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Environmental salinity differentially impacts branchial and hepatic carbohydrate metabolism in tilapia

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Abstract

In response to changes in environmental salinity, euryhaline fishes mobilize energy to support the active transport of ions across osmoregulatory epithelia. Glycogen synthase (GS) and glycogen phosphorylase (GP) are key controllers of carbohydrate metabolism due to their roles in promoting glycogenesis and glycogenolysis, respectively. However, the coordinated responses of GS, GP and glucose transporters (GLUTs) in the liver and gill to changes in salinity remain unresolved. In this study, we initially subjected Mozambique tilapia (*Oreochromis mossambicus*) to unidirectional transfers, either from fresh water (FW) to seawater (SW) or from SW to FW. We then transferred FW- and SW-acclimated tilapia to a tidal regime (TR) where salinity alternated between FW and SW every 6 h. Our goal was to characterize how carbohydrate metabolism is affected by unidirectional and tidal changes in salinity. Whether fish were transferred from SW to FW in a unidirectional manner or to a TR, glucose levels increased after transfer to FW or during the FW phase of the TR (TF). Conversely, hepatic glycogen levels were higher in fish in SW and the SW phase of the TR (TS) than in TF. In both FW and TF, branchial expression of the gill-specific isoform of GP (*gpg*) was downregulated, while *gs* was upregulated. Branchial *gpg* was upregulated in fish unidirectionally transferred from FW to SW or sampled during TS. Hepatic *gp* and *gs* expression increased following transfer from SW to FW. Thus, we consistently found that increases in salinity promoted branchial glycogen breakdown, while decreases in salinity led to hyperglycaemia. Moreover, while branchial *glucose transporter 1* (*glut 1*) expression was downregulated after transfer from FW to SW, *glut1* was transiently upregulated in the liver. In both liver and gill, *glut1* expression was higher in fish in TF compared to TS. Gill filament explants incubated with cortisol exhibited reduced *glut1* expression regardless of medium osmolality. Our collective data indicate that salinity differentially regulates hepatic and branchial carbohydrate metabolism.

KEYWORDS
gill, glucose, glycogen, liver, salinity

1 | INTRODUCTION

Euryhaline fishes can maintain internal osmotic conditions near one-third the level of seawater (SW) when exposed to a wide range of environmental salinities (McCormick, 2001). To maintain internal osmolality within a narrow range, euryhaline fish in fresh water (FW) actively absorb ions from the ambient environment and produce dilute urine. When exposed to SW conditions, euryhaline fish actively excrete ions and conserve water (Evans, 2008). Activation of the ion- and water-transporting processes that maintain osmotic homeostasis

is energetically costly (Abou Anni et al., 2016; Bœuf & Payan, 2001; Tseng & Hwang, 2008) and requires adjustments to carbohydrate metabolism following salinity changes (Guo et al., 2020).

Most of the energy requirements of the gill, which is the primary site for Na^+ and Cl^- exchange (Evans, 2008), is derived from glucose oxidation (Mommsen et al., 1999). In Mozambique tilapia (*Oreochromis mossambicus*), glucose is stored in glycogen-rich cells (GRCs) adjacent to branchial ionocytes (Chang et al., 2007; Tseng et al., 2007). Working in tandem with GRCs, the liver also stores glycogen to meet the energetic needs of ionocytes (Chang et al., 2007). Glycogen synthase (GS) mediates glycogen production, while glycogen phosphorylase controls glycogen breakdown (Mueckler, 1994). Tilapia express gill- and liver-specific isoforms of glycogen phosphorylase, denoted GPG and GP in this manuscript, respectively (Tseng et al., 2007). A review of how glucose is stored, metabolized and transported in euryhaline fishes was provided by Tseng and Hwang (2008).

Both increases and decreases in environmental salinity are associated with elevated plasma glucose levels in Mozambique tilapia (Assem & Hanke, 1979; Zhu et al., 2021). While the breakdown of hepatic glycogen is likely the primary source of glucose (Bollen et al., 1998), information regarding the influence of salinity changes on the pathways that regulate glycogen synthesis and breakdown is limited. In tilapia, glycogen content decreased in the gills and liver following transfers from FW to brackish water (BW) (Angadi et al., 2021; Chang et al., 2007), while branchial *glycogen synthase* (*gs*) expression increased after a transfer from SW to FW (Zikos et al., 2014). When considered together, these patterns suggest that branchial glycogen is inversely related to environmental salinity. Glucose transporters (GLUTs) provide a conduit for the passive transport of glucose across cell membranes. Among the three GLUT isoforms described in Mozambique tilapia, *glucose transporter 1* (*glut1*) is highly expressed in the liver and gill (Angadi et al., 2021). Higher branchial *glut1* and lower hepatic *glut1* expression levels were observed in BW- versus FW-acclimated tilapia (Angadi et al., 2021). Another study, however, found increased expression of *glut1* in both the gill and liver of Nile tilapia (*O. niloticus*) during hypersaline stress (Zhang et al., 2024). Similar to the previous studies in tilapia, branchial *glut1* was upregulated in gilthead sea bream (*Sparus aurata*) following an increase in salinity (Balmaceda-Aguilera et al., 2012). These studies indicate that salinity affects glycogen synthesis and breakdown in the gill and liver, as well as glucose transport in these organs.

Some euryhaline species, particularly those that reside in estuaries, endure frequent, tidally driven changes in salinity. The Mozambique tilapia, for example, originates from regions that experience frequent salinity changes due to tidal cycles (Treweas, 1983). Consequently, the energetic demands required to sustain physiological processes, such as osmoregulation and growth, become particularly challenging under frequently changing conditions. Some physiological consequences associated with tidal conditions have been previously investigated in Mozambique tilapia reared under a simulated tidal regime (TR), where FW and SW conditions alternate every 6 h (Seale & Breves, 2022). Interestingly, tilapia reared in a TR grew faster than those maintained at stable salinities (Moorman et al., 2016). Moreover, unlike the marked changes in plasma osmolality observed in tilapia transferred unidirectionally from FW to SW or vice versa (Seale et al., 2012), tidally reared tilapia maintain plasma osmolality within a narrow range (Moorman et al., 2014). However, the underlying metabolic processes required for maintaining adaptive growth and osmoregulatory performance in a TR are poorly understood.

Salinity challenges activate multiple hormonal systems, including the release of catecholamines from chromaffin cells and the release of cortisol via the hypothalamic–pituitary–interrenal (HPI) axis (Reid et al., 1996; Schreck et al., 2016). While catecholamine release usually occurs within seconds of exposure to a stressor, the full activation of the HPI axis is relatively delayed. Accordingly, catecholamines are released in response to standard blood sampling techniques, therefore cortisol is favoured as an indicator of a stress response to environmental stimuli (Schreck et al., 2016). We recently found that branchial *glucocorticoid receptor* (*gr*) expression decreased when tilapia were unidirectionally transferred from SW to FW or during the FW phase of a TR (Chang et al., 2023). Since cortisol mediates the expression of branchial *gp* in larval tilapia (Wu et al., 2023), these findings imply that cortisol signalling influences carbohydrate availability in the gill during salinity acclimation.

Considering the energetic demands associated with osmoregulation, we hypothesized that adaptive alterations in carbohydrate metabolism would be closely tied to changes in environmental salinity, regardless of the frequency of such changes. We specifically predicted that transient increases in glucose production and transport would occur in close association with salinity challenges. To test this prediction, we transferred Mozambique tilapia from (1) FW to SW and vice versa over a unidirectional 7-day time course and (2) from FW and SW to a TR and sampled at the end of the FW and SW phases of the tidal cycle after 15 days (tidal fresh water [TF] and tidal seawater [TS], respectively). While both experimental paradigms involved transfers between FW and SW, they differed in the duration and frequency of salinity challenges. We subsequently measured plasma osmolality and glucose levels, along with *gpg*, *gp*, *gs* and *glut1* expression in the gill and liver. In fish transferred to a TR, we measured plasma Na^+ and Cl^- concentrations and hepatic glycogen content. Lastly, we examined the effects of extracellular osmolality and cortisol on *gpg*, *gs* and *glut1* expression using an in vitro gill filament system. Using various experimental paradigms with different salinity challenge frequencies, this study offers a targeted evaluation of the role of carbohydrate metabolism in the salinity acclimatization responses of a euryhaline teleost.

2 | MATERIALS AND METHODS

2.1 | Animals

In all experiments, fish were maintained at 25 ± 2°C in flow-through tanks and fed trout chow pellets (Skretting, Tooele, UT) once daily ad libitum for a maximum of 15 min or until they were no longer actively feeding, except for the initial 24 h of Experiment 1, during which food

was withheld. Before each sampling, fish from all experimental and parallel control groups were fasted for at least 24 h to maximize postprandial glucose clearance and minimize the effects of feeding on carbohydrate metabolism (Lin et al., 1995; Liu et al., 2018; Riley et al., 2009). In Experiment 1, all fish were fasted for 24 h prior to the time 0 sampling; fish in experimental tanks continued fasting until the 24 h sampling, after which they were fed daily for the remainder of the experiment, with additional fasting periods of 24 h prior to the 48 h and 7 days samplings. In Experiment 2, all fish were fasted for 24 h before sampling. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee of the University of Hawai'i.

2.2 | Experiment 1: Unidirectional salinity transfers

Adult male Mozambique tilapia (376 g ± 16 g) selected from FW-(municipal water) and SW-acclimated (34‰, Kaneohe Bay, HI, USA) populations were randomly assigned to two tanks per salinity, as previously described (Chang et al., 2023). In this experiment, there were four treatment groups: fish transferred from SW to FW, fish transferred from FW to SW and their respective parallel controls in SW and FW, respectively. Sampling occurred at 6, 12, 24, 48 h and 7 days. Eight fish from each of the four tanks were sampled at time 0. In one SW tank, the inflow was changed from SW to FW after the initial sampling and the fish were sampled 6, 24, 48 h and 7 days post salinity change. In one FW tank, fish were transferred from FW to SW after an initial exposure to BW (25‰) for 48 h (sampled in BW at 6, 24 and 48 h). They were then maintained in SW (34‰) until day 7. The second tank for each initial salinity was kept as a parallel control at its original salinity for the duration of the experiment. Eight fish were sampled per tank at each time point.

2.3 | Experiment 2: Acclimation to a tidal regime

Fish were acclimated to a TR for 15 days and then sampled at the end of the FW and SW phases (TF and TS, respectively) to describe physiological responses in an environmental regime characterized by frequent changes in salinity. This experimental approach was recently described by Chang et al. (2023) and is based on previous studies indicating similar physiological responses in fish reared in a TR for either 7 days or 4 months (Moorman et al., 2014; Pavlosky et al., 2019). Adult male Mozambique tilapia (222.3 ± 11.5 g) were collected from FW and SW brood stock at the Hawai'i Institute of Marine Biology. Fish from each salinity were randomly distributed into two tanks per salinity. On day 0, eight fish from each of the four tanks were sampled. After sampling on day 0, one FW tank and one SW tank were maintained at the same salinity, while the incoming water to one FW tank and one SW tank was adjusted to simulate a TR. TR tanks switched from FW to SW inflow every 6 h (Moorman et al., 2014). Sixteen fish were sampled after 15 days in the TR: eight fish were sampled at the end of the FW phase (TF) of the tidal cycle and the other eight fish were sampled at the end of the SW phase (TS). For FW and SW control tanks at static salinities, eight fish were sampled after 15 days. When fish in FW were transferred to a TR (FW to TF and FW to TS), they experienced mortalities, leaving six fish after 15 days, therefore only three fish were sampled in FW to TF and three fish in FW to TS.

2.4 | Sampling

At the time of sampling, fish were netted from their tanks, anaesthetised with 2-phenoxyethanol (0.3 mL/L; Sigma-Aldrich) and weighed. Blood was collected from the caudal vasculature using a needle and syringe coated with sodium heparin (200 U/mL; Sigma-Aldrich) before rapid decapitation. Plasma was separated by centrifugation and stored at −80°C. Gill and liver tissues were excised, snap-frozen in liquid nitrogen and stored at −80°C. Gill filaments were sampled from the second gill arch.

2.5 | In vitro gill filament incubation

Individual gill filaments collected from three FW-acclimated tilapia (482 ± 69 g) were dissected longitudinally in a balanced salt solution following Watanabe et al. (2016. Once dissected, three filaments were placed in each well of a 24-well plate with 500 µL of Leibovitz's L-15 culture medium (Gibco/Life Technologies), supplemented with 100 IU/mL penicillin and 76.3 IU/mL streptomycin (Inokuchi et al., 2015). The L15 media was adjusted to an osmolality of either 330 mOsm/kg (isosmotic) or 450 mOsm/kg (hyperosmotic), with or without 1 µg/mL of cortisol (Sigma). The cortisol concentration was selected based on a previous study using static gill incubation to identify transcriptional responses to cortisol (Breves et al., 2016). Each treatment was replicated in eight wells. After 6 h, the gill filaments were stored in RNAlater (Invitrogen) for subsequent RNA isolation and quantitative real-time PCR (qRT-PCR) analysis.

2.6 | Plasma osmolality, glucose, Na^+ and Cl^-

Plasma osmolality was measured with a vapour pressure osmometer (Wescor 5100C; Wescor). Plasma glucose was measured using a commercial glucose assay (GAGO-20; Sigma) modified for a microplate reader (BioTek Synergy LX). Blood plasma of fish sampled after 15 days in a TR was analysed by the University of Washington Analytical Service Center. Na^+ and Cl^- were determined using EPA methods 200.7 and 300.0, respectively (Pfaff, 1993; US EPA, 1994).

2.7 | Hepatic glycogen

Hepatic glycogen was measured using a colorimetric glycogen assay (MAK016; Sigma-Aldrich) according to the manufacturer's protocol.

TABLE 1 Specific primer sequences used for qRT-PCR.

Gene	Primer sequence (5′–3′)	Eff. (%)	Accession no.	Reference
18 s	F: GCTACCACATCCAAGGAAGGC R: TTCGTCACTACCTCCCCGAGT	104	AF497908	Magdeldin et al. (2007)
ß-Actin	F: CTCTTCCAGCCTTCCTTCCT R: ACAGGTCCTTACGGATGTCG	85	AB037865	Tipsmark et al. (2011)
ef1a	F: AGCAAGTACTACGTGACCATCATTG R: AGTCAGCCTGGGAGGTACCA	86	AB075952	Breves et al. (2007)
gpg	F: CGAGCCCAGGGAAGCCATCGAA R: TGAAGGCTTAAACCAAACAGGAA	93	DQ010415	Tseng et al. (2007)
gp	F: ACCTCGATAAAATTGCAGCTCTCT R: GCTTTGTGAACCCAGAAATAC	84	DQ010416	Tseng et al. (2007)
gs	F: TCTTCCCAACCTGTGCGTAC R: CTCCACAAAGCAAACCACCG	105	EF565371	Newly designed
glut1	F: CTCCTCCTGGCTTTCCTCTT R: TTTCCTGCATGTCAGCACTC	90	NM_001279727.1	Angadi et al. (2021)

Approximately 0.03 g of liver tissue was homogenized in 500 µL of assay buffer containing trypsin (10 µg/mL). The samples were then centrifuged at 8000×*g* for 10 min at 4°C, after which 400 µL of the supernatant was collected and diluted for the assay.

2.8 | RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from tissue by the TRI Reagent procedure according to the manufacturer's protocols (Molecular Research Center). RNA concentration and purity were assessed by spectrophotometric absorbance (NanoDrop One, Thermo Fisher Scientific). First-strand complementary DNA (cDNA) was synthesized using 500 ng of total RNA with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Relative mRNA levels of reference and target genes were determined by the relative quantification method (Pfaffl, 2001) using the StepOnePlus real-time qRT-PCR system (Life Technologies). qRT-PCR reactions were set up in a 15-µL final volume consisting of 7.5 µL of 2× PowerSYBR Green PCR Master Mix (Life Technologies), 5.9 µL of molecular grade water, 0.3 µL of 10 mM forward and reverse primers, and 1 µL of cDNA (1:10). The following cycling parameters were employed: an initial 10 min denaturation at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A standard curve generated from serial dilutions of pooled cDNA from each tissue was used to convert threshold Ct values into relative concentrations. The geometric mean of three reference genes (*ef1a*, *18 s* and *ß-actin*) was used to normalize target gene levels. Primer sequences and assay efficiencies are provided in Table 1.

2.9 | Statistical analyses

Experiments 1 and 2 were analysed with a two-way ANOVA using time and salinity as the main effects. In Experiment 1, the analysis was separated based on the original salinity of the fish. For the SW tilapia, there were two levels of salinity (SW to FW and SW parallel controls) and five levels of time (0, 6, 24, 48 h and 7 days, $n = 6–8$). Similarly, for the FW tilapia, there were two levels of salinity (FW to SW and FW parallel controls) and five levels of time ($n = 6–8$). In Experiment 2, the analysis consisted of six levels of salinity (FW to FW, FW to TF, FW to TS, SW to SW, SW to TF and SW to TS) and two levels of time (0 and 15 days, $n = 3–8$). Hepatic glycogen and plasma ion concentrations from Experiment 2 were analysed using one-way ANOVA. The in vitro gill incubation data were analyzed with a two-way ANOVA. Significant main and interaction effects ($p < 0.05$) of ANOVA were followed by protected Fisher's least significant difference (LSD) test. When necessary, data were log-transformed to satisfy assumptions of normality and homogeneity of variance. Statistical tests were performed using Prism 9 (GraphPad).

3 | RESULTS

3.1 | Plasma osmolality

For the FW and SW unidirectional transfers, there were significant salinity, time and interaction effects on plasma osmolality (Figure 1a,b). Plasma osmolality did not change in FW control fish throughout the experiment, whereas, for fish transferred from FW to SW, plasma osmolality increased at 6 h following transfer, peaked at 24 h, then remained elevated until day 7. Plasma osmolality was higher in fish transferred from FW to SW at all time points after time 0 (Figure 1a). When SW fish were transferred to FW, plasma osmolality decreased at 24 h and further declined at day 7. The plasma osmolality of fish transferred from SW to FW was lower than time-matched controls at 24 h, 48 h and 7 days (Figure 1b). For the fish transferred to a TR, significant salinity and interaction effects were observed on plasma osmolality (Figure 1c). On day 0, plasma

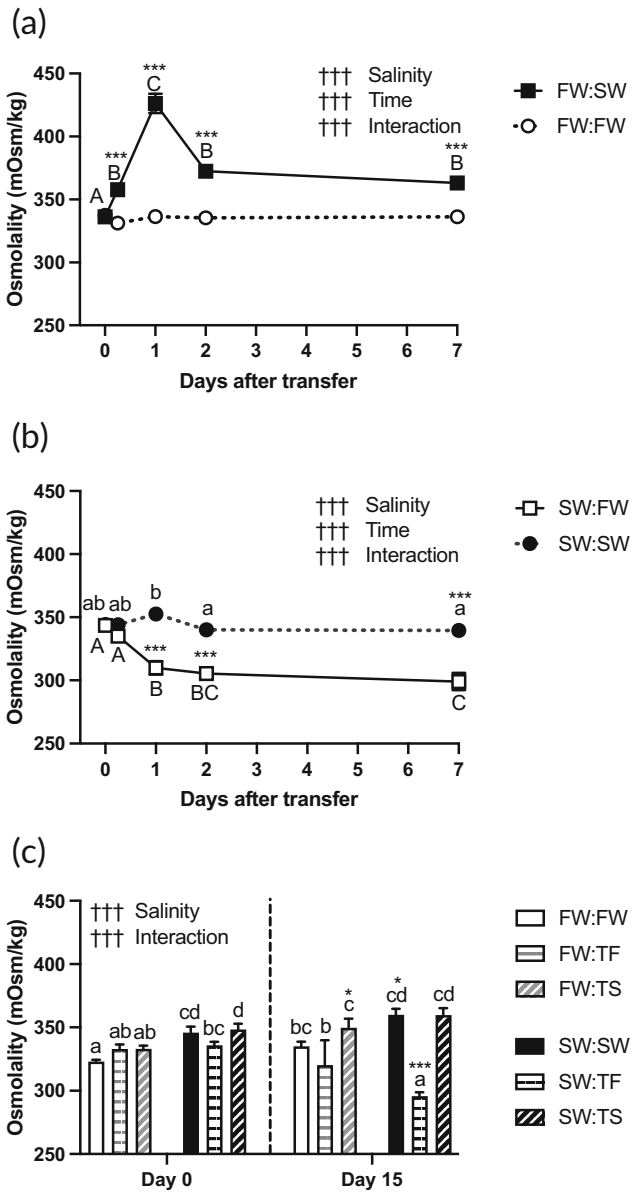

FIGURE 1 Plasma osmolality of fish transferred from FW to SW (a) (n = 6–8), SW to FW (b) (n = 7–8) and from FW or SW to a tidal regime (c) (n = 3–8). Means ± standard error of the mean. Main effects are indicated by †, †† and ††† corresponding to p < 0.05, 0.01 and 0.001, respectively. Significant effects were followed by protected Fisher's least signifcant difference test. Lower-case letters in (a) and (b) indicate significant differences between time points for control fish, and upper-case letters indicate differences between time points for fish transferred to either SW or FW (p < 0.05). Asterisks (*, **, ***) in (a) and (b) indicate differences between control and transferred fish at p < 0.05, 0.01 and 0.001, respectively. In the tidal transfer (c), lower-case letters indicate differences within each time point (p < 0.05), while asterisks indicate differences between time points. FW, fresh water; SW, seawater; TF, tidal fresh water; TS, tidal seawater.

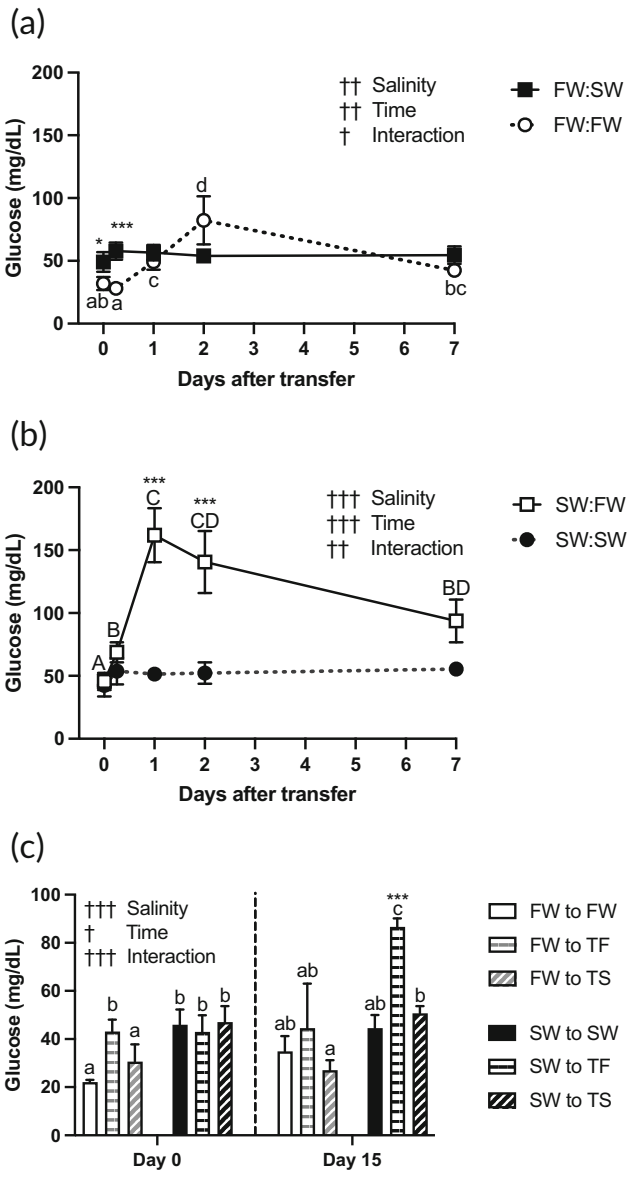

FIGURE 2 Plasma osmolality of fish transferred from FW to SW (a) (n = 7–8), SW to FW (b) (n = 7–8) and from FW or SW to a tidal regime (c) (n = 3–8). Means ± standard error of the mean. Main effects are indicated by †, †† and ††† corresponding to p < 0.05, 0.01 and 0.001, respectively. Significant effects were followed by protected Fisher's least significant difference test. Lower-case letters in (a) and (b) indicate significant differences between time points for control fish, and upper-case letters indicate differences between time points for fish transferred to either SW or FW (p < 0.05). Asterisks (*, **, ***) in (a) and (b) indicate differences between control and transferred fish at p < 0.05, 0.01 and 0.001, respectively. In the tidal transfer (c), lower-case letters indicate differences within each time point (p < 0.05), while asterisks indicate differences between time points. FW, fresh water; SW, seawater; TF, tidal fresh water; TS, tidal seawater.

osmolality was higher in SW versus FW controls. After 15 days in a TR, plasma osmolality was higher in TS versus TF, for both FW- and SW-acclimated tilapia (Figure 1c).

3.2 | Plasma glucose, Na^+ and Cl^-

For the unidirectional transfers, there were significant time, salinity and interaction effects on plasma glucose in both SW and FW fish

TABLE 2 Plasma parameters and hepatic glycogen levels of fish in a tidal regime (Experiment 2).

Salinity (15 d)	Plasma osmolality (mOsm/kg)	Plasma glucose (mmol/L)	Hepatic glycogen (mg/g)	Plasma Na^+ (mmol/L)	Plasma Cl^- (mmol/L)
FW	335.1 ± 3.7^{bc}	1.9 ± 0.4^{bc}	136.7 ± 8.1^{a}	135.9 ± 3.3^{bc}	132.6 ± 4^{b}
FW to TF	320.2 ± 19.8^{c}	2.5 ± 1.0^{bc}	0.6 ± 0.04^{d}	141.5 ± 1.8^{bc}	149.4 ± 6.5^{a}
FW to TS	349.7 ± 7.3^{ab}	1.5 ± 0.2^{c}	2.8 ± 0.1^{d}	133.0 ± 15.9^{bc}	138.2 ± 20.7^{ab}
SW	359.9 ± 4.9^{a}	2.5 ± 0.3^{bc}	123.8 ± 6.6^{a}	155.7 ± 2.1^{a}	153.3 ± 1.7^{a}
SW to TF	295.7 ± 3.0^{d}	4.8 ± 0.2^{a}	88.4 ± 5.9^{c}	130.9 ± 2.1^{c}	126.8 ± 1.9^{b}
SW to TS	359.8 ± 5.4^{a}	2.8 ± 0.2^{b}	105.8 ± 5.1^{b}	144.6 ± 3.8^{b}	148.6 ± 1.9^{a}

Note: Means ± standard error of the mean. Means not sharing lower-case letters are significantly different ($p < 0.05$). Data were analysed by one-way ANOVA, followed by protected Fisher's least significant difference.
Abbreviations: FW, fresh water; SW, seawater; TF, tidal fresh water; TS, tidal seawater.

(Figure 2a,b). Over 7 days, the plasma glucose levels of FW control fish ranged from 30 to 80 mg/dL, showing increases at 24 and 48 h post-transfer compared to 0 h. These increases were transient, and glucose levels on day 7 were similar to those on day 0. When FW fish were transferred to SW, plasma glucose levels were ~50 mg/dL throughout the 7 days (Figure 2a). Glucose levels of SW control fish remained near 50 mg/dL throughout the experimental period. When SW fish were transferred to FW, plasma glucose increased at 6 h and was ~100 mg/dL higher than SW controls at 24 and 48 h, peaking at ~160 and ~140 mg/dL, respectively (Figure 2b). After 15 days in a TR, there were significant salinity, time and interaction effects on plasma glucose levels (Figure 2c). Plasma glucose was higher in fish transferred from SW to TF on day 15 compared to all other groups (Figure 2c). To assess the osmotic contribution of glucose in TF, glucose levels reported in Figure 2c were converted to equivalent units (mmol/L) to facilitate comparisons between glucose, Na^+ and Cl^- (Table 2). We found a significant difference in plasma Na^+ and Cl^- levels between salinity treatments after 15 days in a TR (Table 2). In control fish, plasma Na^+ and Cl^- levels were higher in SW versus FW fish. In FW fish transferred to a TR, plasma Na^+ and Cl^- were similar between TF and TS. In SW fish transferred to a TR, plasma Na^+ and Cl^- were lower in TF relative to TS and SW controls.

3.3 | Hepatic glycogen

Significant differences in hepatic glycogen were observed after 15 days in a TR (Table 2). Although hepatic glycogen content was similar between FW and SW fish, levels were lower in TF and TS fish relative to FW controls. The hepatic glycogen content of fish transferred from SW to a TR was higher in TS compared with TF; glycogen levels in TS and TF fish were lower than those in SW controls (Table 2).

3.4 | Effects of salinity on branchial *gpg* and *gs*

There were significant time, salinity and interaction effects on branchial *gpg* expression in fish transferred to FW and SW (Figure 3a,b). When fish were transferred from FW to SW, *gpg* increased at 6 h, continued to rise, peaked at 24 h and remained elevated at 7 days. Compared to FW controls, *gpg* was higher in fish transferred to SW at all time points following time 0 (Figure 3a). When fish were transferred from SW to FW, *gpg* decreased at 6 h, further decreased at 24 h and remained lower than time 0 through day 7. Following transfer from SW to FW, *gpg* was downregulated compared to SW controls at all time points (Figure 3b). There were significant time, salinity and interaction effects on branchial *gpg* expression in fish transferred to a TR (Figure 3c). At time 0, *gpg* expression was higher in fish acclimated to SW versus FW. After 15 days in a TR, *gpg* expression in fish previously acclimated to FW increased during both TF and TS. In fish previously acclimated to SW, *gpg* increased during TS relative to both TF and SW controls (Figure 3c).

There were significant salinity, time and interaction effects on branchial *gs* expression in fish transferred to FW and SW (Figure 3d,e). Fish transferred from FW to SW increased *gs* expression at 6 and 24 h by 3- and 4-fold, respectively, above FW control levels (Figure 2d). In fish transferred from SW to FW, *gs* expression increased at 6 and 24 h, then returned to day 0 levels by 48 h and remained stable to day 7. The expression of *gs* in fish transferred from SW to FW was higher than that of SW controls at both 6 and 24 h (Figure 3d).

There were significant time, salinity and interaction effects on branchial *gs* expression in fish transferred from either FW or SW to a TR (Figure 3f). Following transfer to a TR, *gs* expression increased during both phases of the tidal cycle compared to their respective FW and SW controls. Additionally, regardless of the initial acclimation salinity, *gs* expression was higher in TF versus TS (Figure 3f).

3.5 | Effects of salinity on branchial *glut1*

For the unidirectional transfers, there were significant salinity, time and interaction effects on branchial *glut1* expression in FW-transferred fish; in SW-transferred fish, there were significant salinity and time effects (Figure 4a,b). Fish transferred from FW to SW decreased *glut1* expression from 48 h until day 7. *glut1* was lower in fish transferred from FW to SW compared with controls from 24 h to

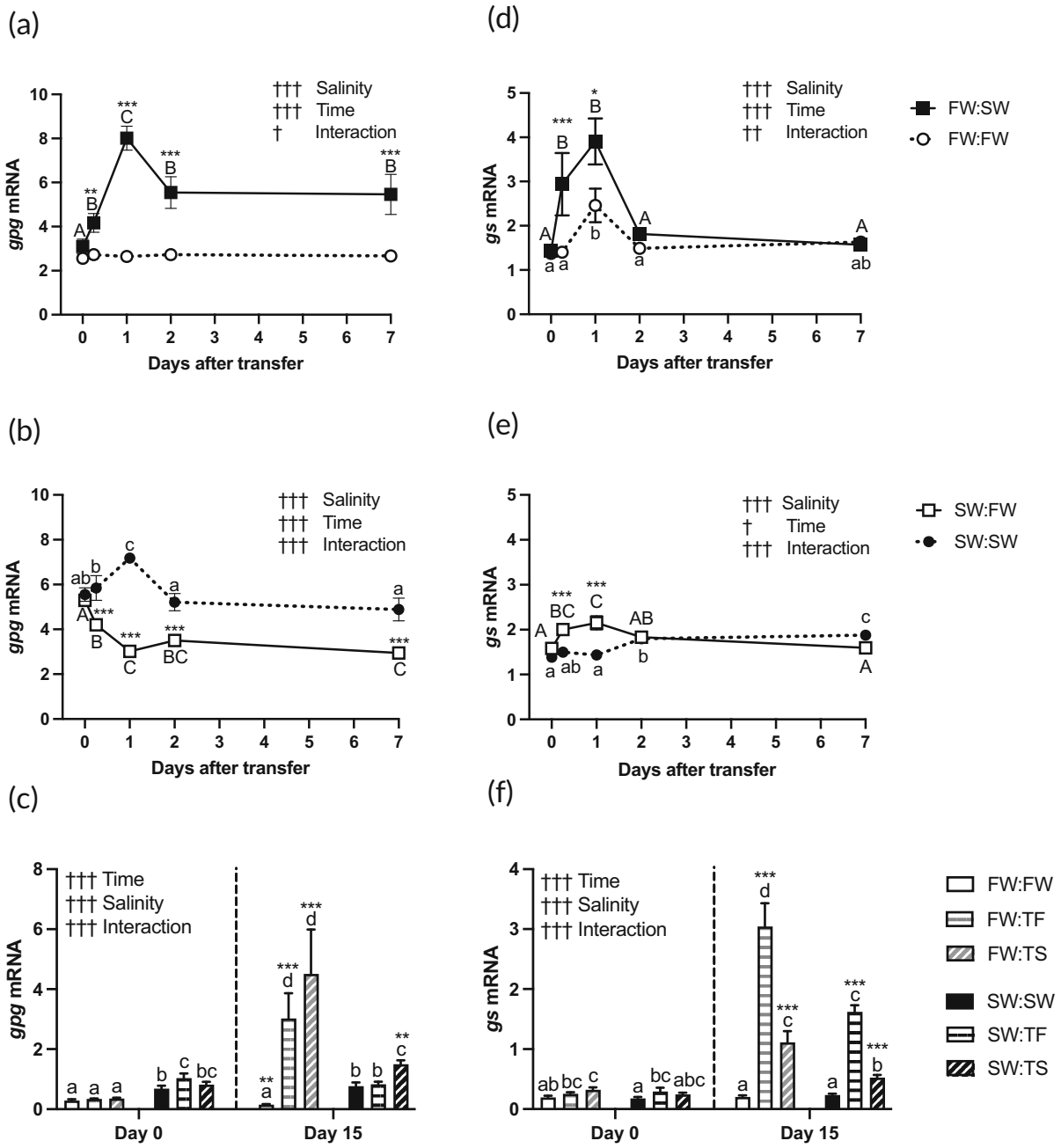

FIGURE 3 Branchial *gpg* (a–c) and *gs* (d–f) expression in fish transferred from FW to SW (a, d) ($n = 6–8$), SW to FW (b, e) ($n = 5–8$) and from FW or SW to a tidal regime (c, f) ($n = 3–8$). Means ± standard error of the mean. Main effects are indicated by †, †† and ††† corresponding to $p < 0.05$, 0.01 and 0.001, respectively. Significant effects were followed by protected Fisher's least significant difference test. Lower-case letters in (a), (b), (d) and (e) indicate significant differences between time points for control fish, and upper-case letters indicate differences between time points for fish transferred to either SW or FW ($p < 0.05$). Asterisks (*, **, ***) in (a), (b), (d) and (e) indicate differences between control and transferred fish at $p < 0.05$, 0.01 and 0.001, respectively. In the tidal transfers (c, f), lower-case letters indicate differences within each time point ($p < 0.05$), while asterisks indicate differences between time points. FW, fresh water; SW, seawater; TF, tidal fresh water; TS, tidal seawater.

the conclusion of the experiment (Figure 4a). In fish transferred from SW to FW, *glut1* was upregulated relative to SW controls at day 7 (Figure 4b). There were significant time, salinity and interaction effects on branchial *glut1* expression in fish transferred from either FW or SW to a TR. Following transfer to a TR, *glut1* was upregulated in TF compared with TS in fish initially acclimated to FW or SW (Figure 4c).

3.6 | Effects of salinity on hepatic *gp* and *gs*

There was a significant time effect on *gp* expression in fish transferred from FW to SW (Figure 5a). Hepatic *gp* levels were similar at 0, 6 and 24 h, then increased approximately 3-fold at 48 h and remained highly expressed until day 7 (Figure 5a). There were significant salinity, time and interaction effects on *gp* expression in fish transferred from SW

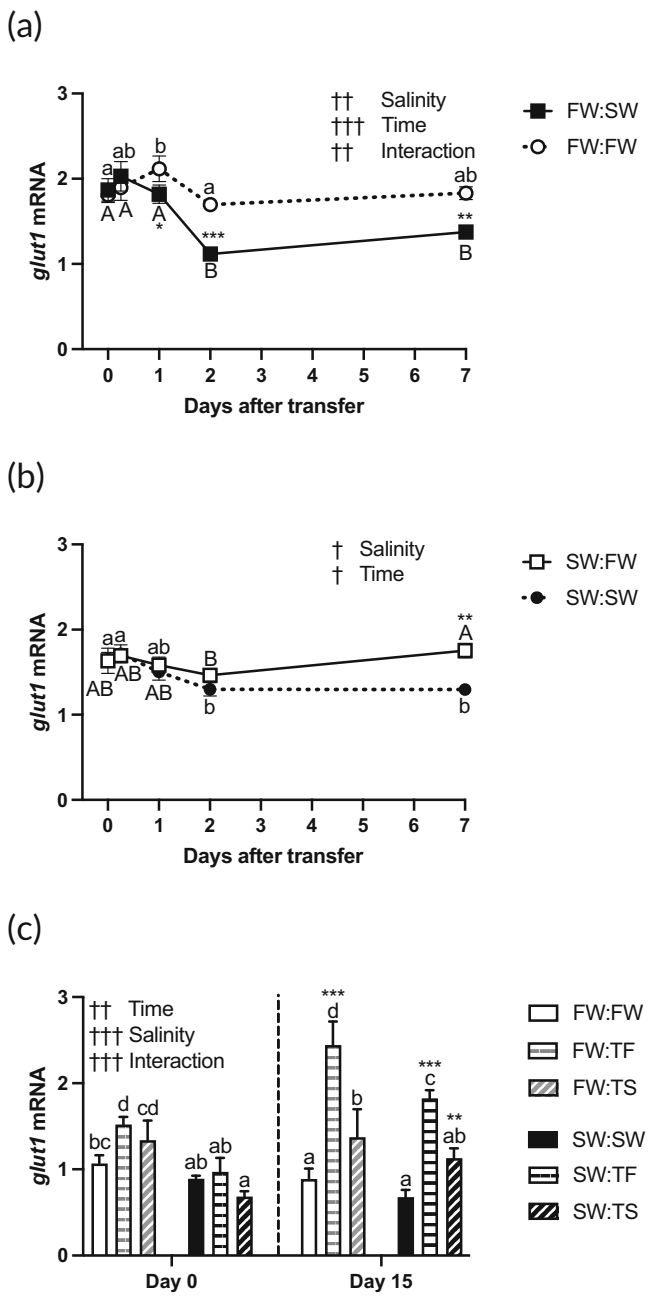

FIGURE 4 Branchial *glut1* expression in fish transferred from FW to SW (a) ($n = 7–8$), SW to FW (b) ($n = 6–8$) and from FW or SW to a tidal regime (c) ($n = 3–8$). Means ± standard error of the mean. Main effects are indicated by †, †† and ††† corresponding to $p < 0.05$, 0.01 and 0.001, respectively. Significant effects were followed by protected Fisher's least significant difference test. Lower-case letters in (a) and (b) indicate significant differences between time points for control fish, and upper-case letters indicate differences between time points for fish transferred to either SW or FW ($p < 0.05$). Asterisks (*, **, ***) in (a) and (b) indicate differences between control and experimentally transferred fish at $p < 0.05$, 0.01 and 0.001, respectively. In the tidal transfer (c), lower-case letters indicate differences within each time point ($p < 0.05$), while asterisks indicate differences between time points. FW, fresh water; SW, seawater; TF, tidal fresh water; TS, tidal seawater.

to FW. SW control fish increased *gp* at 6 h with a further increase at day 7. In comparison, fish transferred from SW to FW showed a decrease in *gp* at 24 h, followed by an increase at 48 h, where it remained elevated until day 7. Additionally, *gp* was downregulated in fish transferred to FW at 6 and 24 h compared to SW controls (Figure 5b). There was a significant salinity effect on *gp* expression when FW and SW fish were transferred to a TR. On day 15, *gp* was upregulated in FW fish sampled in TS versus TF; there were no differences between the TS and TF fish when they were initially acclimated to SW (Figure 5c).

There was a significant time effect on *gs* in fish transferred from FW to SW (Figure 5d). In FW controls, *gs* increased at 48 h and 7 days. In fish transferred to SW, *gs* increased at 24 h, returned to baseline at 48 h and then rose again at 7 days relative to time 0 (Figure 5d). *gs* was upregulated at all time points following a transfer from SW to FW (Figure 5e). In fish transferred from FW or SW to a TR, there were significant salinity, time and interaction effects on *gs* (Figure 5f). On day 15, *gs* was higher in the TF phase compared with the TS phase of the tidal cycle for fish initially acclimated to both FW and SW; *gs* was upregulated in TS compared with respective SW controls (Figure 5f).

3.7 | Effects of salinity on hepatic *glut1*

In both the FW and SW unidirectional transfers, significant salinity, time and interaction effects were observed on hepatic *glut1* expression (Figure 6a,b). Hepatic *glut1* levels in FW control fish increased at 48 h and returned to their initial levels by day 7. When FW fish were transferred to SW, *glut1* peaked at 24 h and returned to day 0 levels by 48 h (Figure 6a). In SW control fish, hepatic *glut1* decreased at 6 h and returned to initial levels by day 7. The expression of *glut1* was higher in fish transferred from SW to FW at 6 and 48 h compared with SW controls (Figure 6b). There were significant salinity, time and interaction effects on *glut1* expression in fish transferred from FW or SW to a TR (Figure 6c). Following transfer to a TR, hepatic *glut1* in both FW- and SW-acclimated tilapia increased by 4- to 6-fold in TF relative to TS and their respective steady-state controls (Figure 6c).

3.8 | Effects of cortisol on branchial *gpg*, *gs* and *glut1*

There were no effects of cortisol or osmolality on *gpg* and *gs* expression in incubated gill filaments (Figure 7a,b). Cortisol reduced branchial *glut1* expression in filaments incubated in both iso- and hyperosmotic media (Figure 7c).

4 | DISCUSSION

The primary goal of this study was to characterize the effects of environmental salinity on hepatic and branchial carbohydrate metabolism

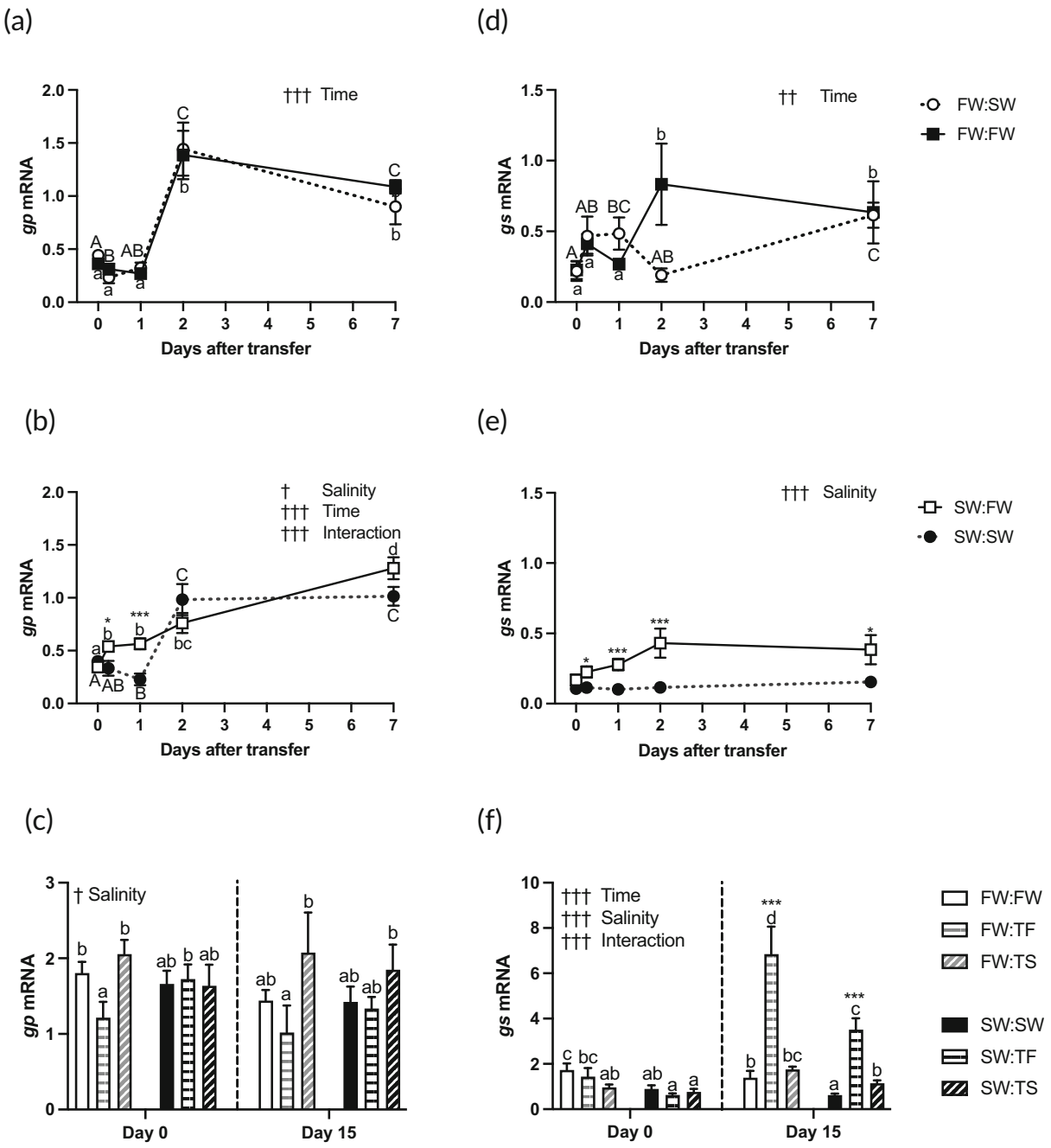

FIGURE 5 Hepatic *gp* (a–c) and *gs* (d–f) expression in fish transferred from FW to SW (a, d) ($n = 6–8$), SW to FW (b, e) ($n = 6–8$) and from FW or SW to a tidal regime (c, f) ($n = 3–8$). Means ± standard error of the mean. Main effects are indicated by †, †† and ††† corresponding to $p < 0.05$, 0.01 and 0.001, respectively. Significant effects were followed by protected Fisher's least significant difference test. Lower-case letters in (a), (b), (d) and (e) indicate significant differences between time points for control fish, and upper-case letters indicate differences between time points for fish transferred to either SW or FW ($p < 0.05$). Asterisks (*, **, ***) in (a), (b), (d) and (e) indicate differences between control and experimentally transferred fish at $p < 0.05$, 0.01 and 0.001, respectively. In the tidal transfers (c, f), lower-case letters indicate differences within each time point ($p < 0.05$), while asterisks indicate differences between time points. FW, fresh water; SW, seawater; TF, tidal fresh water; TS, tidal seawater.

in tilapia. We first utilized unidirectional and dynamic salinity paradigms to describe glucose storage and mobilization following changes in salinity. We then performed in vitro gill filament incubations to probe the involvement of cortisol in these processes. Our findings indicate that (1) plasma glucose levels increase when tilapia acclimate to FW, regardless of whether they are unidirectionally transferred to FW or subjected to a TR; (2) branchial *gpg* expression is elevated in tilapia exposed to SW; (3) branchial and hepatic *gs* and *glut1* expression is higher in tilapia exposed to FW; and (4) cortisol downregulates branchial *glut1* expression. To our knowledge, this is the first

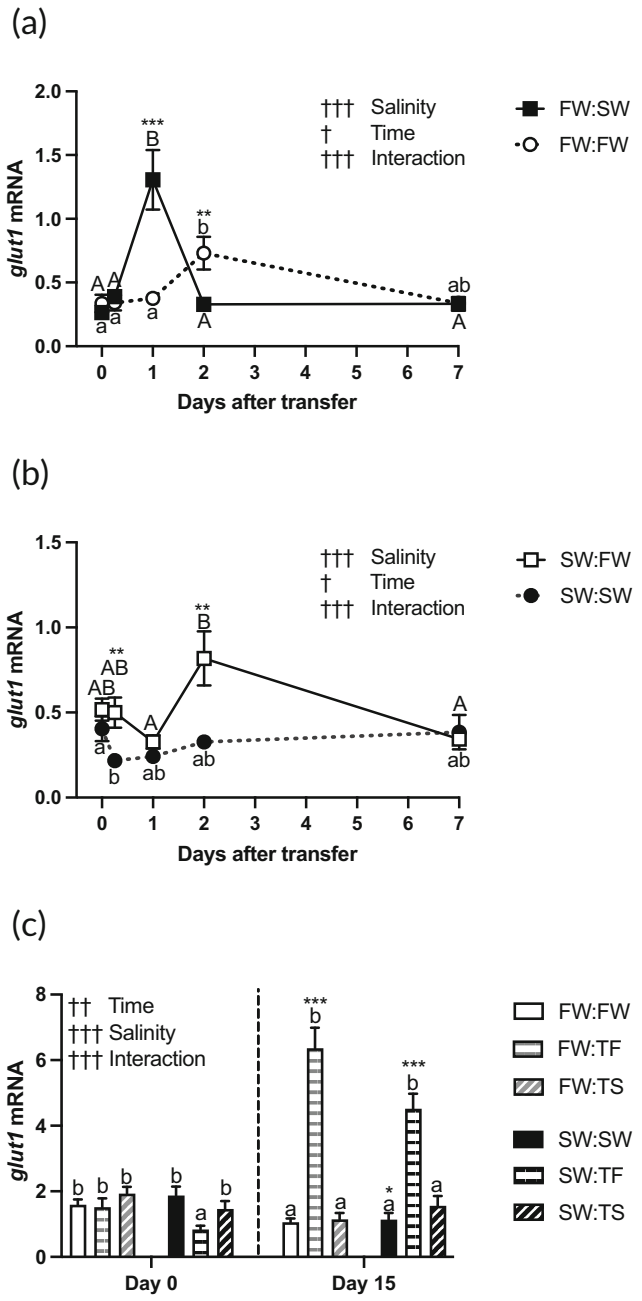

FIGURE 6 Hepatic *glut1* expression in fish transferred from FW to SW (a) ($n = 5–8$), SW to FW (b) ($n = 6–8$) and from FW or SW to a tidal regime (c) ($n = 3–8$). Means ± standard error of the mean. Main effects are indicated by †, †† and ††† corresponding to $p < 0.05$, 0.01 and 0.001, respectively. Significant effects were followed by protected Fisher's least significant difference test. Lower-case letters in (a) and (b) indicate significant differences between time points for control fish, and upper-case letters indicate differences between time points for fish transferred to either SW or FW ($p < 0.05$). Asterisks (*, **, ***) in (a) and (b) indicate differences between control and experimentally transferred fish at $p < 0.05$, 0.01 and 0.001, respectively. In the tidal transfer (c), lower-case letters indicate differences within each time point ($p < 0.05$), while asterisks indicate differences between time points. FW, fresh water; SW, seawater; TF, tidal fresh water; TS, tidal seawater.

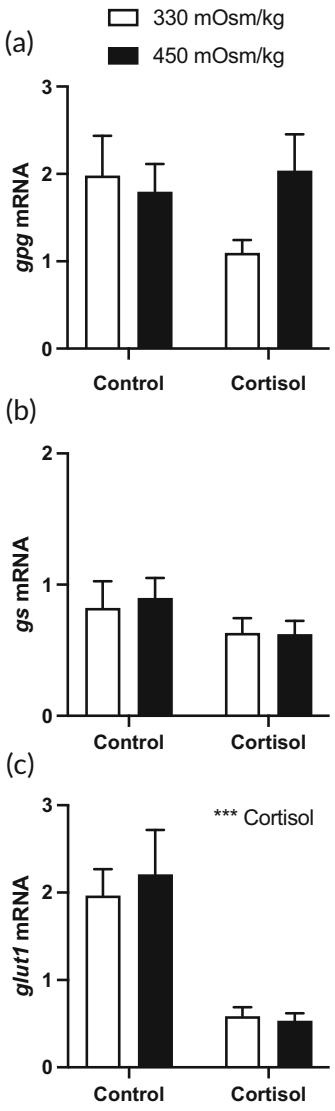

FIGURE 7 Branchial *gpg* (a), *gs* (b) and *glut1* (c) expression following a 6-h incubation in media with and without cortisol adjusted to either 330 or 450 mOsm/kg ($n = 8$). Means ± standard error of the mean. Main effects are indicated by asterisks (***) representing $p < 0.001$.

description of glycogen metabolism in a euryhaline model subjected to tidally changing salinities.

When euryhaline fish transition from FW to SW, they activate branchial ion excretion by increasing the expression of ion channels, transporters and pumps (Kaneko et al., 2008). Higher rates of ion transport involve energetic costs that are met by the mobilization of glucose (Baltzegar et al., 2014; Nakano et al., 1998; Salati et al., 2010; Tsui et al., 2012). Carbohydrates, including glucose and its metabolites such as myo-inositol, can also serve non-energetic roles as compatible osmolytes (Kelly et al., 1999; Sacchi et al., 2013; Yancey, 2005; Zhu et al., 2021). In the current study, a transfer from FW to SW did not elicit a rise in plasma glucose. Transfers from SW to FW, or from SW to TF, elicited robust increases in plasma glucose that coincided with

reductions in plasma osmolality. We did not observe any differences in plasma osmolality or glucose between TF and TS samples from fish that were originally acclimated to FW. While the difference in responses between fish originally acclimated to FW or SW may reflect their initial salinity, the small sample size ($n = 3$) of the FW to TR treatments limits our interpretation. Nonetheless, the increased plasma glucose levels in SW to FW and SW to TF may partially mitigate the decline in plasma Na^+ and Cl^- by providing an energy source for active ion uptake and acting as an osmolyte until hydromineral balance was reestablished. The increased plasma glucose levels in fish transferred from SW to TF coincided with a decrease in hepatic glycogen, suggesting that glycogenolytic processes are activated following transfer to hyposmotic conditions. Successfully acclimating to FW requires the reorganization of ion- and water-transport pathways across osmoregulatory epithelia, which includes the morphofunctional remodelling of branchial ionocytes (Kaneko et al., 2008). However, due to the latency of these functional responses, plasma osmolality typically decreases when fish face a hyposmotic challenge (Seale et al., 2002, 2012), therefore the hyposmotic stress experienced by internal tissues could be mitigated by an extracellular increase in glucose and other osmolytes. An osmotically induced change in glucose is consistent with strategies that employ organic osmolytes rather than inorganic ions to maintain osmotic pressure to protect against macromolecular damage (Yancey, 2005).

For fish initially acclimated to SW, glucose levels were significantly higher in TF than in TS. Since the ionocytes in tilapia acclimated to a TR resemble those of SW-acclimated rather than FW-acclimated tilapia (Moorman et al., 2014; Pavlosky et al., 2019), the hyperosmoregulatory capacity of tilapia in a TR is limited. Tilapia maintained under a TR (TF and TS) express mediators of ion uptake, such as branchial Na^+/Cl^- cotransporter 2, at much lower levels compared with fish acclimated to steady-state FW (Moorman et al., 2014, 2015). The diminished hyperosmoregulatory capacity of fish in a TR is further indicated by the lower plasma osmolalities observed in TF compared with TS and steady-state SW. Hence, tolerance to a TR may be facilitated, at least in part, by rapid changes in plasma glucose concentrations. Recent studies in Nile and Mozambique tilapia indicate that in addition to supplying energy, carbohydrates and carbohydrate-based molecules fulfil non-energetic functions during hypersaline stress (Pan et al., 2023; Zhu et al., 2021). Here, we suggest that carbohydrate metabolism also fulfils non-energetic roles by facilitating osmotic balance during hyposmotic challenges.

Branchial glycogen stores within GRCs are key sources of glucose during bouts of osmotic stress (Tseng et al., 2007). The rapid upregulation of branchial *gpg* during SW acclimation suggests that an increase in glycogen breakdown provides glucose for ionocytes during salinity acclimation. Our findings align with a prior study in which increases in GP content and activity were observed in isolated branchial epithelial cells following transfer from FW to SW (Chang et al., 2007). Furthermore, the expression of *gpg* in the current study was higher in steady-state SW versus FW fish, which is consistent with a previous report indicating a ~2-fold increase in *gpg* expression in tilapia acclimated to SW compared to FW (Tseng et al., 2007). In the present study, branchial *gs* expression increased transiently when fish were transferred from FW to SW. By contrast, branchial GS protein transiently decreased in the branchial epithelial cells of tilapia transferred from FW to SW (Chang et al., 2007). This difference could stem from Chang et al. (2007) using isolated epithelial cells while we used intact gill filaments. In comparison, fish transferred from SW to FW exhibited reduced branchial *gpg* expression, suggesting a reduction in the local breakdown of glycogen under hyposmotic stress. Meanwhile, the expression of branchial *gs* increased transiently, a response that aligns with previous findings (Zikos et al., 2014).

In larval tilapia, cortisol promotes branchial *gpg* expression by acting through GRs (Wu et al., 2023). In our in vitro experiment, cortisol did not affect branchial *gpg* expression. In vivo, however, the decrease in branchial *gpg* in fish transferred from SW to FW resembled the decrease in branchial *gr* expression observed under the same experimental paradigm (Chang et al., 2023). To the extent that diminished *gr* expression attenuates cortisol's capacity to activate *gpg* expression, *gr* levels in FW are consistent with cortisol's action on *gpg* (Wu et al., 2023). In FW to SW transfers, we previously observed a transient increase in pituitary *pomc* expression indicative of HPI-axis activation (Chang et al., 2023). Therefore, when fish transition from FW to SW, branchial *gpg* expression may be systemically enhanced by activating the HPI axis. Conversely, during transitions from SW to FW, cortisol signalling may be reduced at the tissue level due to diminished *gr* expression.

Branchial *gpg*, *gs* and *glut1* expression levels were elevated in a TR compared with steady-state controls, suggesting that carbohydrate turnover rates are elevated under tidal conditions. The upregulation of *gs* and the downregulation of *gp* in TF indicates branchial glycogenesis, a pattern also observed following unidirectional transfer from SW to FW. In TS, 6 h later, the increase in *gp* and the decrease in *gs* expression is indicative of glycogenolysis. These patterns suggest that during the TF phase, when *glut1* expression is also elevated, the gill activates pathways that sequester glucose from the blood to store it as glycogen, which is subsequently broken down during the TS phase. Zebrafish (*Danio rerio*) lacking the GR cleared glucose more rapidly than wild-type fish, suggesting that cortisol signalling inhibits glucose clearance (Faught & Vijayan, 2019). Accordingly, we observed that cortisol decreased branchial *glut1* expression in vitro. Thus, the increase in *glut1* in TF may result from decreased branchial *gr* expression in TF (Chang et al., 2023).

Since the liver plays a crucial role in the storage and metabolism of carbohydrates in fishes (Polakof et al., 2012), we sought to describe the dynamics of hepatic glycogen metabolism and glucose transport. Depending on the nature of the transfers, fish exhibited different hepatic *gs* and *glut1* expression patterns in response to changes in salinity. In fish transferred from SW to FW, *gs* expression rose as early as 6 h and remained elevated through day 7. This increase in hepatic *gs* expression suggests that rising glucose levels in fish transferred from SW to FW trigger glycogenesis. Hepatic *gs* expression also coincided with increases in circulating glucose in hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) and rainbow trout (*Oncorhynchus mykiss*) (Li et al., 2018; Palmer & Ryman, 1972). In the current study,

hepatic *gp* expression was elevated at 6 and 24 h following transfer from SW to FW, suggesting that an initial increase in glycogenolysis coincides with elevated plasma glucose levels.

Both hepatic *gs* and *glut1* were differentially expressed between the two phases of a TR, whereas hepatic *gp* expression resembled steady-state controls. Hepatic *gs* and *glut1* levels were highly upregulated during TF. In the TF phase, the increase in plasma glucose and the decline in hepatic glycogen are consistent with glycogen breakdown and the release of glucose from within hepatocytes into circulation, possibly through GLUT1. On the contrary, *gs* expression increased, which suggests that glycogenesis may also be activated in the liver during TF, possibly due to elevated plasma glucose levels. Additionally, FW-induced hyperglycaemia may also occur through gluconeogenic pathways (Faught & Vijayan, 2016). Future studies investigating the synthesis of organic compatible osmolytes, such as glucose and its metabolites, will further shed light on the systemic mechanisms utilized to both attenuate excursions in plasma osmolality and supply energy to osmoregulatory epithelia during hyposmotic challenges.

In summary, this study describes how alterations in carbohydrate metabolism in the gill and liver of Mozambique tilapia are associated with salinity acclimation under two experimental paradigms: a one-way transfer and a dynamic tidal regime. Regardless of the nature of the salinity challenge, we found consistent differences in markers of glucose production and transport between fish in FW and SW environments. These findings align with our previous investigation into the HPI axis, a known regulator of carbohydrate metabolism, where cortisol signalling was closely tied to salinity changes. The strong salinity-dependent relationship between plasma glucose and salinity, which varies between unidirectional and tidal transfers, further supports glucose's roles as an energetic substrate and an osmolyte. At the tissue level, the regulation of branchial mediators of glycogen metabolism and glucose transport indicates a shift toward glycogenic processes in FW and glycogenolytic processes in SW. By contrast, in liver, evidence of both glycogenic and glycogenolytic processes were found in fish transferred to FW and TF. Together, these findings expand our understanding of how carbohydrate metabolism and transport support the acclimation of euryhaline fishes to dynamic environmental salinities.

AUTHOR CONTRIBUTIONS

RJAC: data collection and analysis; visualization; writing – original draft; writing – review; editing. FTCB: experimental design; sample collection; data collection and analysis; editing; JPB: data collection and analysis; writing – review; editing; funding acquisition; APS: conceptualization; supervision; experimental design; sample collection; data collection and analysis; writing – review; editing; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest could be perceived as prejudicing the impartiality of the research reported.

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