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Research Article

The effects of tidally changing salinities on branchial tight junction protein gene expression in tilapia

G.H.T. Malintha [a,1], Ke Cao [a,1], Fritzie T. Celino-Brady [a,2], Jason P. Breves [b], Andre P. Seale [a,*]

[a] Department of Human Nutrition, Food and Animal Sciences, University of Hawai'i at Mānoa, Honolulu, HI 96822, USA
[b] Department of Biology, Skidmore College, Saratoga Springs, NY 12866, USA

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ABSTRACT

To maintain hydromineral balance during transitions between different environmental salinities, euryhaline teleosts adjust the permeability characteristics of their surface epithelia to align with ambient conditions. Tight junctions (TJs), which form the apical-lateral barriers between epithelial cells, control the paracellular movement of solutes and water. Consequently, euryhaline species must efficiently reorganize branchial TJs when acclimating to freshwater (FW) and seawater (SW) environments. However, little is known about how TJs are regulated under tidally changing salinities. In the current study, we employed RNA-Seq to identify several branchial TJ transcripts that are differentially expressed in Mozambique tilapia (*Oreochromis mossambicus*) acclimated to FW, SW, or a tidal regime (TR). The most abundant and differentially expressed TJ transcripts, which included claudins (*cldn-4 like*, *cldn7b*, *cldn23a*, and *cldn-like ZF-A89*), occludins (*oclna* and *oclnb*), and TJ proteins (*tjp1a* and *tjp3*), were further investigated by qPCR. Generally, the expression of TJ transcripts varied to a greater extent between the FW and SW phases of the TR (denoted TF and TS, respectively) than between steady-state FW and SW conditions. Furthermore, TJ transcripts were usually upregulated in TF compared to TS or SW controls. In contrast, the gene expression of branchial ion transporters did not change as markedly under a TR. Together, these results suggest that TJs play a crucial role in regulating the permeability of branchial epithelia by preventing ion loss in hyposmotic conditions, particularly during short-term tidally changing salinities.

1. Introduction

Euryhaline fishes can maintain osmotic homeostasis in the face of variable environmental salinities. Because of their tolerance to a broad range of salinities, which span from fresh water (FW) to seawater (SW), euryhaline species have been widely employed to study the physiological mechanisms that underlie salinity acclimation in fishes (Evans and Claiborne, 2009; Marshall et al., 1999; Seale et al., 2024). The euryhaline Mozambique tilapia (*Oreochromis mossambicus*) is naturally distributed in water bodies that experience regular fluctuations in salinity, such as the lower Zambezi River and its delta (Shelton and Popma, 2006; Trewavas, 1983). This estuarine system has the highest tidal range in the Western Indian Ocean, with significant salinity variations documented up to 25 km upstream (Banda et al., 2019; Brockway et al., 2006; Hoguane et al., 2020; Sitoe et al., 2024). Thus, the suitability of Mozambique tilapia as a model for studying salinity-driven physiological changes can also be extended to include frequent, tidally driven salinity fluctuations. Importantly, the rate at which salinity rises is crucial in determining whether individuals can tolerate SW and higher salinities. Besides being less likely to survive rapid (3h) unidirectional transitions from FW to SW, the survival rates of fish transferred to SW are inversely related to age (Inokuchi et al., 2021). Mozambique tilapia have been studied using various salinity-change paradigms, including transfers from FW to SW, SW to FW, and a simulated tidal regime (TR), characterized by alternating transitions from FW to SW and vice versa every 6 h (Seale and Breves, 2022). Despite facing constantly changing conditions, Mozambique tilapia subjected to a TR maintained plasma osmolality within a narrow range, between ~320 mOsm/kg during the FW phase of the cycle (TF) and ~ 340 mOsm/kg during the SW phase (TS; Moorman et al., 2015). Moreover, Mozambique tilapia reared in a

* Corresponding author at: Department of Human Nutrition, Food and Animal Sciences, University of Hawai'i at Mānoa, 1955 East-West Road, Honolulu, HI 96822, USA.
E-mail address: seale@hawaii.edu (A.P. Seale).
[1] Authors contributed equally.
[2] Current address: Division of Genetics, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR 97006, USA.

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TR grew faster than those acclimated to steady-state FW or SW (Moorman et al., 2016). In turn, the question arises as to how tilapia regulate the movement of ions and water across epithelial surfaces when exposed to frequently changing salinities.

While various organs, including the kidney, intestine, skin, and gill, are critical for maintaining salt-and-water balance in teleosts, the gill is the primary site of active Na^+ and Cl^- transport (Evans et al., 2005; Kaneko et al., 2008). Within the branchial epithelium, ionocytes are key targets for both endocrine and environmental regulation of ion-transport processes (Breves and Shaughnessy, 2024; Hiroi et al., 2005; McCormick and Bradshaw, 2006). In Mozambique tilapia, for instance, ion transporters and channels involved in ion absorption are upregulated in response to the FW-adapting hormone prolactin or down-regulated under hyperosmotic conditions, which also promote the activation of ion-secretory processes (Breves et al., 2017, 2010; Inokuchi et al., 2015).

Like ion transporters and channels, branchial tight junction (TJ) proteins are also expressed in salinity-dependent manners. TJs form paracellular barriers or pores between cellular junctions to regulate the paracellular movement of solutes across the branchial epithelium (Chasiotis et al., 2012a; Hou et al., 2013). Adjacent cells connect through transmembrane proteins from two major TJ protein families, claudins (Cldns) and TJ-associated MARVEL proteins (TAMPs), which include occludins (Oclns) (Raleigh et al., 2010). Cldns, Oclns, and TJ-associated "scaffold" or "adaptor" proteins play key roles in supporting osmoregulation in fishes (Chasiotis et al., 2012b; González-Mariscal et al., 2003; Günzel and Yu, 2013; Kolosov and Kelly, 2013; Kolosov et al., 2013; Kolosov et al., 2014), with TJ proteins such as Cldn7, Cldn12, and Cldn3a restricting solute loss and water influx during FW acclimation, while Cldn15 aids SW acclimation by forming the paracellular pathway for Na^+ extrusion (Bagherie-Lachidan et al., 2009; Bui and Kelly, 2014; Chasiotis et al., 2009; Chasiotis et al., 2012b, Marshall et al., 2018; Tipsmark et al., 2020; Rosenthal et al., 2020). In Mozambique tilapia, except for *cldn10c* and *cldn10e*, branchial claudins (e.g., *cldn28a*, *cldn30*, *cldn3-like*, and *cldn 4-like*) are generally upregulated when fish transition from SW to FW; these responses align with the restriction of ion permeability (Tipsmark et al., 2008a, 2016). Since these *cldn* expression patterns have been characterized in fish subjected to unidirectional, one-time salinity changes, the transcriptional regulation of TJ protein-encoding genes in response to frequent salinity changes remains unresolved.

The use of tidally changing salinity paradigms that simulate estuarine environments has thus far offered insights into how tilapia respond to dynamic environmental conditions. For example, branchial *Na^+/Cl^- cotransporter 2* (*ncc2*; *slc12a10*), *$Na^+/K^+/2Cl^-$ cotransporter 1a* (*nkcc1a*; *slc12a2a*), and *cystic fibrosis transmembrane conductance regulator 1* (*cftr1*) transcripts are differentially expressed between FW- and SW-acclimated fish, and in many instances, between the FW and SW phases of a TR (Moorman et al., 2014, 2015; Pavlosky et al., 2019; Seale et al., 2019). Moreover, the gene expression of receptors for prolactin, growth hormone, and cortisol also fluctuates in parallel with tidal oscillations in salinity (Chang et al., 2023; Moorman et al., 2016; Seale et al., 2019). To the extent that changes in paracellular permeability contribute to salinity acclimation, TJ protein expression patterns may follow ion channels and transporters that oscillate under a TR. We hypothesized that the abundance of TJ proteins would be presumably adjusted to regulate the permeability of branchial epithelia during rapid, cyclical salinity changes associated with a TR, as part of a strategy that enables euryhaline tilapia to maintain homeostasis. We initially adopted an RNA-Seq approach to identify TJ protein transcripts expressed in the branchial transcriptomes of Mozambique tilapia acclimated to FW, SW, and a TR (in TF and TS). We sought to identify differentially expressed *cldns*, *oclns*, and TJ-associated genes. To further resolve the gene expression patterns identified in the RNA-Seq analysis, we conducted targeted qPCR analyses to measure the most abundant and differentially expressed branchial TJ-associated transcripts.

2. Methods

2.1. Animals

Male Mozambique tilapia were obtained from stocks maintained at the Hawai'i Institute of Marine Biology, University of Hawai'i (Kaneohe, HI). Fish were reared in outdoor tanks with a continuous flow of FW or SW under natural photoperiod and fed to satiety once a day with trout chow pellets (Skretting, Tooele, UT). All experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the University of Hawai'i.

2.2. Experimental design

Adult (222.3 ± 11.5 g) male Mozambique tilapia ($n = 16$/tank) were held at 25 ± 2 °C under natural photoperiod in outdoor 700-L tanks supplied with FW (0.1 ± 0.1 ‰, municipal water) or SW (34 ± 1 ‰, Kaneohe Bay, Hawai'i). Two flow-through SW tanks and one flow-through FW tank were used for this experiment. Briefly, incoming water to one of the SW tanks was adjusted to maintain a TR by alternating the inflow of FW and SW every 6 h (Moorman et al., 2014). In the TR tank, salinity changed completely between SW and FW, and vice versa, within 2 h of switching the water source, as previously reported for this experimental paradigm (Pavlosky et al., 2019; Seale et al., 2019). Control tanks were maintained at a constant salinity throughout the experiment. Fish were held in FW, SW, and TR tanks for 15 days. Eight fish from each of the FW- and SW-controls were sampled 15 days following transfer to a TR. In the TR tank, 8 fish were sampled at the end of TF, and 8 fish were sampled 6 h later, at TS. Fish were fed trout chow pellets (Skretting, Tooele, UT) once daily to satiation throughout the experiment and fasted for 24 h prior to sampling.

At the time of sampling, fish were netted from their respective tanks and anesthetized with 2-phenoxyethanol (0.3 mL/L; Sigma-Aldrich, St. Louis, MO). Following anesthesia, fish were weighed and euthanized. Gill filaments from the second gill arch (left side) were excised, frozen in liquid nitrogen, and stored at −80 °C.

2.3. RNA sequencing

Total RNA was extracted from gill filaments using TRI Reagent following the manufacturer's protocol (MRC, Cincinnati, OH). Three individual total mRNA samples from each salinity group, FW, SW, and SW transferred to TR and sampled in either TF or TS (designated TF and TS, respectively) were provided to Azenta Life Sciences (South Plainfield, NJ) where quality was determined via TapeStation and Qubit Assay before library preparation and sequencing on an Illumina Novaseq 6000. RNA samples with RIN > 8.0 were used in sequencing and the reads were trimmed to remove possible adapter sequences and nucleotides of poor quality using Trimmomatic V.0.36 (Bolger et al., 2014). After passing quality control assessment with FASTQC software (V. 0.11.9, Babraham Bioinformatics - FastQC A Quality Control Tool for High Throughput Sequence Data), the trimmed reads were mapped to the Nile tilapia (O_niloticus_UMD_NMBU) reference genome available on ENSEMBL using the STAR aligner V.2.5.2b (Dobin et al., 2013). Unique gene hit counts were calculated using FeatureCounts (Liao et al., 2014) from the Subread package V.1.5.2. Resulting gene hit counts were used for downstream differential expression analysis. Using DESeq2 (Love et al., 2014), comparisons of gene expression patterns between four groups (FW vs. SW, SW vs. TF, SW vs. TS, and TF vs. TS) were performed. Data quality parameters are described in supporting quality reports available through NCBI GEO (Accession GSE305001). Transcripts representing all TJ protein families, and with the highest copy numbers across all treatment comparisons, were selected for qPCR analysis. ENSEMBL gene IDs were converted to Entrez IDs and common gene IDs using the NCBI Biomart tool (Kasprzyk, 2011).

2.4. Quantitative real-time PCR (qPCR)

Total RNA was extracted from gill samples as described above. Total RNA (500 ng) was reverse transcribed using a cDNA reverse transcription kit (Cat. No.: 4368814; Thermo Fisher Scientific, Waltham, MA). The levels of reference and target genes were determined using the relative quantification method, in which relative expression levels and PCR efficiencies were calculated from standard curves generated from serial dilutions of gill cDNA using a StepOnePlus real-time qPCR system (Thermo Fisher Scientific). The PCR reactions (15 μL) contained SYBR Green PCR Master Mix (Cat. No.: 4367659; Applied Biosystems), 200 nmol/L forward and reverse primers, and 1 μL of cDNA. The PCR cycling parameters were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Gene-specific primers for TJ protein transcripts were designed using NCBI reference sequences and Primer3 (Kõressaar et al., 2018), and their specificities were confirmed by melt curve analysis (Table 1) and through sequencing the purified PCR products. Primer pairs for *ncc2*, *nkcc1a*, and *cftr1* have been previously described (Inokuchi et al., 2008; Moorman et al., 2014). Standard curves were utilized for all genes. The geometric mean of three reference genes (*ef1α*, *18s,* and *β-actin*) was used to normalize the expression levels of all target genes (Breves et al., 2010; Magdeldin et al., 2007; Tipsmark et al., 2011). The relative expression of target genes was calculated employing the Pfaffl method (Pfaffl, 2001).

2.5. Statistical analyses

To identify genes differentially expressed between groups, the Wald test was used in DESeq2 to generate *P*-values and $\log_2$ fold changes. The Benjamini-Hochberg method was applied to control the false discovery rate (FDR) by adjusting multiple comparisons. Genes with an adjusted *P*-value <0.05 and an absolute $\log_2$ fold change >1 were categorized as differentially expressed genes (DEGs). Analyses of relative mRNA expression were conducted by one-way ANOVA with salinity treatment as the main effect. Significant effects ($P < 0.05$) were followed up by protected Fisher's LSD test. Assumptions for one-way ANOVA were confirmed using the Shapiro-Wilk test for normality and the Brown-Forsythe test for equal variances. One dataset (*cldn-like ZF-A89*) was heteroscedastic and was therefore log-transformed to meet the assumption of homogeneity of variances. Data are expressed as mean fold changes ± S.E.M. from the FW control group. Statistical analyses of RNA-Seq data were performed using R Studio, while qPCR data were analyzed with Prism 10 (GraphPad Prism 10, La Jolla, CA).

Table 1
Newly designed primer sequences used in quantitative real-time PCR.

Target Gene	Primer Sequence (5′ to 3′)	Accession No.
cldn4-like	F: CCTCATGGCTGTGATCGGAG R: ATCACTCCAGCCACAATGCA	XM_019363620.2
cldn-like ZF-A89	F: GGACCATCATCATTTGCGCC R: AGATAATCTGCGCCGTCACA	XM_003459444.4
cldn7b	F: GTCTGGGTGTTGCTTGCATG R: ATTCCACCACCCATTGCAGT	XM_019363905.2
cldn23a	F: TACAGCAGCAGTAACCCAGC R: TAACGAAACCGCCCAAGACT	XM_005451396.4
oclna	F: CCGCAGTCCTCAGCACTATT R: CAGGAAGCCAAACACAACGG	XM_013276229.3
oclnb	F: CGCCGATCATCACACAGAGT R: GGCTGGGTTGGACATGACTT	XM_025897007.1
tjp1a	F: GAGAGGTCAAAGGGCAAGCT R: GCTGTCGTCAAGGTCAGGAA	XM_025906015.1
tjp3	F: ATCACCACCAAGCAGTCCAG R: CCCATTCCATATCGTCCGCA	XM_013267128.3

3. Results

3.1. RNA-Seq analysis

Sequencing generated 480,779,740 raw reads, with an average of 90.7 % of bases ≥ Q30, corresponding to ≥99.9 % base call accuracy, indicating high sequencing quality. After QC, RNAseq yielded a total of 448,403,403 trimmed reads, of which 414,811,622 were mapped. Trimmed reads ranged from a minimum of 36 bp to 150 bp. Raw sequencing data, sequencing metrics, per-base quality, GC content plots, and mapping statistics are available in NCBI GEO (Accession GSE305001).

Differential expression analysis of the branchial transcriptomes yielded transcripts that varied significantly between salinity treatments. Among the differentially expressed genes, all transcripts encoding TJ proteins are listed in Table 2. Relative comparisons across salinity treatments are shown in heat maps (Fig. 1). Three TJ protein genes were differentially expressed in the comparison between FW vs. SW, 16 in TF vs. SW; 14 in TS vs. SW; and 14 in TF vs. TF. The TF vs. SW and TF vs. TS comparisons shared 8 differentially expressed transcripts (Fig. 2). Among them, *cldn-like ZF-A89* and *cldn7b* showed similar patterns of expression with TF at levels 4-fold higher than SW or TS. Interestingly, *cldn23a*, the TJ transcript with the highest copy number in the TS vs. SW comparison, was also common to TF vs. SW and TF vs. TS, with a similar fold-change difference. Two occludins, *oclna* and *oclnb*, and a cytosolic protein, *tjp3,* were common to the TF vs. SW and TF vs. TS comparisons. Transcripts that were differentially expressed in more than one of the four comparison groups with more than 1000 hit counts were selected for further gene expression analysis by qPCR.

3.2. Effects of tidally changing salinity on branchial ion transporters

The expression patterns of *ncc2*, *nkcc1a*, and *cftr1* in tilapia reared in FW, SW, and TR for 15 days are provided in Fig. 3. *ncc2* expression was higher in fish acclimated to FW compared with those in SW while *ncc2* levels in both tidal phases of the TR were between FW and SW levels (Fig. 3A). By contrast, *nkcc1a* expression was higher in both phases of TR and in SW-acclimated fish compared with those in FW. Similarly, *cftr1* expression was higher in SW versus FW fish; *cftr1* was also higher in fish sampled in TS compared with TF (Fig. 3C).

3.3. Effects of tidally changing salinity on branchial TJ proteins

Significant effects of salinity were observed in branchial TJ protein transcripts in tilapia reared in FW, SW, and TR for 15 days (Fig. 4). The expression of *cldn4-like* was higher in fish sampled in FW and TF, compared with SW (Fig. 4A). Moreover, *cldn4-like* expression in TS was similar to SW. Branchial expression of *cldn7b*, *cldn23a*, and *cldn-like ZF-A89* was higher in both TR groups compared with FW and SW. Even though there were no significant differences between FW and SW, *cldn7b*, *cldn23a*, and *cldn-like ZF-A89* expression levels in TS were higher than those in the SW group (Figs. 3B, C, D). In addition, *cldn7b*, *cldn23a*, and *cldn-like ZF-A89* were upregulated in fish sampled in TF compared with those in TS (Figs. 3B, C, D). After 15 days, *oclna* expression was similar between FW and SW fish. Both *oclna* and *oclnb* expression levels in TS did not differ from SW. However, *oclna* and *oclnb* were upregulated in the TF group relative to steady-state treatments and TS (Figs. 4E, F). While *tjp1a* expression was similar among the FW, SW, and TS groups after 15 days, it was upregulated in TF (Fig. 4G). Similarly, there were no differences in *tjp3* expression between SW, FW, and TS; however, *tjp3* was upregulated in TF relative to all other groups (Fig. 3H).

4. Discussion

This study characterized the effects of tidally changing salinities on the gene expression of branchial TJ proteins in Mozambique tilapia. A

Table 2
Transcript information and hit counts from the four treatment comparisons included in the differential expression analysis.

Ensemble ID	Gene ID	Gene name	Log_2 Fold Change	Absolute FC of average hit counts
FW vs. SW				
ENSONIG00000001780	*cldnd1a*	claudin domain-containing protein 1	−2.00	4.00
ENSONIG00000011879	*cldn10-like*	claudin-10-like	1.02	2.02
ENSONIG00000021097	*cldn4-like*	claudin-4-like	−2.42	5.34
TF vs. SW				
ENSONIG00000021097	*cldn4-like*	claudin-4-like	3.48	11.16
ENSONIG00000001780	*cldnd1a*	claudin domain-containing protein 1	2.76	6.78
ENSONIG00000020464	*cldna*	claudin-4	1.85	3.62
ENSONIG00000020432	*cldn5a*	claudin 5	−1.52	2.86
ENSONIG00000035101	*cldn15lb*	claudin 1	−2.06	4.16
ENSONIG00000001856	*cldn7b*	claudin-7-A	2.05	4.15
ENSONIG00000011260	*oclna*	occludin	1.58	2.99
ENSONIG00000021405	*cldn8*	claudin-8	1.58	2.98
ENSONIG00000015796	*jam3b*	junctional adhesion molecule C	−1.45	2.74
ENSONIG00000010453	*oclnb*	occludin	1.30	2.46
ENSONIG00000002505	*tjp3*	tight junction protein ZO-3	1.25	2.39
ENSONIG00000021458	*put. cldn24*	putative claudin-24	−2.70	6.56
ENSONIG00000005879	*cldn-likeZF-A89*	claudin-like protein ZF-A89	2.44	5.41
ENSONIG00000020635	*cldn23a*	claudin 23a	2.08	4.22
ENSONIG00000021100	*cldn4*	claudin 4	1.87	3.65
ENSONIG00000021309	*cldn4*	claudin-4	−1.84	3.59
TS vs. SW				
ENSONIG00000021309	*cldn4*	claudin-4	−1.49	2.81
ENSONIG00000035598	*cldn14*	claudin 14	3.28	9.72
ENSONIG00000009315	*cldn15a*	claudin 15	2.86	7.27
ENSONIG00000008756	*cldnc*	claudin c	2.71	6.54
ENSONIG00000001780	*cldnd1a*	claudin domain-containing protein 1	2.52	5.74
ENSONIG00000008477	*cldn10l2*	claudin-10	2.28	4.86
ENSONIG00000009826	*cldni*	claudin i	−1.48	2.79
ENSONIG00000035101	*cldn15lb*	claudin 1	−1.43	2.69
ENSONIG00000020432	*cldn5a*	claudin 5	−1.35	2.56
ENSONIG00000010100	*ocldn*	occludin	−1.08	2.11
ENSONIG00000020635	*cldn23a*	claudin 23a	1.04	2.06
ENSONIG00000015796	*jam3b*	junctional adhesion molecule C	−1.01	2.01
ENSONIG00000008472	*cldn10*	claudin-10	2.13	4.37
ENSONIG00000021458	*put. cldn24*	putative claudin-24	−1.36	2.57
TF vs. TS				
ENSONIG00000021097	*cldn4-like*	claudin-4-like	−2.67	6.37
ENSONIG00000009315	*cldn15a*	claudin 15	2.04	4.16
ENSONIG00000005879	*cldn-likeZF-A89*	claudin-like protein ZF-A89	−1.74	3.34
ENSONIG00000011260	*oclna*	occludin	−1.63	3.09
ENSONIG00000002505	*tjp3*	tight junction protein ZO-3	−1.57	2.96

Table 2 (*continued*)

Ensemble ID	Gene ID	Gene name	Log_2 Fold Change	Absolute FC of average hit counts
ENSONIG00000009826	*cldni*	claudin i	−1.55	2.93
ENSONIG00000001856	*cldn7b*	claudin-7-A	−1.49	2.81
ENSONIG00000005621	*cldn17*	claudin-8-like	1.46	2.76
ENSONIG00000006221	*tjp1a*	tight junction protein ZO-1	−1.45	2.73
ENSONIG00000021458	*put. cldn24*	putative claudin-24	1.36	2.59
ENSONIG00000010453	*oclnb*	occludin	−1.29	2.44
ENSONIG00000021100	*cldn4*	claudin 4	−1.14	2.20
ENSONIG00000020464	*cldna*	claudin-4	−1.03	2.04
ENSONIG00000020635	*cldn23a*	claudin 23a	−1.01	2.01

transcriptomic approach was employed to identify TJ-associated transcripts differentially expressed between steady-state and tidally changing salinities. By comparing the expression of selected target genes, we found that the most abundant TJ-associated transcripts responded differently between steady-state and TR salinities. TJ protein transcripts were strikingly sensitive to the frequent salinity shifts associated with a TR and were generally upregulated in TF compared with TS. Our results indicate that TJ proteins are transcriptionally regulated in a fashion that reflects the rapid shifts in salinity that occur with a TR, suggesting that, collectively, they play key roles in salinity acclimation.

Our previous studies started to resolve the physiological responses of tilapia to tidal conditions (Seale and Breves, 2022). Mozambique tilapia can maintain their internal osmolality within a tighter range when held under tidal conditions compared with fish subjected to unidirectional transfers from SW to FW or from FW to 80 % SW (Moorman et al., 2014, 2015; Pavlosky et al., 2019). In both phases of the TR, tilapia maintain ionocyte populations that most closely resemble those found in SW-acclimated fish and regulate the expression of ion transporters and channels in ways that support ion uptake and secretion during TF and TS, respectively (Moorman et al., 2014). Plasma osmolality in TR tends to follow steady-state trends, remaining higher in TS compared to TF regardless of initial salinity (Chang et al., 2025). Consistent with its expression in FW-type ionocytes (Hiroi et al., 2008), we observed that *ncc2* was upregulated in FW relative to SW, while *ncc2* levels in TF and TS were intermediate to those observed in FW- and SW-acclimated fish. By contrast, both *nkcc1a* and *cftr1* were upregulated in SW-acclimated fish relative to fish in FW, which reflects the expression of their encoded proteins in SW-type ionocytes (Kaneko et al., 2008); *nkcc1a* and *cftr1* levels in TF resembled those of SW-acclimated fish, while expression levels in TS were even higher than those in SW. These transcriptional patterns align with previous studies that characterized branchial effectors of ion transport in tilapia acclimated to FW, SW, and a TR (Moorman et al., 2014, 2015; Pavlosky et al., 2019; Seale et al., 2019). Generally, the expression levels of the TJ proteins analyzed in the present study were higher in TF compared with TS. The labile nature of TJ proteins in a TR suggests they underlie key functional changes in the branchial epithelium when salinity changes are frequent and abrupt.

In the present study, and consistent with previous reports (Tipsmark et al., 2008a, 2008b), we found that branchial *cldn-4 like* expression was higher in FW- versus SW-acclimated tilapia. *cldn-4 like* varied between the TF and TS phases, with levels in TF rising beyond those of FW-fish and levels in TS decreasing to those of SW-fish, indicating precise and timely regulation of branchial TJs under tidal conditions. These patterns support the idea that Cldn-4 like and Cldn-4 restrict ion movements by tightening branchial TJs in FW and ion-poor conditions (Kolosov et al., 2020; Tipsmark et al., 2008a). Similar responses were observed for *cldn-4* in Atlantic killifish (*Fundulus heteroclitus*) in hyposmotic conditions (Whitehead et al., 2012). In contrast to *cldn-4 like*, *cldn-like ZF-A89* expression was upregulated in both phases of the TR, although there was higher expression in TF than in TS. Since the specific functions of ZF-A89 are currently unknown, ZF-A89 warrants future investigation in tilapia

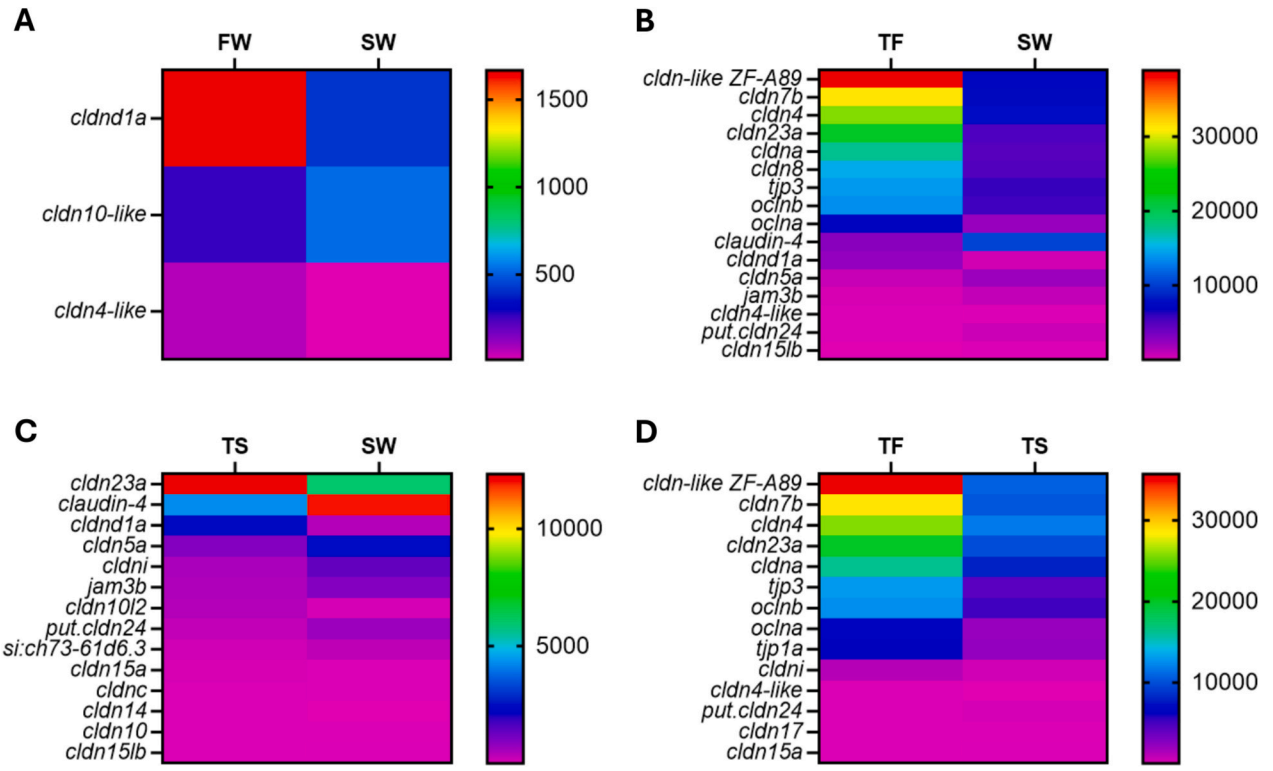

Fig. 1. Heat maps showing TJ-associated transcripts differentially expressed between FW vs. SW (A), TF vs. SW (B), TS vs. SW (C), and TF vs. TS (D) in branchial transcriptomes of Mozambique tilapia in steady-state FW, steady-state SW, or the FW- and SW-phases (TF and TS) of a tidal regimen (TR) for 15 days (n = 3). Transcript expression levels are presented as mean count number per treatment.

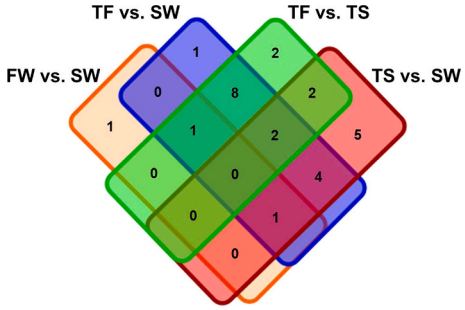

Fig. 2. Venn diagram showing differentially expressed TJ transcripts shared among the treatment comparisons of FW vs. SW, TF vs. SW, TF vs. TS, and TS vs. SW in the branchial transcriptomes of Mozambique tilapia.

and other estuarine species. Both *cldn7b* and *cldn23a* are abundant in teleost gills (Kolosov et al., 2014; Loh et al., 2004), irrespective of the acclimation salinity. Here, both *cldn7b* and *cldn23a* did not differ between SW and FW, but in a TR, both transcripts were upregulated during TF and downregulated during TS, suggesting they contribute to the tilapia's tolerance of tidal conditions. A similar response to environmental salinity was observed for branchial *cldn23* in puffer fish (*Tetraodon nigroviridis*) (Bui and Kelly, 2011, 2015). In mammals, Cldn23 reduces paracellular ion permeability by forming epithelial barriers through heteromeric and heterotypic complexes with Cldn3 and Cldn4; different combinations yield distinct porous structures and ion permeability characteristics (Raya-Sandino et al., 2022).

Oclns constitute another category of transmembrane TJ proteins that regulate branchial permeability by forming epithelial barriers (González-Mariscal et al., 2003). A previous study reported that Ocln is involved in gill barrier formation and that branchial *ocln* expression was elevated in goldfish (*Carassius auratus*) exposed to ion-poor water (Chasiotis et al., 2009). Accordingly, *oclna* and *oclnb* were elevated in the TF group compared with other groups. Furthermore, the higher expression of *oclna* and *oclnb* in TF relative to FW suggests that an acute response to salinity change requires a rapid minimization of solute and water movement. Similarly, rapid upregulation of *ocln* was observed in Atlantic killifish gills following transfer to hyposmotic conditions, indicating its possible role in modifying branchial ionic permeability during physiological transitions (Whitehead et al., 2012). Epithelial tightening due to Ocln abundance was previously reported in goldfish and rainbow trout (*Oncorhynchus mykiss*) (Chasiotis and Kelly, 2008, 2011). In the present study, *oclnb* expression in SW was lower than in FW. While *oclnb* expression in TF was higher than FW, in TS it was lowered to the levels observed in SW, suggesting that Oclna and Oclnb play parallel roles during transitions to tidal environments.

Tjp1a and Tjp3 are TJ "adaptor" proteins that provide structural support by connecting Oclns and Cldns through the cytoskeleton (Bauer et al., 2010; González-Mariscal et al., 2003; Furuse et al., 1994; Haskins et al., 1998). The involvement of Tjp1 in gill structures has been described previously by Kato et al. (2007). In agreement with our observations of *oclna* and *tjp1a* expression, it was reported that both Ocln and Tjp1 are involved in maintaining branchial structural integrity in grass carp (*Ctenopharyngodon idella*) (Feng et al., 2017). Although no differences were observed between FW- and SW-controls, the branchial expression of *tjp1a* was higher in TF than in TS. Similarly, *tjp3* expression was upregulated in TF and decreased in TS compared with FW- and SW-controls. Both *tjp* isoforms were similarly expressed between FW and SW, and the drastic changes in their expression between TF and TS suggest potential roles of these isoforms in epithelial tightening during short-term FW acclimation. Roles for Tjp1 in remodeling gill epithelia have been previously suggested in Atlantic salmon (*Salmo salar*) (Engelund et al., 2012) and goldfish (Chasiotis et al., 2012b). Although the specific roles of Tjp1 and Tjp3 in TJ complexes are not well-defined, downregulating their gene transcripts in TS might be necessary for

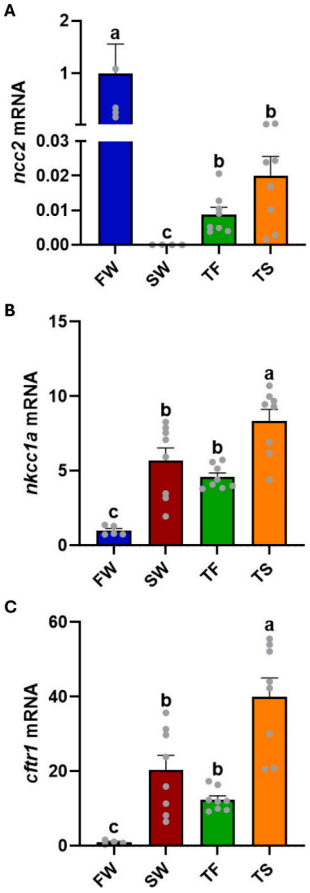

Fig. 3. Branchial expression of *ncc2* (A), *nkcc1a* (B), and *cftr1* (C) in Mozambique tilapia in FW, SW, and transferred to a TR for 15 days. In the TR, fish were sampled at the end of the FW (TF) and SW phases (TS). Data are expressed as normalized mean fold-change from day 15 FW-controls ± S.E.M ($n = 8$). Data were analyzed by one-way ANOVA followed up by protected Fisher's LSD test. Means that do not share a letter are different between salinity treatments ($P < 0.05$).

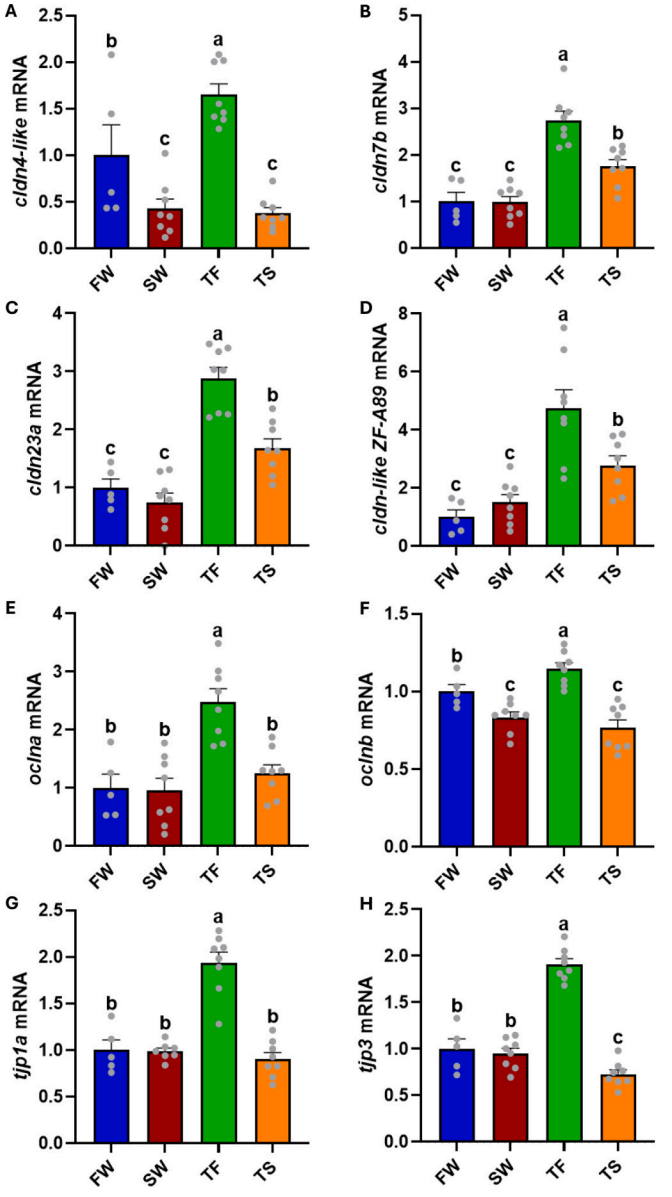

Fig. 4. Branchial expression of *cldn4-like* (A), *cldn7b* (B), *cldn23a* (C), *cldn-like ZF-A89* (D), *oclna* (E), *oclnb* (F), *tjp1a* (G), and *tjp3* (H) in Mozambique tilapia in FW, SW, and transferred to a TR for 15 days. In the TR, fish were sampled at the end of the FW (TF) and SW phases (TS). Data are expressed as normalized mean fold-change from day 15 FW-controls ± S.E.M ($n = 8$). Data were analyzed by one-way ANOVA followed up by protected Fisher's LSD test. Means that do not share a letter are different between salinity treatments ($P < 0.05$).

tilapia to quickly "loosen" the tight junctions between SW-type ionocytes and accessory cells to enhance the removal of excess Na^+ from the blood (Hwang et al., 2011; Hwang and Lee, 2007). In a TR, plasma Na^+ concentrations were higher in fish sampled in TS compared with TF (Chang et al., 2025). Since Na^+ must be extruded through paracellular routes to mitigate increased plasma levels during TS, the diminished expression of specific TJ-associated transcripts seems to be a key response to the hyperosmotic conditions experienced during tidally changing conditions.

In conclusion, our results indicate that TJ-associated proteins are transcriptionally regulated under tidally changing salinities, contributing to the suite of molecular responses that maintain hydromineral balance in estuarine fishes. Notably, by 15 days, most TJ-associated proteins were upregulated in TF relative to TS, a response consistent with the tightening of paracellular pathways to restrict ion permeability and water influx in hyposmotic environments. These findings enhance our understanding of how transmembrane and structural support proteins are essential for remodeling the epithelial barrier, enabling agile responses to dynamic salinities.

CRediT authorship contribution statement

G.H.T. Malintha: Writing – original draft, Investigation, Formal analysis, Data curation. **Ke Cao:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Fritzie T. Celino-Brady:** Writing – review & editing, Methodology, Investigation, Data curation. **Jason P. Breves:** Writing – review & editing. **Andre P. Seale:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Data availability

Data will be made available on request.

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