Regulation of gill claudin paralogs by salinity, cortisol and prolactin in Mozambique tilapia
(*Oreochromis mossambicus*)

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Abstract

In euryhaline teleosts, reorganization of gill tight junctions during salinity acclimation involves dynamic expression of specific claudin (Cldn) paralogs. We identified four transcripts encoding Cldn tight junction proteins in the tilapia gill transcriptome: *cldn10c*, *cldn10e*, *cldn28a* and *cldn30*. A tissue distribution experiment found *cldn10c* and *cldn10e* expression levels in the gill to be 100-fold higher than any other tissues examined. *cldn28a* and *cldn30* levels in the gill were 10-fold greater than levels in other tissues. Expression of these genes in Mozambique tilapia was examined during acclimation to fresh water (FW), seawater (SW), and in response to hormone treatments. Transfer of tilapia from FW to SW elevated *cldn10c* and *cldn10e*, while *cldn28a* and *cldn30* were stimulated following transfer from SW to FW. In hypophysectomized tilapia transferred to FW, pituitary extirpation induced reduced expression of *cldn10c*, *cldn10e* and *cldn28a*; these effects were mitigated equally by either prolactin or cortisol replacement. *In vitro* experiments with gill filaments showed that cortisol stimulated expression of all four *cldns* examined, suggesting a direct action of cortisol *in situ*. Our data indicate that elevated *cldn10c* and *cldn10e* expression is important during acclimation of tilapia to SW possibly by conferring ion specific paracellular permeability. On the other hand, expression of *cldn28a* and *cldn30* appears to contribute to reorganization of branchial epithelium during FW acclimation. Hormone treatment experiments showed that particular FW- and SW-induced *cldns* are controlled by cortisol and prolactin.

Key words: epithelia, growth hormone, hypophysectomy, osmoregulation, tight junctions.

1. Introduction

Euryhaline teleosts like Mossambique tilapia (*Oreochromis mossambicus*) must regulate the permeability of their surface epithelia when they acclimate to changes in environmental osmolality and ion conditions. As the main interface with the surrounding water, the gill epithelium mediates not only essential iono- and acid/base-regulatory functions but is also the main site of external respiration in the adult fish (Evans et al., 2005). In turn, passive transepithelial fluxes of solutes and water are continuously counteracted to maintain internal plasma osmolality at about one-third that of full-strength seawater (SW) in both concentrated marine and dilute freshwater (FW) environments.

Tight junction strands regulate paracellular solute and water movement by making up the apical-lateral barriers, or gates, between epithelial cells (Hou et al., 2013). In the gill of fish acclimated to FW, tight junctions between adjacent pavement cells and ionocytes, and in between individual pavement cells, are deep with multi-stranded connections (Sardet et al., 1979). In SW, shallow tight junctions connect accessory cells with adjacent ionocytes permitting “leaky” paracellular pathways between these cells (Sardet et al., 1979; Cozzi et al., 2015). In SW-type ionocytes, NaCl secretion involves transcellular chloride transport via a basolateral $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter (Nkcc) and an apical cystic fibrosis transmembrane conductance regulator anion channel (Evans et al., 2005). Chloride transport is secondarily coupled to the activity of Na^+, K^+ -ATPase (Nka) found in the extensive tubular network which extends from the basolateral membrane (Karnaky et al., 1976). The active Cl^- secretion is thought to generate a serosal positive transepithelial potential driving Na^+ extrusion via a paracellular pathway (Degnan and Zadunaisky, 1980; Evans et al., 2005). In contrast with hyposmoregulating marine fishes, FW teleosts are subject to passive ion losses and osmotic water gain. The gill epithelium does not readily exhibit complexes of ionocytes and accessory cells with shallow tight junctions as seen in marine teleosts, and is considered very “tight” with respect to electrical resistance (Marshall, 1977; Sardet et al., 1979; Wood et al., 2002; Zhou et al., 2003; Chasiotis et al., 2012). FW-type ionocytes actively absorb NaCl from the dilute environment via transcellular routes that exchange mucosal Na^+ and Cl^- with serosal H^+ and HCO_3^- (see Dymowska et al., 2012; Kwong and Perry, 2012) or by the action of an apical Na^+ and Cl^- cotransporter (Ncc; Hiroi and McCormick, 2012).

Transmembrane tight junction proteins include claudins (Cldns), occludin and tricellulin, all of which have four trans-membrane spanning domains, two extracellular loops, and a cytosolic carboxy-terminal linked to the cytoskeleton through adaptor molecules (Hou et al., 2013). Members of the Cldn superfamily, with 27 mammalian isoforms, appear to determine the permeability and ion selectivity of tight junctions (Furuse et al., 1998; Hou et al., 2013). Site-directed mutagenesis and overexpression studies have shown that paracellular permeability of specific ions is controlled by charged amino acids in the first extracellular loop (Collegio et al., 2003; Hou et al., 2013). Therefore, the expression of specific Cldn isoforms controls the overall permeability and selective barrier functions critical to a specific tissue. Accordingly, the expression levels of various Cldns often change during development in tissue- and cell-specific manners (Loh et al., 2004; Tipsmark et al., 2008a; Baltzegar et al., 2013; Madsen et al., 2015). In Atlantic salmon (*Salmo salar*), spotted green puffer fish (*Tetraodon nigroviridis*) and Japanese medaka (*Oryzias latipes*), SW-acclimation entails the elevated expression of branchial *cldn10c*, *cldn10d* and *cldn10e* (Tipsmark et al., 2008a; Bui and Kelly, 2014; Bossus et al., 2015). Based on the amino acid sequences of their first extracellular loops, these paralogs could be involved in forming the cation-selective pathway associated with Na⁺ extrusion (Günzel et al., 2009; Bossus et al., 2015). Mammalian Cldn4 shares a common ancestor with 13 teleost Cldns (Baltzegar et al., 2013), some of which (Cldn28a and Cldn30) have salinity-dependent expression in the gill of Atlantic salmon and Mozambique tilapia. In both species, *cldn28a* and *cldn30* levels are elevated in FW-acclimated animals (Tipsmark et al., 2008a; Tipsmark et al., 2008b). Cldn30 is directly involved in the tightening of fish epithelia (Engelund et al., 2012; Kwong and Perry, 2013), while the sequence of Cldn28a suggests a similar capacity as its barrier-forming mammalian homologs (Hou et al., 2013; Bossus et al., 2015). Cortisol and prolactin directly decrease the permeability of primary gill cultures of trout epithelia (Kelly and Wood, 2002; Zhou et al., 2003). Accordingly, blood plasma levels of both hormones are elevated during the acute phase of FW acclimation in surveyed teleosts (McCormick, 2001), including tilapia (Kajimura et al., 2004). Furthermore, both cortisol (Atlantic salmon: Tipsmark et al., 2009; goldfish, puffer fish: Bui et al., 2010; Chasiotis and Kelly: 2011) and prolactin (Atlantic salmon: Tipsmark et al., 2009) up-regulate expression of specific gill *cldn* paralogs suggesting close endocrine control of epithelial permeability.

In the present study, we first identified *cldn* transcripts in the gill transcriptome of the congeneric Nile tilapia (*O. niloticus*) in order to probe how tight junctions are regulated by environmental salinity and hormones in Mozambique tilapia. To date, only one previous study has addressed the molecular physiology of tight-junctions in a cichlid (Tipsmark et al., 2008b). Our approach was to examine changes in gene expression of identified *cldns* following transfer from FW to SW, and *vice versa*. Then, hypophysectomy and subsequent replacement therapy trials with prolactin (Prl), growth hormone (Gh) and/or cortisol were conducted to assess the contribution of endocrine signaling to the expression of identified *cldns*. Lastly, we tested the *in vitro* effects of cortisol on *cldn* expression in incubated gill filaments.

2. Materials and Methods

2.1. Fish

For tissue distribution analyses, salinity-challenge experiments, and *in vivo* hormone replacement experiments, male Mozambique tilapia (*O. mossambicus*) were selected from a population maintained at the Hawai'i Institute of Marine Biology (HIMB; University of Hawai'i). Fish were maintained outdoors at 24-26 °C with a continuous flow of FW (municipal water: 0.98 mM Na⁺, 0.25 mM Ca²⁺, 0.01 mM Mg²⁺, 0.04 mM K⁺) under natural photoperiod and fed daily (Silver Cup Trout Chow, Nelson & Sons, Murray, UT). Some fish were acclimated to SW (Kāne'ohe Bay, HI; 34 ‰) for 4 weeks before the start of the FW-transfer experiment. The SW-transfer experiment was conducted with SW-naïve animals. The Institutional Animal Care and Use Committee of the University of Hawai'i approved all experimental protocols conducted at HIMB. For *in vitro* experiments, gill filaments were collected from tilapia maintained within the Department of Biological Sciences (University of Arkansas, Fayetteville). These fish were maintained indoors with recirculated FW at 22-24°C (dechlorinated municipal water: 0.34 mM Na⁺, 0.64 mM Ca²⁺, 0.09 mM Mg²⁺, 0.03 mM K⁺) under artificial photoperiod (14L:10D) and fed daily (Aquamax Starter Fingerling 300; PMI Nutrition International, Brentwood, MO). The Institutional Animal Care and Use Committee of the University of Arkansas approved all experiments at the University of Arkansas.

2.2. Experiments

2.2.1. Experiment 1: SW challenge

Fish (160-170 g) were held in 700 L tanks and salinity was changed from FW to brackish water (BW; 23 ‰) by opening a SW valve and adjusting the flow of incoming FW; 70% SW was reached after about 15 min. After 24 h, the FW valve was turned off to gradually raise the salinity to full-strength SW. This mode of SW-acclimation was adopted to reduce handling stress and to ensure survival (Breves et al., 2010). Control groups were maintained in FW for the entire experiment. Fish were sampled (n = 8) at 0, 1, 7 and 14 d after the initial transition to BW. All fish were fasted for the duration of the experiment. At the time of sampling, fish were netted and anesthetized with a lethal dose of 2-phenoxyethanol (2-PE; 2 mL L⁻¹, Sigma, St. Louis, MO). Fish were rapidly decapitated and 15-20 filaments from the first gill arch were frozen in liquid nitrogen, and stored at -80 °C until RNA isolation and analyses of gene expression.

2.2.2. Experiment 2: FW challenge

Fish (75-180 g) were held in 700 L tanks and acclimated to SW for more than 4 weeks. At the start of the experiment, salinity was changed to FW with minimal disturbance by switching inflow from a SW valve to a FW valve as described previously (Breves et al., 2010). FW conditions were reached after 60 min. Control groups were maintained continuously in SW. Fish were sampled at 0, 0.25, 1, 2 and 14 d after transition to FW (n = 8) as described above. Fish were fasted for the duration of the experiment.

2.2.3. Experiment 3: Hypophysectomy and hormone replacement in FW and SW

Hypophysectomy was performed by the transorbital technique developed by Nishioka (1994). First, tilapia were anesthetized by immersion in 2-PE (0.3 mL L⁻¹) 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) and fish were allowed to recover in BW (12 ‰). Following recovery, fish were transferred to re-circulating experimental aquaria containing aerated BW and treated with kanamycin sulfate (National Fish Pharmaceuticals, Tucson, AZ). Sham operations were carried out in the same manner, but without aspiration of the pituitary. Following a 3 d post-operative period in BW (12 ‰), fish (n = 6-14) were administered ovine Prl (oPrl; 5 µg g⁻¹ body weight), ovine Gh (oGh; 5 µg g⁻¹) or cortisol (1 µg g⁻¹), alone or in combination, by intraperitoneal injection concurrently with FW or SW acclimation. FW- and

SW-acclimations were conducted by adding FW or SW directly to re-circulating aquaria. In the first group, FW conditions were reached after 90 min and maintained for the duration of the experiment. In the second 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. oPrl and cortisol were purchased from Sigma; oGh was obtained from the National Hormone and Peptide Program (NIDDK-oGh-15, Torrance, CA). Hormones were delivered in saline vehicle (0.9% NaCl; 1.0 $\mu\text{L g}^{-1}$ body weight). The first injection occurred immediately prior to transfer to either FW or BW (23 ‰). 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 previously described. Sham-operated fish were injected with saline vehicle only. Fish were not fed for the duration of the recovery and transfer periods. At sampling, all fish were checked for completeness of hypophysectomy by post-mortem inspection of the hypothalamic region.

2.2.4. Experiment 4: *In vitro* gill incubation with cortisol

Incubation of gill filaments collected from FW-acclimated tilapia (see section 2.1) was performed as described by McCormick and Bern (1989) with only minor modifications (Tipsmark et al., 2009). Gill blocks of two to three filament pairs were dissected from the cartilage arch. Before incubation, initial gill samples (pre-incubation) were frozen in liquid nitrogen and stored at -80 °C until further analyses. Gill filaments were incubated for 16 h in 6-well Falcon plates with gentle shaking at room temperature in 3 mL Leibovitz's L15 medium (Gibco, Grand Island, NY) containing 10% FBS (HyClone Laboratories Inc., Logan, UT) and penicillin (100 IU mL^{-1} ; Gibco) and streptomycin (100 $\mu\text{g mL}^{-1}$; Gibco). Media contained cortisol-hemisuccinate sodium salt at 0, 1 or 10 $\mu\text{g mL}^{-1}$ (Sigma).

2.3. Analytical techniques

2.3.1. Extraction of RNA and cDNA synthesis

For the salinity transfer and hormone replacement experiments, total RNA was extracted by the Tri-Reagent procedure (MRC, Cincinnati, OH) according to the manufacturer's recommendation. Total RNA concentrations were determined by measuring A_{260} in duplicate with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

cDNA was synthesized by reverse transcribing 0.6 µg total RNA using qScript cDNA Supermix (Quanta, Gaithersburg, MD) according to the manufacturer's instructions. For *in vitro* experiments, total RNA was extracted using 500 µL of Tri-Reagent. Quantity and quality of RNA were assessed with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized from 1 µg of total RNA for each sample with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol.

2.3.2. Quantitative PCR

For the salinity transfer and hormone replacement experiments, quantitative PCR (qPCR) assays were run on a StepOnePlus real-time PCR system (Applied Biosystems). Reactions were carried out with Power SYBR Green PCR Master Mix (Applied Biosystems), cDNA amount equivalent to 15 ng total RNA, and 200 nM forward and reverse primers in a total volume of 15 µL. For *in vitro* experiments, mRNA levels were quantified by qPCR using a SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) kit in a C1000 Thermal Cycler, CF96 Real Time System (BioRad, Hercules, CA). All reactions were performed with an initial activation step of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. All primer sets were designed using Primer3 software (Rozen and Skaletsky, 2000) and checked using NetPrimer software (Premier Biosoft International, CA). Primer sequences were tested for non-specific product amplification and primer-dimer formation by melting curve analysis. Primers for *elongation factor 1a (ef1a)*, *acidic ribosomal phosphoprotein p0 (arp)* and *beta actin (actb)* have been previously described (Tipsmark et al., 2011). Additional primers were: *cldn10c* (Acc. No. XM_005452862; forward, TTCTGGTCTGCTCCACAATG; reverse, ATGCAGTCCTTCCACAGGTT; amplicon = 106 nt), *cldn10e* (Acc. No. XM_003447356; forward, GGCAATGGACTATTGGAGGA; reverse, CAGCCGCAGAATCTGTGTAA; amplicon = 116 nt), *cldn28a* (Acc. No. EU344800; forward, TCTCTGCTGGCTCTTCCTC; reverse, CAGCAATGCCCAGGATAACT; amplicon = 98 nt), and *cldn30* (Acc. No. EU344801; forward, GAGGGAAGTGCACCAACTGT; reverse, ACTGGGACAAGGCACAAAAC; amplicon = 106 nt). These four *cldn* transcripts were chosen for further analysis because they were specifically detected in the gill transcriptome of the Nile tilapia. Two of the paralogs, *cldn28a* and *cldn30*, have been described in a previous study on Mozambique tilapia (Tipsmark

et al., 2008b). The two remaining paralogs, *cldn10c* and *cldn10e*, were classified according to their sequence identities with their orthologs in Atlantic salmon (Tipsmark et al., 2008a) and Fugu (Loh et al., 2004) when using the tblastx function in GenBank. The phylogenetic relationships were further ascertained by maximum likelihood analysis (see Supplementary Data), as described previously (Tipsmark, 2008; Tipsmark et al., 2008a), using the SEQBOOT, PROML and CONSENSE, all programs in the PHYLIP package (Felsenstein, 1989). The GeNorm (version 3.5) tool was applied to determine a normalization factor from the examined reference genes (*ef1a*, *arp* and *actb*) and used to normalize expression of target genes (Vandesompele et al., 2002). Relative copy numbers of the target genes were calculated as E_a^{-Ct} , where Ct is the threshold cycle number and E_a is the amplification efficiency. Normalized units were obtained by dividing relative copy number of target genes with the determined normalization factor. Primer-dimer association was controlled with no template controls (NTC). As an alternative to DNase treatment, absence of genomic DNA amplification by qPCR was verified using total RNA samples instead of cDNA, and a no reverse transcriptase control (NRT control). Even organs with low Ct values represent expression, since controls were negative within the 40 PCR cycles applied.

2.4. Statistics

The tissue distribution, hypophysectomy, and *in vitro* experiments were analyzed with one-way analysis of variance (ANOVA), and differences between group means were assessed by Tukey's HSD test. The salinity transfer experiments were analyzed by a two-factorial ANOVA followed by Bonferroni-adjusted Fisher's LSD test. When required, data were transformed to meet the ANOVA assumption of homogeneity of variances. All statistical analyses were performed using GraphPad Prism 4.0 software (San Diego, CA) and significance was accepted when $P < 0.05$.

3. Results

3.1. Tissue distribution

A screening of the transcriptome for transmembrane tight junction proteins of the Cldn superfamily in the currently available Nile tilapia database (GenBank) revealed four Cldn paralogs: *cldn10c*, *cldn10e*, *cldn28a* and *cldn30*. All four *cldn* paralogs exhibited the highest

expression in gill tissue (Fig. 1). While these transcripts were detected in all tissues examined, branchial levels were 1-4 orders of magnitude higher than anywhere else. Like in FW-acclimated fish, these 4 paralogs also showed the highest expression levels in the gill of SW-acclimated fish (data not shown).

3.2. SW challenge

When FW-acclimated tilapia were transferred to SW, *cldn10c* expression increased 2-3 fold after 7 and 14 d (Fig. 2A). *cldn10e* expression increased 4 fold after 1 d and remained higher than FW controls for the remainder of the experiment (Fig. 2B). Transfer to SW induced a gradual decline in *cldn28a* expression that was significantly less than FW controls by 14 d (Fig. 2C); no significant changes in *cldn30* expression were observed (Fig. 2D).

3.3. FW challenge

When SW-acclimated tilapia were transferred to FW, *cldn10c* and *cldn10e* expression decreased by about 50% by 14 d (Fig. 3A,B). Significant increases in *cldn28a* and *cldn30* expression were observed by 2 d after transfer to SW; expression levels were markedly higher (10-15 fold) by 14 d (Fig. 3C,D).

3.4. Hypophysectomy and hormone replacement

In FW, hypophysectomy elicited decreases in *cldn10c*, *cldn10e* and *cldn28a* expression from sham-operated controls (Fig. 4A-C); *cldn30* was unaffected by hypophysectomy (Fig. 4D). Replacement with oPrl and cortisol, alone or in combination, rescued the effects of hypophysectomy on *cldn10c*, *cldn10e* and *cldn28a* transcripts; there were no significant effects of oGh alone or in combination with cortisol (Fig. 4A-C). The effects of oPrl and cortisol were neither additive nor synergistic. In SW, no significant effects of hypophysectomy or hormone treatment were detected for any *cldn* transcript (Fig. 5).

3.5. In vitro gill incubation with cortisol

In control gill filaments incubated for 16 h, we observed drops in *cldn10c* and *cldn10e* expression to about 10% and 20% of pre-incubation values, respectively (Fig. 6A,B). Meanwhile, *cldn28a* and *cldn30* were unchanged in controls compared with pre-incubation

expression (Fig. 6C,D). Consistent with *in vivo* patterns (Fig. 4), cortisol at 10 $\mu\text{g mL}^{-1}$ induced 2-5 fold increases in *cldn10c*, *cldn10e* and *cldn28a* (Fig. 6A-C); there was also an *in vitro* effect of cortisol on *cldn30* (Fig. 6D).

4. Discussion

This study examined transcriptional regulation of four tilapia Cldn paralogs in the gill following salinity changes and hormone treatments. We showed that *cldn10c* and *cldn10e* exhibit markedly higher expression in the gill of SW-acclimated fish suggesting a prominent role in ionocyte function in the marine environment. Alternatively, clear stimulation of *cldn28a* and *cldn30* levels after FW transfer implicates these Cldn paralogs as particularly important mediators of gill remodeling during FW acclimation. With respect to unraveling the hormonal control of these responses, our combined data suggest that *cldn10c*, *cldn10e* and *cldn28a* expression in FW is controlled, at least in part, by both Prl and cortisol.

While 27 Cldns have been described in mammals (Günzel and Yu, 2013), gene duplication events have led to more than 50 different paralogs in teleost fishes (Loh et al., 2004; Baltzegar et al., 2013). A number of these paralogs exhibit highly tissue-specific expression patterns (Loh et al., 2004; Baltzegar et al., 2013). Therefore, we used a targeted approach to select Cldn paralogs represented in the Nile tilapia gill library (GenBank). This provided us with four target genes for further analyses: *cldn10c*, *cldn10e*, *cldn28a* and *cldn30*. Our tissue distribution screening on gill samples from Mozambique tilapia confirmed robust expression of these four paralogs in tilapia gill (Fig. 1). Branchial expression of *cldn28a* and *cldn30* was previously observed in Mozambique tilapia (Tipsmark et al., 2008b), as similarly reported in both stenohaline zebrafish (*Danio rerio*; Baltzegar et al., 2013) and anadromous Atlantic salmon (Tipsmark et al., 2008a). The expression patterns of *cldn10c* and *cldn10e* in the current study are consistent with the branchial expression of several *cldn10* paralogs, including *cldn10c* and *cldn10e*, in a number of other teleosts (Loh et al., 2004; Tipsmark et al., 2008a; Bui et al., 2010; Kolosov et al., 2014). In euryhaline fishes, several Cldn10 paralogs are hypothesized to be involved in forming the paracellular conduit for Na^+ associated with SW-type ionocytes (Tipsmark et al., 2008a; Bui and Kelly, 2014; Bossus et al., 2015). Additional roles for Cldn10 paralogs in FW epithelia, and possibly in association with ionocyte function, was suggested by

studies of the FW stenohaline zebrafish, where *cldn10c* and *cldn10e* transcripts were only detected in gill tissue (Baltzegar et al., 2013).

If we look to the Cldn paralogs that may form cation selective pores, such as *cldn10c* and *cldn10e* (Günzel et al., 2009; Bossus et al., 2015), we observe elevations by 3-4 fold at 1-7 d after SW transfer (Fig. 2A,B). Meanwhile, FW transfer did not induce a drop in expression of *cldn10c* and *cldn10e* until 14 days (Fig. 3A,B). The SW-induced stimulation of *cldn10c* and *cldn10e* has not been previously reported in a cichlid fish. Compared with other teleosts, these observations parallel findings of *cldn10e* induction by SW-transfer, and during smoltification, of Atlantic salmon (Tipsmark et al., 2008a; Nilsen et al., 2013), *cldn10d* and *cldn10e* mRNA and protein expression in spotted green puffer (Bui and Kelly, 2014) and *cldn10c*, *cldn10d* and *cldn10e* in Japanese medaka (Bossus et al., 2015). This suggests that elevated expression of specific *cldn10* paralogs is a conserved response underlying SW acclimation by euryhaline teleosts.

The results of our SW to FW transfer experiments suggest that *cldn28a* and *cldn30* are involved in the transition of the gill epithelium from an ion-secreting, to an ion-absorptive tissue, as indicated by strong (6-15 fold) increases in these two transcripts within 1-2 d (Fig. 3C,D). This matches elevated *cldn28a* and *cldn30* expression previously observed 1 d after FW transfer of Mozambique tilapia (Tipsmark et al., 2008b). It seems these Cldn paralogs are key tight junction proteins in the gill of several fishes, given that both are found in gill transcriptomes of evolutionarily distant teleosts, such as Atlantic salmon (Tipsmark et al., 2008a) and Nile tilapia (GenBank). Furthermore, the zebrafish Cldn30 ortholog was localized to tight junctions between pavement cells and Na⁺ wasting is associated with partial knock-down of *cldn30* (Kwong and Perry, 2013). Similarly, salmon Cldn30 forms tight junctions that limit paracellular Na⁺ movement (Engelund et al., 2012). Cldn28 and Cldn30 are orthologous to Class VI claudins, which are related to mammalian Cldn3 and -4 (Loh et al., 2004; Baltzegar et al., 2013). Cldn3 and -4 are known to form cation selective sealing junctions; this function depends on the amino acid compositions of their first extracellular loops (Hou et al., 2013). These amino acid sequences are conserved in medaka Cldn28a and Cldn30 (Bossus et al., 2015), as well as Atlantic salmon and Nile tilapia Cldn28a and Cldn30. Thus, we suggest a similar physiological role for these teleost paralogs as their mammalian orthologs. Cldn paralogs with the capacity to form tight seals are expected to dominate between individual pavement cells and in the junctions

of pavement cells and ionocytes in both FW- and SW-acclimated fish. Putative “leaky” shallow tight junctions are only observed in SW-acclimated fish between neighboring ionocytes and ionocytes associated with accessory cells (Sardet et al., 1979). These junctions may specifically contain Cldn10 paralogs given the stimulated expression of *cldn10s* after SW transfer (Tipsmark et al., 2008a; Bui and Kelly, 2014; Bossus et al., 2015). Since the majority of surface cells in the gill epithelium are pavement cells, irrespective of salinity, the expression of putative “tightening” Cldns like *cldn28a* and *cldn30* may remain stable when measuring overall gill expression since “tight” inter-pavement cell junctions would be adaptive in both FW and SW residing fish. In this regard, Cldns28a and -30 would limit passive solute and water fluxes. In the current study (Fig. 3C,D), and in previous experiments (Tipsmark et al., 2008b), we saw an increase in gill *cldn28a* and *cldn30* expression after FW transfer similar to observations in Atlantic salmon (Tipsmark et al., 2008a). By contrast, salinity changes had no effect on these orthologs in Japanese medaka (Bossus et al., 2015). It therefore seems likely that species-specific differences exist in the regulation of *cldn28a* and *cldn30*. At least in tilapia, we suggest that *cldn28a* and *cldn30* play critical roles in both FW and SW, and that rapidly elevated expression after FW transfer contributes to reorganization of branchial epithelium during the initial phase of FW acclimation.

An interesting pattern observed in the current study was that hypophysectomized animals in FW exhibited reduced expression of *cldns* that are induced by both SW (*cldn10c* and *cldn10e*) and FW (*cldn28a*; Fig. 4A-C). The Prl and cortisol effects on SW-induced *cldns* suggest that baseline *cldn10c* and *cldn10e* expression in FW is highly dependent on pituitary factors. In zebrafish and rainbow trout (*Oncorhynchus mykiss*), indirect evidence suggests that Cldn10 paralogs are primarily expressed in FW-type ionocytes (Baltzegar et al., 2013; Kolossov et al., 2014), which is confirmed by direct localization studies in puffer fish (Bui and Kelly, 2014). The apparent regulation of *cldn10c* and *cldn10e* by Prl in tilapia parallels the regulation of *ncc* and *nka a1a* (Breves et al., 2010; Tipsmark et al., 2011; Breves et al., 2014; Inokuchi et al., 2015). Thus, we suggest that while *cldn10c* and *cldn10e* paralogs play a role in SW, they may also be co-expressed in FW-type ionocytes. Their specific role here remains speculative at present but non-canonical roles in the microtubular network or basolateral membrane are plausible (Alshbool and Mohan, 2014; Tipsmark, unpublished observations). Alternatively, their role in SW-ionocytes in regard to cation conduction may differ from their barrier functions in FW-ionocytes due to heterotypic trans or heteromeric cis interactions with other Cldn paralogs

(Coyne et al., 2003; Hou et al., 2013). Hypophysectomy did not impact *cldn10c* and *cldn10e* following SW transfer (Fig. 5A,B). This pattern is in accord with our previous findings that expression of *nkcc* and *nka α1b*, hallmarks of SW-type ionocytes, is insensitive to hypophysectomy (Breves et al., 2010; Tipsmark et al., 2011). In other words, Mozambique tilapia have the capacity to induce expression of particular genes underlying SW acclimation without pituitary mediation. Studies in cultured epithelia derived from FW-acclimated fish have shown that cortisol consistently induces, and maintains, high epithelial conductance (Kelly and Wood, 2002; Zhou et al., 2003; Sandbichler et al., 2011). The effects of Prl in these systems is less straightforward, but Prl generally stimulates early barrier function of the surrogate epithelia and enhances cortisol-related effects (Kelly and Wood, 2002; Zhou et al., 2003). Our gill incubations revealed enhanced gene expression of putative barrier-forming Cldns (*cldns28a* and *cldns30*) by cortisol (Fig. 6C,D). Interestingly, *cldn10c* and *cldn10e* expression plummeted during overnight incubation; this effect was either partially (*cldn10c*), or fully (*cldn10e*), counteracted by cortisol. These patterns parallel *in vitro* studies employing Atlantic salmon gill filaments where *cldn10e* and *cldn30* were stimulated by cortisol. Moreover, overnight incubation without cortisol induced a similar drop-off in the expression of the salmon *cldn10e* (Tipsmark et al., 2009). We propose that *cldn10c* and *cldn10e* may be expressed in ionocytes because of a similar dependence upon cortisol for the maintenance of ionocyte populations, in both surrogate cell culture and explant systems (McCormick and Bern, 1989; Tipsmark et al., 2002; Zhou et al., 2003; Kiilerich et al., 2007). Future work entailing promoter analysis is required to also assess whether *cldn* expression is activated via steroid response elements.

In summary, our present data suggest important roles for *cldn28a* and *cldn30* during FW-acclimation, and *cldn10c* and *cldn10e* during SW acclimation, in a euryhaline cichlid. While Cldn10 paralogs are strongly up-regulated in SW suggestive of a putative role in forming cation pores associated with SW-type ionocytes, they also appear important to the function of branchial epithelium in FW. The yet to be defined roles of *cldn10c* and *cldn10e* in FW-type ionocytes are likely under the control of cortisol and Prl. It will be imperative for future studies to examine subcellular Cldn localization in combination with protein pull-down approaches to establish possible trans and cis intramolecular interactions in both FW- and SW-type ionocytes.

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Figure Captions

Figure 1. Relative expression of *cldn10c* (A), *cldn10e* (B), *cldn28a* (C) and *cldn30* (D) mRNA in gill, esophagus, stomach, anterior intestine (Ant-int), middle intestine (Mid-int), posterior intestine (Post-int), kidney, skeletal muscle (Muscle), liver, and brain in FW-acclimated tilapia. Groups that do not share letters are significantly different ($P < 0.05$). Means $\pm$ S.E.M. ($n = 4-5$) are presented as a percent of gill expression levels.

Figure 2. Effects of transfer from FW to SW on gill mRNA expression of *cldn10c* (A), *cldn10e* (B), *cldn28a* (C) and *cldn30* (D). FW-acclimated tilapia were transferred to SW (FW-SW; filled circle and solid line), while control fish were maintained in FW (FW-FW; open circle and dotted line). For *cldn10c*, there was an overall effect of time (**), salinity (***) and a significant interaction between factors (*). For *cldn10e*, there was an overall effect of time (***), salinity (**) and a significant interaction between factors (*). For *cldn28a*, there was an overall effect of time (*), salinity (*) and a significant interaction between factors (*). For *cldn30*, there was an overall effect of time (*) and a significant interaction between factors (*). Asterisks indicate significant differences from time-matched controls (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Means $\pm$ S.E.M. ($n = 7-8$) are presented as a percent of the FW control at time 0.

Figure 3. Effects of transfer from SW to FW on gill mRNA expression of *cldn10c* (A), *cldn10e* (B), *cldn28a* (C) and *cldn30* (D). SW-acclimated tilapia were transferred to FW (SW-FW; filled circle and solid line), while control fish were maintained in SW (SW-SW; open circle and dotted line). For *cldn10c*, there was an overall effect of time (**) and a significant interaction between factors (**). For *cldn10e*, there was a significant interaction between factors (**). For *cldn28a*, there was an overall effect of time (***), salinity (***) and a significant interaction between factors (***). For *cldn30*, there was an overall effect of time (***), salinity (***) and a significant interaction between factors (**). Asterisks indicate significant differences from time-matched controls (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Means $\pm$ S.E.M. ($n = 7-8$) are presented as a percent of the SW control at time 0.

Figure 4. Effects of hypophysectomy (Hx: shaded bars) and hormone replacement with oPrl, oGh and/or cortisol (Cort) on gill mRNA expression of *cldn10c* (A), *cldn10e* (B), *cldn28a* (C) and *cldn30* (D) in tilapia transferred to FW. Fish received two intraperitoneal injections of saline (sham operated (open bars) and control groups), oPrl (5 µg g⁻¹) or oGh (5 µg g⁻¹) alone or in combination with Cort (1 µg g⁻¹). The first injection occurred immediately prior to transfer from brackish water (12 ‰) to FW, and the second occurred after 24 h. Fish were sampled 3 days after the initial transfer to FW. Groups that do not share letters are significantly different (P < 0.05). Means + S.E.M. (n = 6-8) are presented as a percent of the sham group.

Figure 5. Effects of hypophysectomy (Hx) and hormone replacement with oPrl, oGh and/or cortisol (Cort) on gill mRNA expression of *cldn10c* (A), *cldn10e* (B), *cldn28a* (C) and *cldn30* (D) in tilapia transferred to SW. Fish received two intraperitoneal injections of saline (sham and control groups), oPrl (5 µg g⁻¹) or oGh (5 µg g⁻¹) alone or in combination with Cort (1 µg g⁻¹). The first injection occurred immediately prior to transfer from brackish water (12 ‰) to SW, and the second occurred after 24 h. Fish were sampled 3 days after the initial transfer to SW. Groups that do not share letters are significantly different (P < 0.05). Means + S.E.M. (n = 6-8) are presented as a percent of the sham group.

Figure 6. *In vitro* effects of cortisol (Cort) on *cldn10c* (A), *cldn10e* (B), *cldn28a* (C) and *cldn30* (D) mRNA expression in gill filaments incubated for 16 h. Treatments that do not share letters are significantly different (P < 0.05). Means + S.E.M. (n = 9-10) are presented as a percent of the pre-incubation (Pre-inc) levels.

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