coworkers (Inokuchi *et al.* 2015). These four osmotic conditions were selected on the basis that Mozambique tilapia are exposed to, and readily tolerate, such plasma osmolalities during the acute phases of acclimation to FW or SW (Breves *et al.* 2010*a*, 2011). Filaments were incubated for 3 and 6 h. Incubations were terminated by collecting filaments in TRI Reagent.

To confirm that hyperosmotic conditions *in vitro* elicit comparable transcriptional responses *in vivo*, FW-acclimated tilapia (26.6±4.6 g; mixed sex) were transferred to ~85% SW (30‰) and sampled at 0, 3 and 6 h after transfer (n=6). Food was withheld for the duration of the experiment. At sampling, blood plasma and gill filaments were collected as described earlier.

RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from gill filaments by the TRI Reagent procedure according to the manufacturer's protocol. RNA concentration and purity were assessed by spectrophotometric absorbance (Nanodrop 1000, Thermo Scientific). First strand cDNA was synthesized by reverse-transcribing 50–100 ng total RNA with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Relative levels of mRNA were determined by qRT-PCR using the StepOnePlus real-time PCR system (Life Technologies). Primer sequences for *ncc2*, *clc-3* and *ef1*α have been previously described (Inokuchi *et al.* 2008, Breves *et al.* 2010*b*, Tang & Lee 2011). Primers for a tilapia *clc-2c* sequence (Acc. No. XM_003459762) identified by Pérez-Ruis *et al.* (2015) were designed to span a predicted exon–exon junction: F: AGAAGGTCAGTCAGCCAAGC and R: AGCGAAATGGGCCGAACTT (product=72 bp), and to not amplify *clc-2a* (XM_019347600) or *clc-2b* (XM_005461001). The qRT-PCR reactions were set up as previously described (Inokuchi *et al.* 2015). Briefly, in Power SYBR Green PCR Master Mix (Life Technologies), 200 nmol/L of each primer and 3 µL cDNA were added to a 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. After verification that *ef1*α mRNA levels did not vary across treatments, *ef1*α levels were used to normalize target genes. Reference and target genes were calculated by the relative quantification method with PCR efficiency correction (Pfaffl 2001). Standard curves were prepared from serial dilutions of gill filament cDNA and included on each plate to calculate the

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PCR efficiencies for target and normalization gene assays. Relative gene expression ratios between groups are reported as a fold change from controls.

Statistics

Multiple group comparisons were performed by one-way ANOVA followed by Tukey's HSD test. Significance for all tests was set at $P<0.05$. Transfer experiments were analyzed by two-way ANOVA. Significant effects of treatment, time or an interaction ($P<0.05$) are indicated in figures: *$P<0.05$, **$P<0.01$ and ***$P<0.001$. When significant main or interaction effects were detected, Student's *t*-tests were employed at each time point. Significant differences between groups at a given time point are also indicated in figures: †$P<0.05$, ††$P<0.01$ and †††$P<0.001$. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software).

Results

Tissue distribution of *clc-2c* gene expression

We first established that *clc-2c* mRNA was expressed in the gill and determined the relative amounts of expression across tissues collected from FW-acclimated tilapia (Fig. 1A). *clc-2c* was highly expressed in gill and kidney, with lower expression in other examined tissues. Branchial *clc-2c* mRNA levels were markedly higher in long-term FW- vs BW- and SW-acclimated tilapia (Fig. 1B).

Effects of salinity transfer on branchial *ncc2*, *clc-2c* and *clc-3* gene expression

In the SW to FW transfer experiment, a significant effect of treatment was detected for *ncc2* (Fig. 2A). *ncc2* expression was elevated in FW-transferred animals above controls maintained in SW at both 3 and 7 days after transfer. For *clc-2c*, there were significant main effects of treatment and time, and a significant interaction (Fig. 2B). Similar to *ncc2*, *clc-2c* expression was enhanced in FW-transferred animals from controls at both 3 and 7 days after transfer. On the other hand, there were no significant main effects (or interaction) detected for *clc-3* expression (Fig. 2C). In the subsequent FW to SW transfer experiment, there was a significant main effect of treatment for both *ncc2* and *clc-2c* (Fig. 3A and B). Both transcripts were reduced at 3 and 7 days following transfer to SW. There were no significant main effects (or interaction) on *clc-3* expression levels (Fig. 3C).

Effects of oPrl on plasma parameters and branchial *ncc2*, *clc-2c* and *clc-3* gene expression in Hx animals

To assess whether Prl impacts *clc-2c*, we injected Hx tilapia held in BW with oPrl and compared *clc-2c* levels with sham-operated and Hx tilapia injected with saline vehicle. First, plasma osmolality and plasma Cl^- levels were reduced in saline-injected Hx fish compared with sham-operated animals; both reductions were rescued by oPrl (Fig. 4A and B). oPrl stimulated branchial *ncc2* and *clc-2c* compared with sham-operated and Hx tilapia injected with saline (Fig. 4C and D). Hx fish injected with oPrl showed higher *clc-3* levels when compared with saline-injected sham-operated animals, but not when compared with saline-injected Hx animals (Fig. 4E).

Effects of $tPrl_{177}$ and $tPrl_{188}$ on *clc-2c* and *clc-3* gene expression in incubated gill filaments

To then determine whether Prl stimulates *clc-2c* expression in a gill-autonomous fashion, we incubated gill filaments

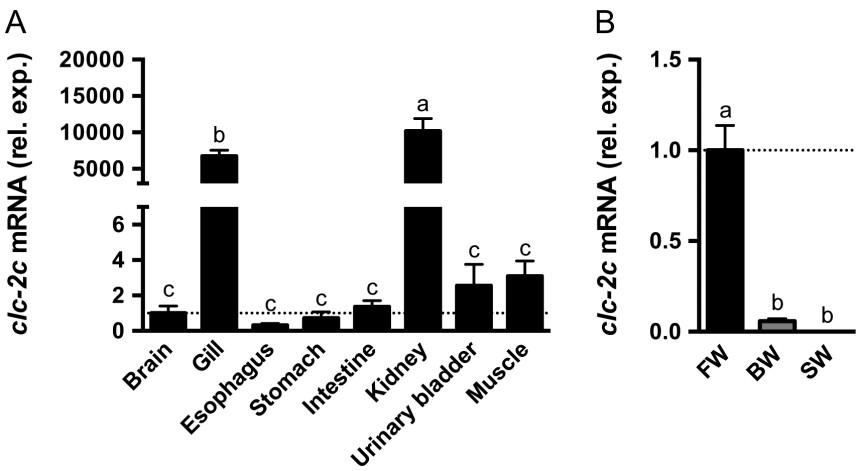

Figure 1
Gene expression of *clc-2c* in various tissues of fresh water (FW)-acclimated tilapia (A). Data were normalized to *ef1α* as a reference gene and are presented relative to brain expression levels. Branchial *clc-2c* expression in FW-, (BW; 12‰)- and seawater (SW)-acclimated tilapia (B). Gene expression is presented as a fold change from FW. Mean ± S.E.M. ($n=6$). Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD test, $P<0.05$).

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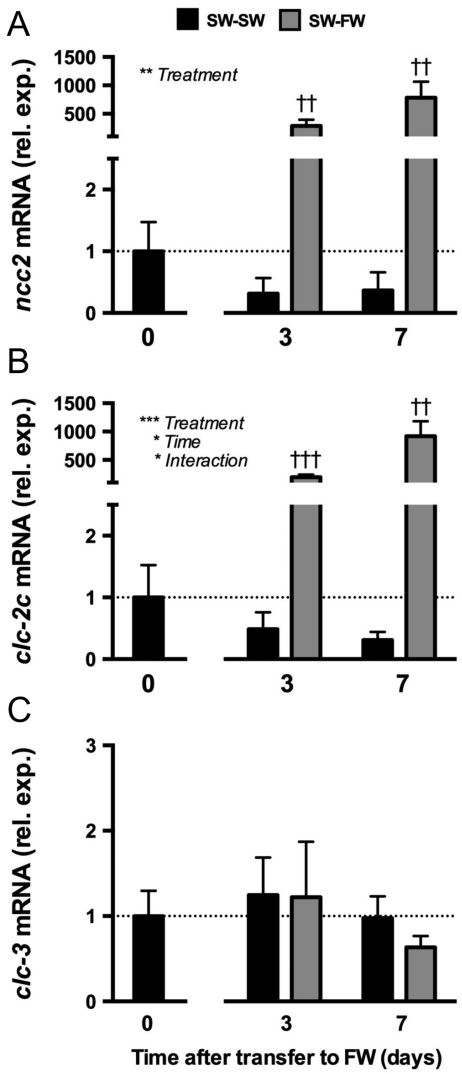

Figure 2
Changes in branchial *ncc2* (A), *clc-2c* (B) and *clc-3* (C) gene expression at days 3 and 7 after transfer of tilapia from seawater (SW) to fresh water (FW; shaded bars). Mean ± S.E.M. ($n = 8$). Time-matched control fish were maintained in SW (solid bars). Gene expression is presented as a fold change from time 0. Differences among groups were evaluated by two-way ANOVA. Significant effects of treatment, time or an interaction are indicated in respective panels (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). When there was a significant treatment effect, *post hoc* comparisons (Student's *t*-tests) were made between groups at each time point (††$P < 0.01$ and †††$P < 0.001$).

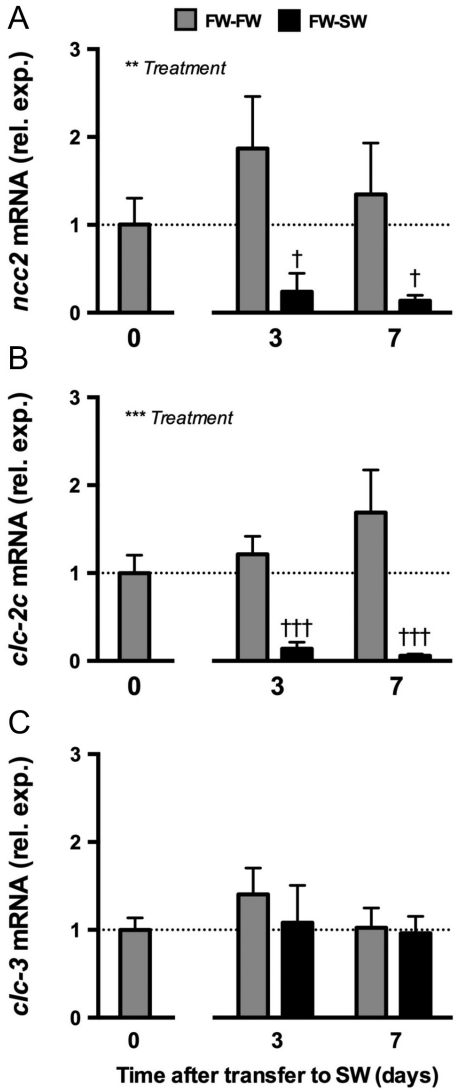

Figure 3
Changes in branchial *ncc2* (A), *clc-2c* (B) and *clc-3* (C) gene expression at days 3 and 7 after transfer of tilapia from fresh water (FW) to seawater (SW; solid bars). Mean ± S.E.M. ($n = 8$). Time-matched control fish were maintained in FW (shaded bars). Gene expression is presented as a fold change from time 0. Differences among groups were evaluated by two-way ANOVA. A significant effect of treatment is indicated in respective panels (**$P < 0.01$ and ***$P < 0.001$). When there was a significant treatment effect, *post hoc* comparisons (Student's *t*-tests) were made between groups at each time point (†$P < 0.05$ and †††$P < 0.001$).

in the presence of various concentrations of $tPrl_{177}$ or $tPrl_{188}$ for 24 h. While there was no apparent effect of $tPrl_{177}$ (Fig. 5A), $tPrl_{188}$ at 1.0 µg/mL stimulated *clc-2c* by 5.4-fold relative to 0 µg/mL controls (Fig. 5B). For *clc-3*, there were no significant differences between any tested $tPrl_{177}$ or $tPrl_{188}$ concentration and associated controls (Fig. 5C and D).

Effect of extracellular osmolality on the *clc-2c* gene expression

We incubated gill filaments for 3 and 6 h under conditions ranging from 280 to 450 mosmol/kg to identify direct effects of extracellular osmolality on *clc-2c* expression. At 3 and 6 h, *clc-2c* levels in the 450 mosmol/kg groups were 0.3-fold and 0.5-fold the levels observed in control groups

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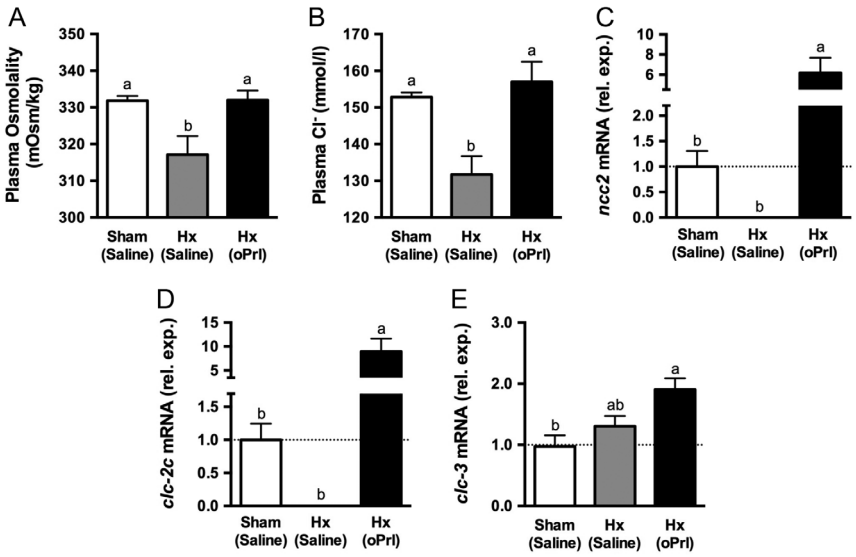

Figure 4
Effects of hypophysectomy (Hx) and ovine Prl (oPrl) on plasma osmolality (A) and Cl^- (B) and branchial gene expression of *ncc2* (C), *clc-2c* (D) and *clc-3* (E). Mean ± S.E.M. (n = 8–9). Gene expression is presented as a fold change from the saline-injected sham group (open bars). While held in brackish water (12‰), fish received three intraperitoneal injections of oPrl (5 μg/g body weight) (solid bars) over 5 days (see 'Materials and methods' section). Sham-operated (open bars) and Hx fish (shaded bars) received saline injections. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD test, P < 0.05).

(330 mosmol/kg), respectively (Fig. 6). In FW-acclimated fish transferred to 30‰, plasma osmolality increased in parallel with reductions in *ncc2* and *clc-2c* levels; *ncc2* and *clc-2c* were significantly reduced from FW controls by 6 h (Fig. 7).

Discussion

In the current study, we addressed how extrinsic and intrinsic factors regulate branchial *clc-2c* in a euryhaline teleost. Following a combination of *in vivo* and *in vitro* experiments, we propose that (1) Prl acts directly on the gill to regulate salinity-dependent *clc-2c* expression in support of acclimation to FW, and (2) hyperosmotic conditions constitute a proximate cue for the rapid (hours) inhibition of branchial *clc-2c* expression during SW acclimation. The transcriptional control of *clc-2c* reported here reveals a novel role for Prl in teleost osmoregulation, and, moreover, represents the first identification of this regulatory connection in any vertebrate.

Given the recently defined role for Clc-2c in mediating Cl^- transport by zebrafish ionocytes (Pérez-Ruis *et al.* 2015, Wang *et al.* 2015), we started by characterizing the expression pattern of *clc-2c* across tissues collected from tilapia acclimated to FW (Fig. 1A). Consistent with a putative role for tilapia Clc-2c in Cl^- uptake, we observed high *clc-2c* gene expression in the gill. Interestingly, we observed comparable expression levels in kidney, a pattern that differs from *clc-2c* patterns in zebrafish (Pérez-Ruis *et al.* 2015, Wang *et al.* 2015). Future studies should investigate whether Clc-2c supports Cl^- transport by renal tubules. To date, only Clc-K has been implicated in Cl^- reabsorption by kidney of FW-acclimated tilapia (Miyazaki *et al.* 2002). Importantly, branchial *clc-2c* levels were clearly suppressed in fish acclimated to BW and SW (Fig. 1B) in accord with the low density of Ncc2-expressing ionocytes in these salinities (Hiroi *et al.* 2008, Inokuchi *et al.* 2008). Further suggesting a functional link between Ncc2 and Clc-2c, we observed parallel changes in *ncc2* and *clc-2c* expression under both SW to FW and FW to SW transfer paradigms (Figs 2A, B and 3A, B). Resembling the increase in *clc-2c* when tilapia are transferred from SW to FW, zebrafish likewise activate *clc-2c* expression when challenged with low-Cl^- environments, a response corresponding with the recruitment of ionocytes which coexpress Ncc2 and Clc-2c (Wang *et al.* 2015). Immunohistochemical detection of Clc-2c (presumably within Ncc2-expressing ionocytes) is required to resolve the relationship between *clc-2c* levels and ionocyte recruitment in tilapia. While Clc-3 has actually been localized to Ncc2-expressing ionocytes (Tang & Lee 2011), *clc-3* levels were not responsive to either SW or FW transfers (Figs 2C and 3C). These data are in agreement with patterns reported by Tang & Lee (2011) who proposed that Clc-3 mediates Cl^- absorption from severely Cl^--depleted environments. In any event, given the salinity-dependent expression of *clc-2c* in tilapia, we next focused on identifying a systemic regulator for this gene.

Mozambique tilapia, like other euryhaline fishes, exhibit robust changes in plasma Prl following salinity changes. Plasma Prl levels and ambient salinity are inversely related (Yada *et al.* 1994). We previously linked the expression of genes underlying branchial phenotypes associated with FW, such as *ncc2*, *Na^+/K^+-ATPase$_{\alpha 1a}$* (*nka$_{\alpha 1a}$*) and *aquaporin-3*, to Prl levels through Hx and hormone replacement (Breves *et al.* 2010*c*,

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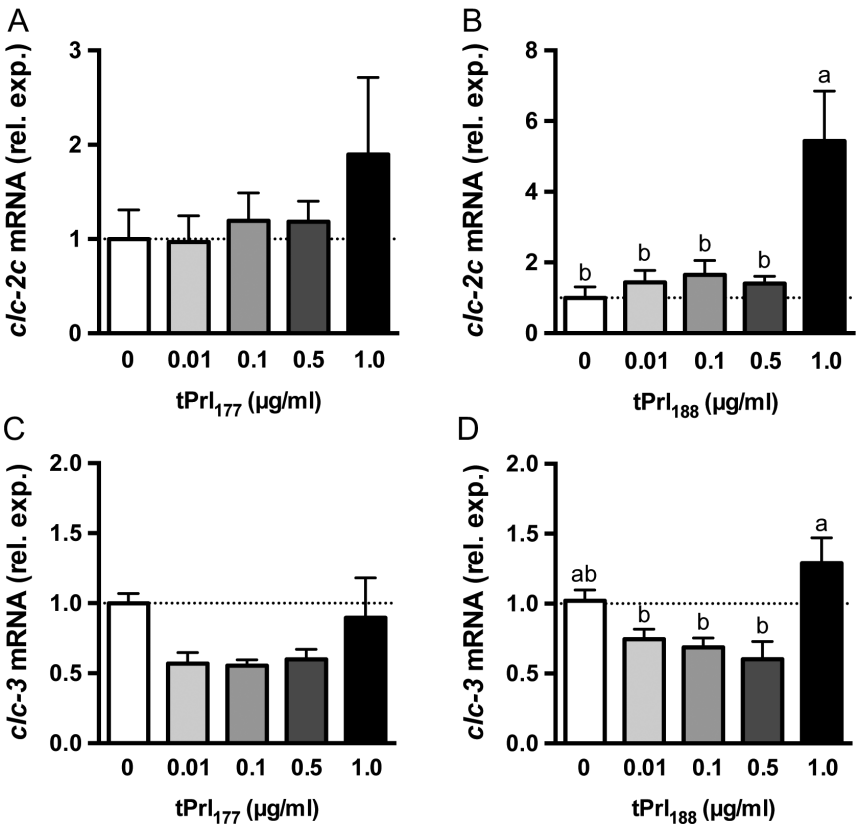

Figure 5
Effects of homologous $tPrl_{177}$ (A and C) and $tPrl_{188}$ (B and D) concentration on *clc-2c* and *clc-3* gene expression in gill filaments incubated for 24 h. Mean ± S.E.M. ($n = 8$). Gene expression is presented as a fold change from the 0 concentration groups. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD, $P < 0.05$).

2016, Tipsmark *et al.* 2011). Here, we again leveraged pituitary extirpation to probe a link between endocrine Prl and branchial *clc-2c*. Hx clearly affected osmotic and Cl^- balance (Fig. 4A and B) as previously demonstrated (Dharmamba & Maetz 1972). Saline-injected Hx tilapia exhibited reductions in plasma osmolality and Cl^- that are explained, at least in part, by their inability to maintain branchial ionocytes employing Ncc2-mediated ion uptake (Breves *et al.* 2010*c*, 2014*b*). It was intriguing that saline-injected Hx fish showed drops in plasma osmolality and Cl^- when held under slightly hyperosmotic conditions. Tilapia held in BW (1/3 SW) simultaneously maintain both FW- and SW-type ionocytes (Inokuchi *et al.* 2008). When we abolished Prl signaling via Hx, we disrupted ion uptake by Ncc2 cells and ion secretion became dominant via SW-type ionocytes, and in turn, plasma osmolality/Cl^- dropped. Replacement with oPrl successfully restored plasma osmolality and Cl^- levels to control (sham) levels (Fig. 4A and B) while inducing both *ncc2* and *clc-2c* (Fig. 4C and D). In light of the reduced expression of *clc-2c* in BW compared with FW (Fig. 1B), it is noteworthy that Prl stimulated *clc-2c* in a salinity where it would be otherwise suppressed. This effect resembles the strong induction of *ncc2* by Prl in Hx animals held in SW (Breves *et al.* 2010*c*). On the other hand, there was no clear effect of Hx on *clc-3* levels. Thus, compromised Cl^- balance, at least in 12‰ BW, was not correlated with *clc-3* levels (Fig. 4E). These results are consistent with a regulatory connection between coincident increases in plasma Prl and branchial *clc-2c* levels in tilapia undergoing FW acclimation.

Watanabe and coworkers recently described a gill filament incubation technique suited to investigate how tilapia ionocytes respond to hormones (Watanabe *et al.* 2016). Filament incubations sustain gill-autonomous functions for several days depending upon the technique employed (McCormick & Bern 1989, Kiilerich *et al.* 2007, Watanabe *et al.* 2016). Using this *in vitro* approach, we probed the sensitivity of *clc-2c* and *clc-3* to purified tPrls for three major reasons. First, we sought to determine whether the *in vivo* effect of oPrl was mediated through gill-autonomous processes, or alternatively, whether Prl stimulated *clc-2c* through an intermediary factor(s) only present in the whole organism. $tPrl_{188}$ stimulated *clc-2c* levels in isolated filaments (Fig. 5B), an effect mediated by Prl receptors in the gill (Weng *et al.* 1997, Fiol *et al.* 2009). Second, because we injected heterologous Prl under our Hx paradigm, we assessed whether oPrl elicits similar effects on *clc-2c* as homologous tPrls. $tPrl_{188}$ exerted a similar stimulatory effect as oPrl on *clc-2c* (Fig. 5B). Third, tPrl occurs in two forms, $tPrl_{177}$ and $tPrl_{188}$, which share

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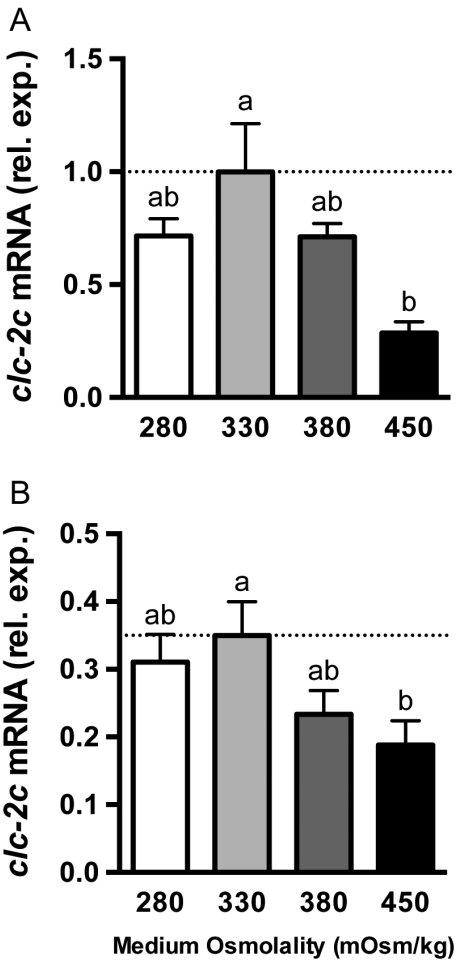

Figure 6
Effects of medium osmolality on the *clc-2c* gene expression in gill filaments incubated in various osmolalities for 3 (A) and 6 h (B). Mean ± S.E.M. (n = 8). Gene expression is presented as a fold change from the 330 mosmol/kg group at 3 h. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD test, $P < 0.05$).

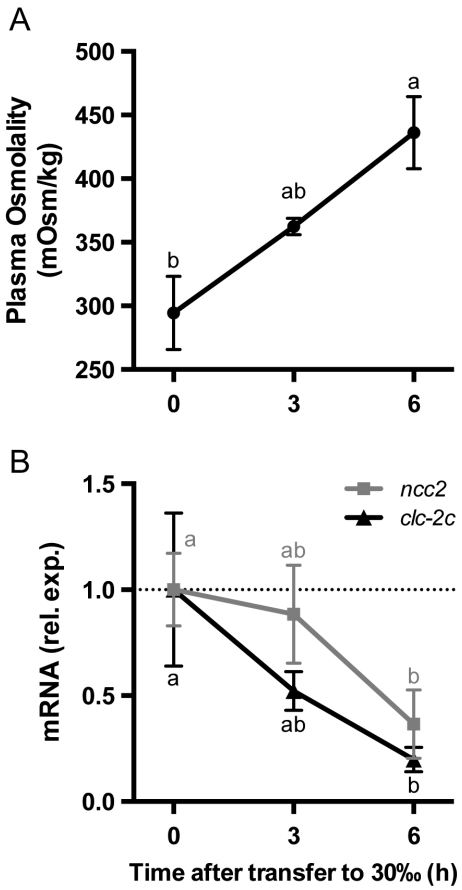

Figure 7
Changes in plasma osmolality (A) and branchial *ncc2* and *clc-2c* gene expression (B) at 3 and 6 h after transfer of tilapia from fresh water to 30‰ brackish water. Mean ± S.E.M. (n = 6). Gene expression is presented as a fold change from time 0. For a given parameter, means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD test, $P < 0.05$).

69% amino acid identity and are encoded by separate genes (Specker *et al.* 1985, Yamaguchi *et al.* 1988). While both tPrls support hyperosmoregulation (Specker *et al.* 1985), we recently identified differences in their osmosensitivity as it related to secretion patterns from the *rostral pars distalis* (Seale *et al.* 2012). We found that $tPrl_{188}$, but not $tPrl_{177}$, stimulated *clc-2c* (Fig. 5A and B). This differs from Prl control of *ncc2*, where under identical incubation conditions, *ncc2* was stimulated by both tPrls, albeit $tPrl_{188}$ had a more robust effect than $tPrl_{177}$ (Inokuchi *et al.* 2015). The control of *clc-2c* by Prl reported here provides a mechanism for the ion-retaining activity of Prl first identified in tilapia by Dharmamba & Maetz (1972). Furthermore, induction of *clc-2c* provides a means for Prl to exert deleterious effects in tilapia inhabiting SW when the promotion of a Cl^- uptake pathway would be maladaptive (Herndon *et al.* 1991, Pisam *et al.* 1993). Given that Prl affects multiple osmoregulatory tissues (Hirano 1986), additional extrabranchial pathways are likely to contribute to Prl's maladaptive effects in SW.

Through *in vitro* incubations of gill filaments, we uncoupled the osmotic changes that occur during salinity acclimation from the hormonal milieu to investigate the independent effects of plasma osmolality and Prl on *clc-2c*. The tested osmotic conditions reflect the range of plasma osmolalities that *O. mossambicus* readily tolerate during the acute phases (initial 24 h) of FW and SW acclimations (Breves *et al.* 2010*a*, 2011). When compared with isosmotic controls (330 mosmol/kg), hyperosmotic conditions (450 mosmol/kg) inhibited *clc-2c* (Fig. 6). We previously reported that *ncc2* and $nka_{\alpha 1a}$ respond in an identical manner as shown here for *clc-2c* (Inokuchi *et al.* 2015), thereby indicating that multiple gene transcripts

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supportive of FW acclimation are jointly inhibited by hyperosmotic conditions. On the other hand, *Na⁺/K⁺/2Cl⁻ cotransporter 1a* (*nkcc1a*) expressed exclusively in SW-type (ion secretory) ionocytes was induced by hyperosmotic conditions (Inokuchi *et al.* 2015). When we induced hyperosmotic stress *in vivo* by transferring FW-acclimated fish to 30‰ BW, *clc-2c* was suppressed by 6 h (Fig. 7B). Our collected experiments indicate that tilapia ionocytes are controlled by the interplay of osmotic and endocrine cues; long-term regulation of *clc-2c* via endocrine Prl is preceded by short-term (and local) control via plasma osmolality/ions. Given the sensitivity of mammalian Clc-2 currents to cell volume, extracellular pH and intracellular [Cl^-] (Foskett 1998, Bi *et al.* 2013), post-translational regulation of teleost Clc-2c should be addressed in future investigations.

Hiroi & McCormick (2012) surveyed ionocytes across teleosts and suggested that Ncc2-mediated ion uptake occurs in at least some Ostariophysi and Acanthoterygii species. While Ncc2 has not yet been colocalized with Clc-2c in any species beyond zebrafish, it will be interesting to learn the extent to which Prl and Clc-2c are linked. In this regard, teleost ionocytes will continue to provide a platform from which to identify novel and potentially conserved targets of Prl in vertebrates and enable the further characterization of these activities.

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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