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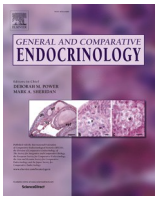

Changes in cortisol and corticosteroid receptors during dynamic salinity challenges in Mozambique tilapia

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ABSTRACT

In estuarine environments, euryhaline fish maintain a narrow range of internal osmolality despite daily changes in environmental salinity that can range from fresh water (FW) to seawater (SW). The capacity of euryhaline fish to maintain homeostasis in a range of environmental salinities is primarily facilitated by the neuroendocrine system. One such system, the hypothalamic-pituitary-interrenal (HPI) axis, culminates in the release of corticosteroids such as cortisol into circulation. Cortisol functions as both a mineralocorticoid and glucocorticoid in fish because of its roles in osmoregulation and metabolism, respectively. The gill, a key site for osmoregulation, and the liver, the primary storage site for glucose, are known targets of cortisol's actions during salinity stress. While cortisol facilitates acclimation to SW environments, less is known on its role during FW adaptation. In this study, we characterized the responses of plasma cortisol, mRNA expression of pituitary pro-opiomelanocortin (*pomc*), and mRNA expression of liver and gill corticosteroid receptors (*gr1, gr2,* and *mr*) in the euryhaline Mozambique tilapia (*Oreochromis mossambicus*) under salinity challenges. Specifically, tilapia were subjected to salinity transfer regimes from steady-state FW to SW, SW to FW (experiment 1) or steady state FW or SW to tidal regimen (TR, experiment 2). In experiment 1, fish were sampled at 0 h, 6 h, 1, 2, and 7 d post transfer; while in experiment 2, fish were sampled at day 0 and day 15. We found a rise in pituitary *pomc* expression and plasma cortisol following transfer to SW while branchial corticosteroid receptors were immediately downregulated after transfer to FW. Moreover, branchial expression of corticosteroid receptors changed with each salinity phase of the TR, suggesting rapid environmental modulation of corticosteorid action. Together, these results support the role of the HPI-axis in promoting salinity acclimation, including in dynamically-changing environments.

1. Introduction

Vertebrates maintain tight hydromineral balance by actively regulating the exchange of ions and water between their internal and external environment. In aquatic environments, where salinities can range from fresh water (FW) to above that of seawater (SW), euryhaline fish consistently maintain their internal osmolality at roughly one-third that of SW (McCormick, 2001). To maintain osmotic equilibrium, fish in FW must actively uptake ions and excrete dilute urine while those in SW must actively extrude ions (Evans, 2008), a process largely mediated by the endocrine system. Acting through various pathways, hormones affect ion transport across osmoregulatory epithelia including intestine, kidney, and gill (Breves et al., 2011; Breves et al., 2021; Inokuchi et al., 2015; McCormick, 2001; Sakamoto and McCormick, 2006; Seale et al., 2014; Seale et al., 2021; Whittamore, 2012), enabling euryhaline fish to withstand rapid changes in salinity (Seale and Breves, 2022).

One such hormonal pathway in fish, the hypothalamic- pituitary-interrenal (HPI) -axis, facilitates the part of the neuroendocrine stress response that culminates in the synthesis and release of corticosteroids into circulation (Schreck et al., 2016; Wendelaar Bonga, 1997). Environmental cues, such as changes in salinity, are perceived by the brain where hypothalamic corticotropin releasing hormone triggers the release of adrenocorticotropic hormone (ACTH) from the pituitary gland (Aruna et al., 2015a; Flik et al., 2006; Kageyama and Suda, 2009; Pepels et al., 2004). ACTH is the mature peptide hormone cleaved from pro-opiomelanocortin (POMC), encoded by the *pomc* gene (Pepels and Balm, 2004). Following release from the pituitary, ACTH stimulates the interrenal cells of the head-kidney to synthesize and release cortisol into

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circulation (Balm et al., 1994). Cortisol then binds to nuclear glucocorticoid and/or mineralocorticoid receptors in target tissues to enact its effects, including the alteration of branchial ionocyte morphology and hepatic metabolic pathways (McCormick, 1995; Faught and Vijayan, 2016). In tilapia, glucocorticoid receptors -1 and -2 (GR-1, -2), and mineralocorticoid receptor (MR) were identified and localized in various organs including the gill (Aruna et al., 2012). Moreover, non-genomic actions of cortisol have been described, indicating direct interactions with the plasma membrane that elicit effects that are more rapid than classical steroid hormone action (Borski et al., 2001). In branchial ionocytes, cortisol affects both ion uptake and secretion, facilitating FW and SW osmoregulation, respectively (McCormick, 2001). Although the exact mechanisms are unclear, cortisol has been shown to increase the upper range of SW tolerance, stimulate ionocyte Na^+/K^+ ATP-ase (NKA) activity, and promote ionocyte differentiation in several euryhaline fish (McCormick, 1990; McCormick, 1995).

Several studies have analyzed the effects of salinity stress on the HPI axis. Pituitary *pomc* expression was upregulated when FW-acclimated Nile tilapia (*Oreochromis niloticus*) and hybrid tilapia (*O. mossambicus* × *O. niloticus*) were transferred to brackish water (BW) (Aruna et al., 2015b; Rengmark et al., 2007), suggesting an increase in circulating ACTH and cortisol secretion during SW acclimation. In Mozambique tilapia (*O. mossambicus*), however, pituitary ACTH release transiently increased in response to a hyposmotic challenge *in-vitro* (Seale et al., 2002) and transfer of fish from FW to SW had variable effects on plasma cortisol levels (Breves et al., 2010; Kammerer et al., 2010; Morgan et al., 1997). The effects of salinity on branchial corticosteroid receptors also remain unclear, though these receptors appear to be supportive of SW-acclimation. In one study, a transfer of Mozambique tilapia from FW to 2/3 SW increased the number of intracellular branchial GR1 (Dean et al., 2003). In another study, when hybrid tilapia (*O. mossambicus* × *O. niloticus*) were transferred from FW to SW, branchial *gr1* mRNA levels remained unchanged following transfer, while *gr2* and *mr* increased 1 and 4 days post-transfer, respectively (Aruna et al., 2012). The relationship between salinity and corticosteroid receptors in the liver of euryhaline fish also remains unclear. In Mozambique tilapia kept in FW or transferred to 2/3 SW, specific binding of cortisol to cytosolic liver preparations were unchanged, suggesting a lack of salinity-specific differences in hepatic corticosteroid receptor dynamics (Dean et al., 2003). Similarly, in rainbow trout (*Oncorhynchus mykiss*) hepatic *gr* transcripts were unchanged during hypersaline stress while the amount of GR protein transiently increased (Singer et al., 2007). In the Japanese Medaka (*Oryzias latipes*), however, hepatic *gr1* expression was highest in brackish water (BW; 11 ‰) compared with either FW (0 ‰) or SW (33 ‰) (Ogoshi et al., 2012).

While previous studies on the effects of salinity on the stress response have focused primarily on responses to hyperosmotic environments, the response of the HPI-axis to hyposmotic environments is less understood. For example, it remains to be investigated whether the transcriptional responses of *gr*'s and *mr* to SW-acclimation can be reversed when tilapia are transferred to FW. We expect SW acclimated tilapia to decrease branchial corticosteroid receptor expression upon transfer to FW as these receptors appear supportive of SW adaptation in the gill.

Most studies of salinity acclimation focus on one-time transfers to higher or lower salinities, but some euryhaline fish inhabit estuaries that are subject to dynamically changing salinities produced by ocean tides. Mozambique tilapia are native to near-shore waters of southeast Africa and typically found near river mouths exposed to such variations in salinities (Trewavas, 1983). Some of the physiological adaptations of this and other euryhaline species exposed to conditions that simulate a tidal environment have been recently reviewed (Seale and Breves, 2022). Under these tidally changing conditions, however, the response of the HPI axis remains uninvestigated. When acclimated to a simulated tidal regime (TR), where the salinity changes between FW and SW every 6 h, Mozambique tilapia exhibit growth and osmoregulatory patterns that are distinct from fish reared in steady-state FW and SW (Moorman et al., 2014, 2016; Pavlosky et al., 2019). Despite continuously shifting between FW and SW, Mozambique tilapia in a TR could maintain plasma osmolality within a narrow range (Moorman et al., 2014; Seale et al., 2019) and grow faster than both FW and SW steady-state fish (Moorman et al., 2016). In previous salinity acclimation studies employing tilapia subjected to one-way transfers, circulating levels of prolactin (Prl) and growth hormone (GH), both known to play roles in FW- and SW-acclimation respectively (Sakamoto and McCormick, 2006), increased following transfer from SW to FW and FW to SW, respectively (Breves et al., 2010; Seale et al., 2002). Interestingly, plasma levels of both PRL and GH did not change between FW and SW phases of a tidal cycle while transcription of their receptors in target tissues did, suggesting a shift from systemic to local endocrine regulation under a TR (Moorman et al., 2014, 2015, 2016; Seale et al., 2019). We hypothesize that this shift from ligand to receptor modulation of hormone action under dynamically changing conditions may also be observed in other endocrine systems involved in osmoregulation, including cortisol and its receptors.

In this study, we investigated changes in the HPI-axis in Mozambique tilapia transferred from FW to SW, SW to FW and acclimated to a TR. Specifically, we measured circulating cortisol, mRNA levels of *pomc* in the pituitary, and mRNA levels of *gr1, gr2*, and *mr* in gill and liver. Inasmuch as a change in salinity may induce a stress response regardless of the direction of transfer, we hypothesized that the HPI-axis would be activated by all salinity transfers, including a TR. Of particular interest, by employing these distinct salinity challenge paradigms we further clarified the role of the HPI-axis during hyposmotic stimuli under both steady-state and dynamically changing conditions.

2. Materials and methods

2.1. Animals

Mature male Mozambique tilapia (*O. mossambicus*) of mixed sizes 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, University of Hawai'i.

2.2. Experiment 1 - One-way transfers

Adult (376 ± 16.20 g) male Mozambique tilapia were held under natural photoperiod in outdoor 700L tanks holding roughly 525 L of FW (0.1 ± 0.1 ‰, municipal water) or SW (34 ± 1 ‰, Kāne'ohe Bay, Hawai'i, USA) maintained at 25 ± 2 °C. Eighty SW-acclimated fish, which were spawned and reared in SW, having no prior exposure to FW, were randomly distributed into two replicate SW tanks; Eighty FW-acclimated fish, spawned and reared in FW, were divided into two replicate FW tanks. Fish were fasted 24 h prior to initial sampling and fed trout chow pellets (Skretting, Tooele, UT) once daily to satiation throughout the experiment. Ahead of the experimental trial, each of the 4 tanks held 40 fish for 1 week prior to day 0 sampling. On day 0 of the experiment, 8 fish from each of the four tanks were sampled. After initial sampling, one tank from each salinity was transferred to the opposite salinity, by changing the water intakes from FW to SW or vice-versa, and one tank was maintained at the same salinity. The fish transferred from FW to SW were initially acclimated to brackish water (BW; 25 ‰) by turning on both FW and SW inflows, yielding a transition from 0 to 25 ‰ by 1 h. Fish were then held at 25 ‰ until the 48 h sampling time (sampled in BW at 6 h, 1 d, 2 d), after which they were transitioned to full strength SW (34 ‰). The fish transferred from SW to FW were transitioned by changing the inflow from SW to FW, yielding a change from 35 to 0 ‰ by 1 h. Eight fish were sampled from each tank at 6 h (0.25 d), 1 d, 2 d, and 7 d post-transfer.

Table 1
Gene targets and primer sequences for RT-qPCR. Abbreviations: *pomc* – pro-opiomelanocortin; *mr* – mineralocorticoid receptor; *gr* – glucocorticoid receptor; *18 s* – 18 s ribosomal RNA; *ß-actin* – Actin Beta; *ef1a* – elongation factor 1-alpha.

Gene	Primer Sequence (5′-3′)	R^2	Eff.%	Accession no.	Reference
18 s	F: GCTACCACATCCAAGGAAGGC R: TTCGTCACTACCTCCCCGAGT	0.98	104	AF497908	Magdeldin et al., 2007
ß-actin	F: CTCTTCCAGCCTTCCTTCCT R: ACAGGTCCTTACGGATGTCG	0.99	85	AB037865	Tipsmark et al., 2011
ef1a	F: TAAAACCCTGCCTGACTTCC R: AATCCTCATTAGCCCCAAAA	0.99	86	AB075952	Breves et al., 2010
pomc	F: CAAGCGCTCCTACTCAATGGA R: ACGCCGTTGGAGGTGTACA	0.99	90	AF116240	Aruna et al., 2015b
mr	F: TGGTACGCATGGTGAAATGG R: TCAGGGTGATTTGGTCCTCAAT	0.99	106	HM7699565	Aruna et al., 2015b
gr1	F: TCCCTCGTACCCAAGTGCAT R: TGTCTGGCTCTATCGCCTTCA	0.99	98	GU296354	Aruna et al., 2015b
gr2	F: CAGGAGCATGCCCCAACT R: GCCCGAGTAGATGATCTCTGGTT	0.98	106	HQ704389	Aruna et al., 2015b

2.3. Experiment 2 - tidal transfer

Adult (222.3 ± 11.5 g) male Mozambique tilapia were held under natural photoperiod in outdoor 700 L tanks holding roughly 525 L with FW (0.1 ± 0.1 ‰, municipal water) or SW (34 ± 1 ‰, Kaneohe Bay, Hawai'i USA) maintained at 25 ± 2 °C. Two replicate flow-through SW tanks, and two replicate flow-through FW tanks were used for this experiment. One tank per salinity was used for control fish that were maintained in the salinity for 15 days and the other tank was transferred to a TR for 15 days. Fish were fed trout chow pellets (Skretting, Tooele, UT) once daily to satiation throughout the experiment and fasted 24 h prior to each sampling. On Day 0 of the experiment, 48 total fish, consisting of 24 fish from both FW and SW, were sampled to account for each salinity condition tested (n = 8 per group: steady state control, tidal freshwater, tidal seawater). Following initial sampling, there were 20 fish per tank. Incoming water to one of the FW tanks and one of the SW tanks was adjusted to simulate a TR by alternating FW and SW inflow every 6 h (Moorman et al., 2014; Seale and Breves, 2022) while the control tanks were maintained at the same salinity. Eight fish from each of the FW and SW controls were sampled 15 days following transfer. As a result of mortalities in the FW to TR tank, only 6 fish remained available for sampling on day 15; therefore, 3 fish were sampled at the end of the FW phase (TF) and 3 fish were sampled at the end of the SW phase (TS) of the TR. In the SW to TR tank, there were no mortalities and 8 fish were sampled at the end of the FW phase (TF) of the TR, and 8 fish were sampled 6 h later at the end of the SW phase (TS) of the TR.

2.4. Sampling

In both experiments, fish were sampled in the same manner. Four fish were initially netted from their respective tanks and transferred to five gallon buckets at 2 fish per bucket. Fish were then anesthesized in the buckets with 2-phenoxyethanol (0.3 mL/L, Sigma Aldrich, St. Louis, MO). Following anesthesia, fish were weighed, blood was drawn with a needle and syringe coated with sodium heparin (200 U/ml, Sigma-Aldrich, St. Louis, MO), then euthanized. The total time taken from fish capture to plasma sampling was between 2 and 5 min from first to last of the 4 fish sampled. This sampling procedure was then repeated to total 8 fish sampled per salinity per time point. Plasma was separated by centrifugation and stored at −80 °C. Gill filaments from the second gill arch on the left side of the fish, pituitary gland, and liver was collected. All tissue samples were frozen in liquid nitrogen and stored at −80 °C until further analyses.

2.5. Plasma cortisol

Plasma cortisol was measured with a cortisol enzyme immunoassay (EIA) modified from Carey and McCormick, 1998 and validated for tilapia plasma. To validate the assay, standard curves for EIA were generated using cortisol (Sigma) at 500, 400, 200, 100, 50, 25, 10, 5, and 2.5 ng/mL dissolved in either Ringer's solution or in pooled tilapia plasma stripped of hormones using dextran coated charcoal (Sigma-C6241). Briefly, dextran coated charcoal was added to the pooled tilapia plasma at 20 g/L and the mixture was shaken overnight at 4 °C. The following day, plasma was separated from the charcoal by centrifugation at 2000 × g for 15 min, and the supernatant was collected and used for validation. Ninety-six-well microplates were first loaded with rabbit anti-cortisol (Fitzgerald Ind. Int'l, MA; 150 µl/ well) diluted to 1:30,000 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 100 mL ddH_2O). Plates were then incubated for 4 h at 37 °C. Following incubation, the cortisol antibody solution was discarded, and the plates were rinsed 5 times with 250 µl/ well of 1X wash solution (0.15 M NaCl, 0.05% Tween 20, dd H_2O to 1 L) at 250 µl/well. Then, 250 µl of EIA buffer (19.5 mL of 0.2 M $NaH_2PO_4H_2O$, 30.5 mL of 0.2 M Na_2HPO_4, 0.15 M NaCl, 0.1% BSA, 50 mL dd H_2O) was loaded into each well and left to incubate at room temperature for 30 min. The EIA buffer was then discarded, and 150 µl of fresh EIA buffer was loaded into each well. Then, 100 µl of Cortisol-HRP conjugate (Fitzgerald Ind. Int'l, MA; diluted at 1:6,000 in EIA buffer) and standards or samples were added to each well and incubated overnight at 25 °C. Following incubation, the solution was discarded, and the wells were rinsed 5 times with 250 µl/ well of wash solution. Then, 200 µl of TMB Peroxidase Substrate (Sigma T0440) was added to each well and incubated for 10 min at room temperature with gentle shaking (60–70 rpm). Finally, 50 µl of 0.5 M HCL was added to each well and absorbance was read at 450 nm using a multimode microplate reader (Synergy LX, BioTek). Standards were run in 4 replicates per concentration. Both standard curves were plotted using a 4-parameter curve fit in Gen5 (Biotek) software yielding an R^2 of 0.996 and 0.999 for cortisol standards dissolved in Ringer's solution and stripped plasma, respectively (Supplemental Fig. 1). Pooled plasma samples, both stripped and unstripped, were also loaded in duplicate at dilutions of 1, 1:2, 1:4, and 1:8. For quantifying cortisol in experimental plasma samples, 2.5 µl of each standard and plasma samples (diluted 1:4 in 0.1% BSA/ringers) were run in triplicates and duplicates, respectively.

2.6. mRNA expression

Total RNA was extracted from tissues with TRI Reagent following the manufacturer's protocol (Molecular Research Center, Cincinnati, OH).

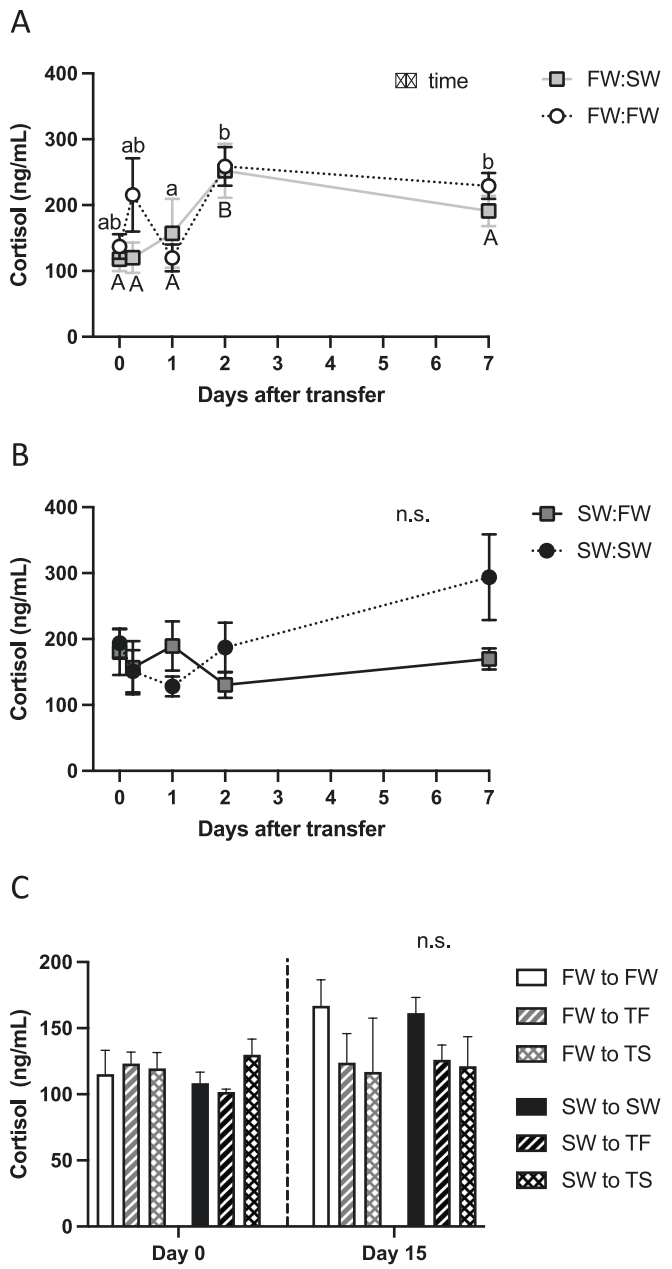

Fig. 1. Plasma cortisol levels of Mozambique tilapia after transfer from FW to SW (A) (n = 6–8), SW to FW (B) (n = 6–8), and from FW or SW to a TR (C) (n = 3–8). Main effects are denoted with † representing $P < 0.05$, †† representing $P < 0.01$, ††† representing $P < 0.001$. Significant main effects were followed by protected Fisher's LSD test. In one-way transfers (A, B), lower case letters represent differences between time points within a salinity transfer for control fish (FW:FW and SW:SW) and upper case letters represent differences between time points within a salinity for experimental transfers (FW:SW and SW:FW) ($P < 0.05$). At the corresponding time points, *, **, *** indicate significant differences between control and experimental groups ($P < 0.05$, $P < 0.01$, $P < 0.001$ respectively). N.s. stands for no significant effects. In tidal transfers (C), different lowercase letters denote significant differences between salinities within a time point ($P < 0.05$) while asterisks indicate significant differences between time points within a salinity (*, **, ***; $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively).

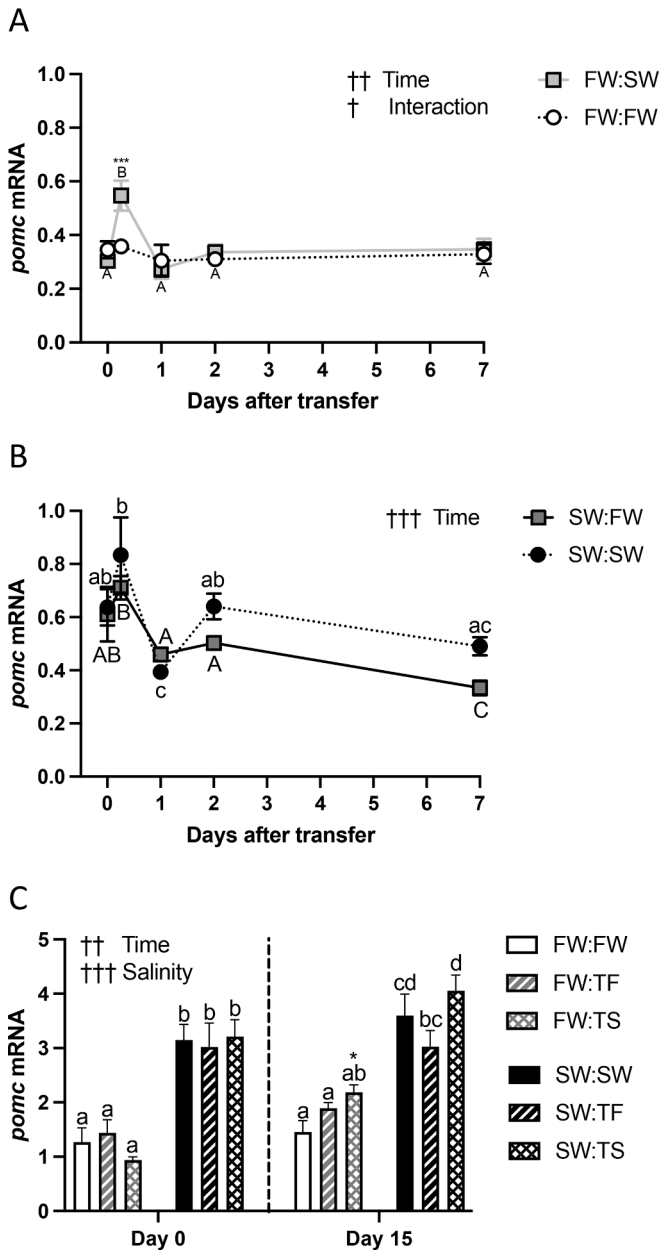

Fig. 2. Pituitary *pomc* levels of Mozambique tilapia after transfer from FW to SW (A) (n = 6–8), SW to FW (B) (n = 6–8), and from FW or SW to a TR (C) (n = 3–8). Target gene transcripts were normalized to the geometric mean of *18 s, ef1a,* and *β-actin* and graphed as normalized expression. Main effects are denoted with † representing $P < 0.05$, †† representing $P < 0.01$, ††† representing $P < 0.001$. Significant main effects were followed by protected Fisher's LSD test. In one-way transfers (A, B), lower case letters represent differences between time points within a salinity transfer for control fish (FW:FW and SW:SW) and upper case letters represent differences between time points within a salinity for experimental transfers (FW:SW and SW:FW) ($P < 0.05$). At the corresponding time points, *, **, *** indicate significant differences between control and experimental groups ($P < 0.05$, $P < 0.01$, $P < 0.001$ respectively). N.s. stands for no significant effects. In tidal transfers (C), different lowercase letters denote significant differences between salinities within a time point ($P < 0.05$) while asterisks indicate significant differences between time points within a salinity (*, **, ***; $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively).

RNA concentration and purity were assessed using a NanoDrop (NanoDrop One, Thermo Fisher Scientific, Waltham, MA). Total RNA (500 ng) was reverse transcribed into cDNA with the High Capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA). The StepOnePlus real-time PCR system (Thermo Fisher Scientific) was used to determine the mRNA levels of reference and target genes. The qPCR reaction mix (15 μl/well) consisted of 7.5 μl of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 5.9 μl of molecular grade H_2O, 0.3 μl of 10 uM forward and reverse primers, and 1 μl cDNA. PCR cycling parameters

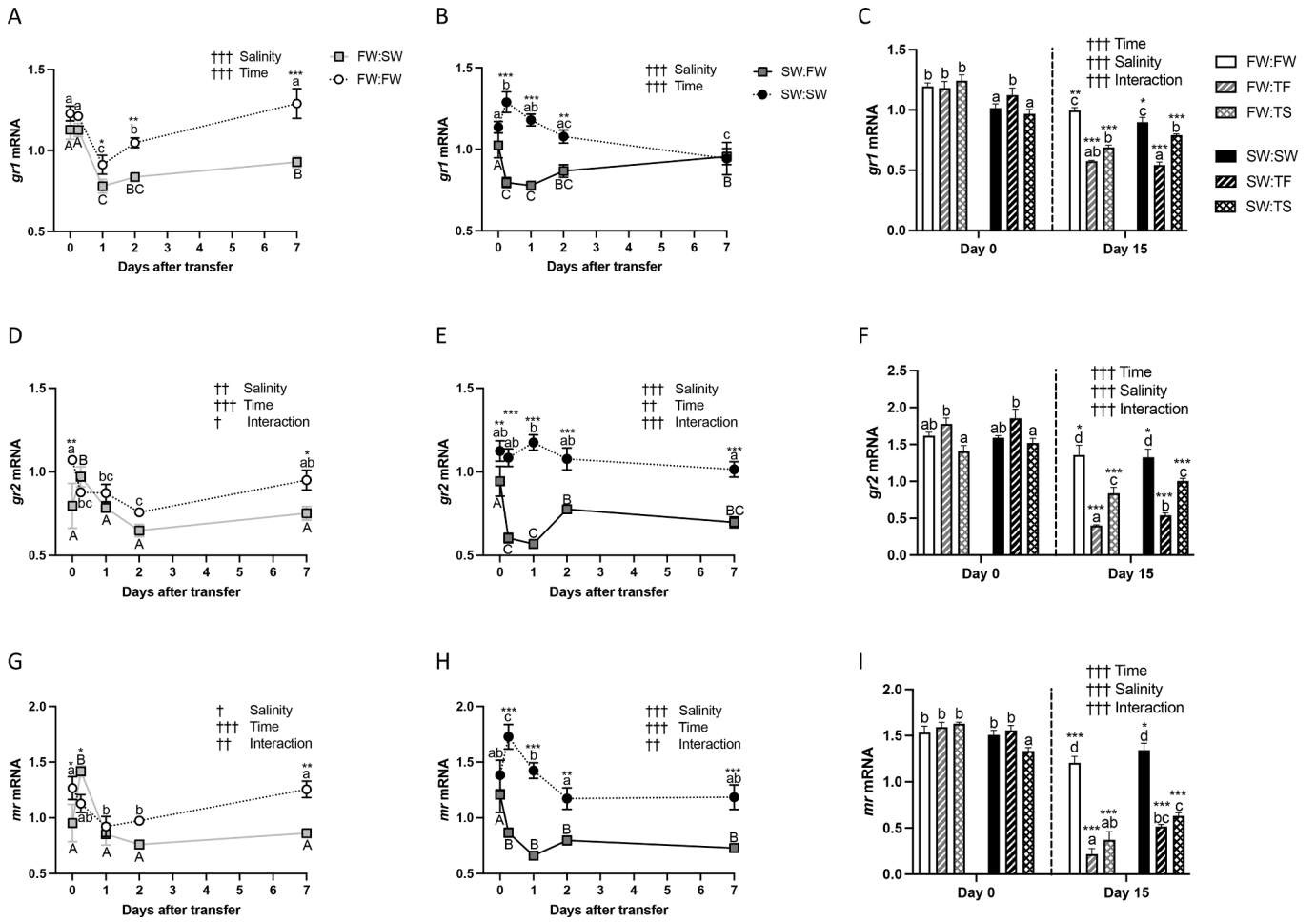

Fig. 3. Branchial *gr*1 (A, B, C), *gr2* (D, E, F), and *mr* (G, H, I) mRNA levels following transfer of Mozambique tilapia from FW to SW (A, D, G) (n = 6–8), SW to FW (B, E, H) (n = 6–8), and from FW or SW to a TR (C, F, I) (n = 3–8). Target gene transcripts were normalized to the geometric mean of *18 s, ef1a*, and *β-actin* and graphed as normalized expression. Main effects are denoted with † representing $P < 0.05$, †† representing $P < 0.01$, ††† representing $P < 0.001$. Significant main effects were followed by protected Fisher's LSD test. The legends for figures A, B and C are applicable to the figures below (For example: A, D, and G share a legend). In one-way transfers (A, B), lower case letters represent differences between time points within a salinity transfer for control fish (FW:FW and SW:SW) and upper case letters represent differences between time points within a salinity for experimental transfers (FW:SW and SW:FW) ($P < 0.05$). At the corresponding time points, *, **, *** indicate significant differences between control and experimental groups ($P < 0.05$, $P < 0.01$, $P < 0.001$ respectively). N.s. stands for no significant effects. In tidal transfers, different lowercase letters denote significant differences between salinities within a time point ($P < 0.05$) while asterisks indicate significant differences between time points within a salinity (*, **, ***; $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively).

were: 2 min at 50 °C, 10 min at 95 °C, then 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Threshold Ct values were converted to concentration using a standard curve generated from serial dilutions of pooled cDNA from each tissue. The geometric mean of three reference genes (*ef1a, 18 s, β-actin*), measured using the same method as target gene transcripts, was used to normalize target gene expression. Data is presented as the concentration of target genes divided by the concentration of the geometric mean of the three reference genes. Specific primers were employed as previously described for pituitary *pomc* and branchial and hepatic *mr, gr1,* and *gr2.* All primer sequences are listed in Table 1.

2.7. Statistical analysis

In both experiments, data were analyzed using two-way ANOVA with time and salinity as main effects. Significant effects ($P < 0.05$) of ANOVA were followed by protected Fisher's LSD test. When necessary, data were log or square root transformed to satisfy the underlying assumptions of normality and homogeneity of variance. Statistical analysis was performed using Prism 9 (GraphPad).

3. Results

3.1. Plasma cortisol

Time had a significant effect on plasma cortisol levels in fish transferred from FW to SW, but there were no main effects in those transferred from SW to FW (Fig. 1A, B). Plasma cortisol levels of FW control fish were similar to day 0 at all time points despite a decrease on day 1 relative to days 2 and 7. When FW fish were transferred to SW, their plasma cortisol levels increased by 2 d. This increase was temporary, with plasma cortisol returning to day 0 levels by day 7 (Fig. 1A). Plasma cortisol did not differ between FW- and SW-acclimated fish nor was it affected by transfer to a TR (Fig. 1C).

3.2. Pituitary pomc expression

There were significant effects of time and interaction on pituitary *pomc* in fish transferred from FW and a significant effect of time in fish transferred from SW (Fig. 2A, B). Pituitary *pomc* expression remained unchanged from day 0 to day 7 post transfer in FW controls. In fish transferred from FW to SW, pituitary *pomc* expression transiently

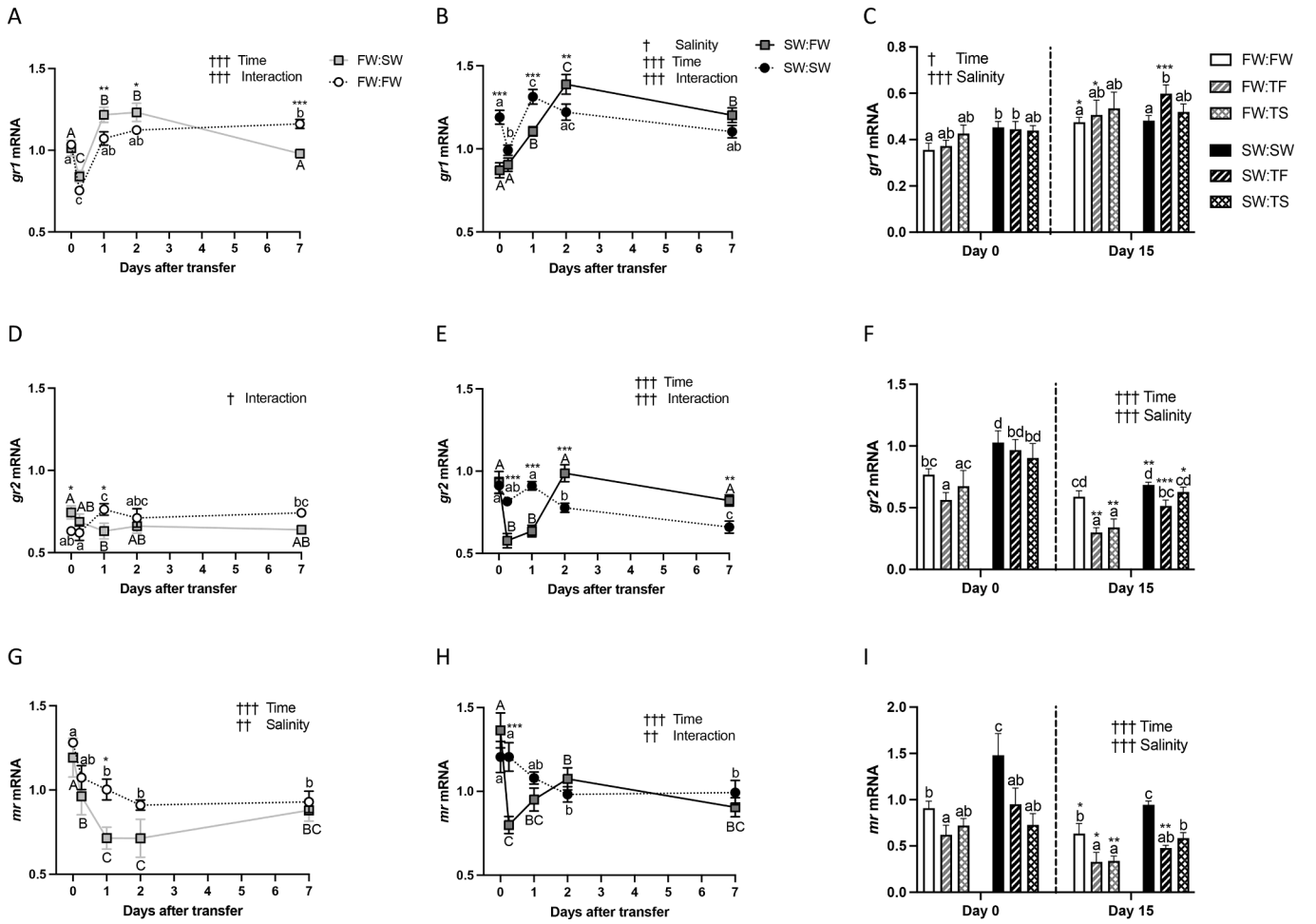

Fig. 4. Hepatic *gr*1 (A, B, C), *gr2* (D, E, F), and *mr* (G, H, I) mRNA levels following transfer of Mozambique tilapia from FW to SW (A, D, G) (n = 6–8), SW to FW (B, E, H) (n = 6–8), and from FW or SW to a TR (C, F, I) (n = 3–8). The legends for figures A, B and C are applicable to the figures below (For example: A, D, and G share a legend). Target gene transcripts were normalized to the geometric mean of *18 s, ef1a*, and *ß-actin* and graphed as normalized expression. Main effects are denoted with † representing $P < 0.05$, †† representing $P < 0.01$, ††† representing $P < 0.001$. Significant main effects were followed by Fisher's protected LSD test. In one-way transfers (A, B), Lower case letters represent differences between time points within a salinity transfer for control fish (FW:FW and SW:SW) and upper case letters represent differences between time points within a salinity for experimental transfers (FW:SW and SW:FW) ($P < 0.05$). At the corresponding time points, *, **, *** indicate significant differences between control and experimental groups ($P < 0.05$, $P < 0.01$, $P < 0.001$ respectively). N.s. stands for no significant effects. In tidal transfers, different lowercase letters denote significant differences between salinities within a time point ($P < 0.05$) while asterisks indicate significant differences between time points within a salinity (*, **, ***; $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively).

increased by 6 h, then returned to control levels at 1, 2, and 7 d post transfer (Fig. 2A). In SW fish, comparing day 0 with day 7, the SW control group maintained similar *pomc* levels while the SW to FW transferred fish decreased *pomc* expression by 7 d (Fig. 2B). In the transfers from either FW or SW to a TR, salinity and time had significant effects on pituitary *pomc* (Fig. 2C). At day 0, *pomc* expression was higher in fish acclimated to SW than those in FW by roughly 2.5-fold. After 15 days in a TR, *pomc* was lower in SW fish sampled in TF relative to those in TS. Although there were no differences between TF and TS in FW fish at day 15, fish sampled in TS expressed more *pomc* at day 15 than at day 0 (Fig. 2C).

3.3. Branchial mRNA levels of corticosteroid receptors

In the gill, expression of three corticosteroid receptors (*gr1, gr2*, and *mr*) was analyzed. In one-way transfers, time and salinity had significant effects on *gr1* expression in both FW and SW fish (Fig. 3A, B). In FW fish, *gr1* decreased at 1 d and 2 d post transfer in both the FW control group and SW transfer group. At 1,2, and 7 d post transfer, the FW-SW fish expressed lower *gr1* than the FW-FW controls. By 7 d post transfer, *gr1* expression in the FW control group returned to day 0 levels while expression levels in the FW-SW group remained at low levels (Fig. 3A). Seawater control fish increased *gr1* by 6 h post transfer, returned to day 0 levels by 1, 2, and 7 d. On the other hand, *gr1* decreased in fish transferred from SW to FW by 6 h and remained low through 7 d. Expression levels of *gr1* were significantly lower in SW-FW fish than in SW-SW fish at 6 h, 1 d, and 2 d post transfer (Fig. 3B). In the tidal transfer, there were significant effects of time, salinity, and interaction of branchial *gr1* expression (Fig. 3C). By day 15, *gr1* was downregulated in the fish in a TR relative to steady-state controls. In fish transferred from SW to a TR, *gr1* was upregulated in fish sampled in a TS relative to TF. All groups downregulated *gr1* by day 15 compared with their respective day 0 controls (Fig. 3C).

There were significant effects of time, salinity, and interaction on *gr2* expression in FW and SW fish in one-way transfers (Fig. 3D, E). Control FW fish decreased *gr2* levels by 6 h, 1 d, and 2 d. Fish transferred from FW to SW transiently increased *gr2* by 6 h then returned to day 0 levels by day 7. The SW control fish expressed *gr2* at higher levels than the fish transferred from SW to FW at all time points. At 6 h, *gr2* expression in the fish transferred from SW to FW decreased relative to day 0 and remained lower than day 0 throughout the experiment (Fig. 3E). When fish were transferred to a TR, there were significant effects of time, salinity, and

interaction on branchial *gr2* expression (Fig. 3F). By day 15, *gr2* was downregulated in the fish in a TR relative to steady-state controls; *gr2* was upregulated in fish sampled in a TS relative to TF. All groups downregulated *gr2* by day 15 compared with their respective day 0 controls (Fig. 3F).

In one-way transfers, significant effects of time, salinity, and interaction were found on *mr* expression in FW and SW fish (Fig. 3G, H). When FW fish were transferred to SW, *mr* expression transiently increased by 6 h. When comparing salinity treatments, *mr* was higher in fish transferred from FW to SW compared with FW controls by 6 h (Fig. 3G). In fish transferred from SW to FW a decrease in *mr* was observed as early as 6 h; *mr* remained low through day 7. Expression of *mr* was lower in fish transferred from SW to FW at all time points past day 0 compared with SW controls (Fig. 3H). In fish transferred to a TR, time, salinity, and interaction had significant effects on *mr* expression (Fig. 3I). At day 15, fish from both FW and SW transferred to a TR downregulated *mr* compared with steady-state controls; unlike *gr1* and *gr2*, there were no transcriptional differences between TF and TS. Expression of *mr* on day 15 was lower in all groups compared to their respective day 0 controls (Fig. 3I).

3.4. Hepatic mRNA levels of corticosteroid receptors

The expression levels of *gr1*, *gr2*, and *mr* were also analyzed in the liver. There were significant effects of time and interaction on *gr1* expression in one-way transfers from FW to SW (Fig. 4A). Following transfer from FW to SW, hepatic *gr1* expression transiently decreased by 6 h then increased by 1 and 2 d before returning to day 0 levels by day 7. Expression of *gr1* in FW control fish followed a similar pattern, decreasing by 6 h and increasing by 7 d when compared to day 0. By 6 h and 1 d following transfer, *gr1* was upregulated relative to FW controls (Fig. 4A). In fish transferred from SW to FW there were significant effects of salinity, time, and interaction on *gr1* expression (Fig. 4B). For SW-SW control fish, *gr1* initially decreased by 6 h post transfer then increased by 1 d post transfer when compared to day 0. Expression of *gr1* was upregulated in fish transferred from SW to FW by 1 d, peaked by 2 d, then subside to levels observed at 1 d by day 7. There were significant effects of time and salinity on *gr1* expression in tidal transfers (Fig. 4C). Expression of *gr1* in fish transferred from SW to a TR increased in the TF group relative to SW controls by day 15. (Fig. 4C).

There was an interaction effect on *gr2* expression in fish transferred from FW to SW (Fig. 4D). A rise in *gr2* was observed by 1 d in FW control fish. In fish transferred from FW to SW, *gr2* decreased by 1 d relative to day 0. In fish transferred from SW to FW, there were significant effects of time and interaction on *gr2* expression (Fig. 4E). In SW control fish, *gr2* decreased by 2 d then further decreased by 7 days. The fish transferred from SW to FW downregulated *gr2* by 6 h followed by a return to day 0 levels by 2 d. Compared with SW controls, *gr2* expression in fish transferred from SW to FW decreased by 6 h and 1 d, then increased by 2 and 7 d (Fig. 4E). Time and salinity had significant effects on *gr2* expression in tidal transfers (4F). Following transfer to a TR, FW fish downregulated *gr2* in both TF and TS relative to FW controls while *gr2* in SW fish decreased only in TF. With the exception of FW controls, *gr2* expression was lower on day 15 than day 0 (Fig. 4F).

Salinity and time had significant effects on hepatic *mr* expression in fish transferred from FW to SW (Fig. 4G). Hepatic *mr* decreased in both FW controls and transferred fish over the course of the experiment. Although expression patterns were similar, *mr* was lower in fish transferred from FW to SW than FW controls by 1 d (Fig. 4G). There were significant effects of time and interaction on *mr* expression in fish transferred from SW to FW, (Fig. 4H). Expression of *mr* drastically decreased in fish transferred from SW to FW by 6 h, remaining lower than day 0 until day 7. Meanwhile, *mr* in SW control fish only decreased by 2 d and 7 days compared with day 0. Compared with SW controls, *mr* was lower in fish transferred from SW to FW by 6 h (Fig. 4H). In tidal transfers, salinity and time had significant effects on hepatic *mr* expression (Fig. 4I). By 15 days in a TR, *mr* was lower in both TF and TS relative to steady-state controls. A similar pattern, however, was observed prior to transfer on day 0. Except for fish in SW and those transferred from SW to TS, all groups decreased *mr* expression levels by 15 days compared with day 0 (Fig. 4I).

4. Discussion

The objective of this study was to characterize the response of the HPI axis to changes in salinity regime. Following our recent findings on the effects of the dynamically changing TR on Prl- and GH- mediated osmoregulation and growth (Seale and Breves, 2022), we have employed a novel approach to investigating the effects of salinity challenges on the HPI axis by combining both one-way transfers between steady-state salinities and to a TR in a euryhaline fish model. In a previous study it was found that by 7 d following transfer of adult fish from either FW or SW to a TR, osmoregulatory parameters were similar to those of fish raised in a TR since yolk-sac absorption (Pavlosky et al., 2019). Inasmuch as osmoregulatory acclimation from a steady-state salinity to a TR occurred within a 7-day period, we expected that by 15 days the endpoints involved in the mediation of the HPI axis would represent those of fish fully acclimated to a TR. We found that: (1) plasma cortisol levels increased following a one-way transfer from FW to SW but did not change between fish acclimated to FW and SW or between the two phases of a TR; (2) pituitary *pomc* expression was upregulated in SW fish; and (3) branchial *gr1, gr2*, and *mr* expression was strongly downregulated in FW in both one-way and tidal transfers.

4.1. Survival of fish after transfer to a tidal regime

Previous studies have consistently reported that Mozambique tilapia cannot survive a direct transfer from FW to SW without spending some time in an intermediate salinity (Breves et al., 2010; Inokuchi et al., 2021; Seale et al., 2002). Nevertheless, Mozambique tilapia can withstand a direct transfer from steady-state FW to a tidal regime, despite experiencing a full transfer to SW within two hours in the TS phase (Pavlosky et al., 2019). In this study, however, the survival rate of FW fish transferred to a tidal regime was limited to 30.0% (6 out of 20 fish) by 15 d following transfer to a TR. This difference in survivability compared with previous studies could be related to age, since survival following a transfer from FW to SW was reported to be lower in older animals of this species (Inokuchi et al., 2021). Nonetheless, 100% of the fish transferred from SW to TR survived, enabling, for the first time, a thorough characterization of the HPI-mediated stress response of fish subjected to a TR.

4.2. Plasma cortisol

Plasma cortisol transiently increased by 2 d following the transfer of fish from FW to SW. This finding is consistent with another study where an increase in plasma cortisol was observed following the transfer of fish from FW to SW, though in that study, levels ranged between 50 and 100 ng/mL (Kammerer et al., 2010). An elevation in cortisol during a transfer from FW to SW corroborates the role of this hormone in hyposmoregulation described in other euryhaline species (Madsen, 1990; Marshall et al., 2005; Richman et al., 1987; Veillette et al., 2007). Baseline cortisol levels in Mozambique tilapia have also been previously reported under ~ 25 ng/mL (Kajimura et al., 2004), further reflecting the variability of values across studies. In the current study, and in a previous report where effects of salinity on cortisol were not as evident, plasma cortisol levels ranged between 100 and 300 ng/mL and 2–400 ng/mL respectively (Breves et al., 2010). It is possible that distinct approaches for the detection of plasma cortisol could produce variable baseline levels observed across studies, though handling and sampling stress may also contribute to variations in cortisol levels. For example, plasma cortisol increased as soon as 4 min following netting stress in this

species (Foo and Lam, 1993). It is also possible that the use of large fish contributed to higher cortisol levels across treatments, inasmuch as baseline cortisol was found to be positively correlated with fish size in Nile tilapia (Alfonso et al., 2023). Other salinity comparisons in this study, for example, including between FW- and SW-acclimated fish and those transferred to TR did not elicit any changes in circulating cortisol. Whether the lack in consistency between changes in cortisol and salinity reflect uncontrolled effects or the distinct nature of experimental paradigms employed in this study, other components of the HPI-axis could further clarify cortisol's regulation and actions.

4.3. Pituitary pomc expression

Encoded by *pomc*, ACTH is synthesized by the pituitary and controls corticosteroid synthesis and release from the interrenal cells. In response to salinity transfers, pituitary *pomc* levels transiently increased 6 h after transfer from FW to SW and were higher in SW and TS compared with FW and TF, respectively. Moreover, *pomc* expression decreased by 7 days in fish transferred from SW to FW. These findings indicate that *pomc* is not only activated in fish transferred and acclimated to SW but is also labile, responding to rapid changes in salinity brought about by a TR. The increase in *pomc* expression in SW environments could be tied to an increase in ACTH production and subsequent trigger for corticosteroid production and release to increase salinity tolerance. *In-vitro* incubations of whole pituitaries revealed that ACTH release was unresponsive to hyperosmotic medium *per se* (Seale et al., 2002), suggesting that release of this hormone may be exclusively responsive to hypothalamic corticotropin releasing hormone (CRH) rather than direct osmotic control. Nonetheless, at day 0, *pomc* levels were higher in fish acclimated to SW compared with those in FW. A similar difference between FW and SW acclimated tilapia was found in pituitary *pomc* expression and through *in-situ* hybridization (Aruna et al., 2015b). The different rates of change in *pomc* expression observed in fish transferred from FW to SW and vice-versa could also suggest that the factors controlling *pomc* expression respond more quickly to hyposmotic than to hyperosmotic conditions. This is also evidenced by the dynamic changes in *pomc* in TR fish, where downregulation is apparent in the TF group relative to TS counterparts. This dynamic salinity-dependent expression pattern, where pituitary *pomc* is downregulated more quickly in fish in FW than it is upregulated in fish in SW, suggests that downstream targets of *pomc* play an adaptive role in attenuating hyperosmoregulation.

4.4. Branchial corticosteroid receptor expression

A further assessment of cortisol's effect in target tissues during salinity acclimation was also estimated through the analysis of corticosteroid receptor expression. In the gill, transient increases in branchial *gr2* and *mr* by 6 h in fish transferred from FW to SW and increase in *gr1*, and *gr2* in TS coincides with respective increases in *pomc* expression. Together, transient increases in *pomc, gr2*, and *mr* in SW environments suggests the activation of cortisol's upstream and downstream effectors during hyperosmotic conditions. Conversely, all three receptors in the gill responded similarly to a transfer from SW to FW, by rapid downregulation as early as 6 h post-transfer. All three corticosteroid receptors were downregulated in a TR compared with steady state controls, while *gr1* and *gr2* were downregulated in TF compared with TS. The branchial downregulation of corticosteroid receptor levels in FW in both one-way and tidal transfers suggest a decrease in the gill's sensitivity to cortisol's steroidogenic actions in hyposmotic environments. This rapid and sustained downregulation of receptors further corroborates the notion that cortisol's steroidogenic actions are more supportive of SW- than of FW-acclimation. In addition to its known SW-adapting effects, cortisol also inhibits the release and action of the FW-adapting hormone prolactin (Prl) (Borski et al., 2001; Breves et al., 2016; Hyde et al., 2004). For example, cortisol antagonizes the Prl-induced upregulation of the water channel aquaporin 3 (*aqp3*) (Breves et al., 2016), possibly through traditional steroid actions via GR and MR receptors. The SW-induced transient rise in branchial *gr2* and *mr*, therefore, may be contributing to the inhibition of hyperosmoregulatory effects by bolstering the actions of cortisol. In summary, during SW-acclimation, cortisol could be acting through its receptors in the gill to attenuate the FW-adapting effects of Prl before Prl levels decrease. During FW acclimation, the downregulation of branchial *gr*s and *mr* could mitigate cortisol's inhibitory effects on Prl's hyperosmoregulatory actions. Interestingly, despite being equivalently exposed to both FW and SW in a TR, fish in both TF and TS downregulated branchial corticosteroid receptors relative to steady-state controls. These data, taken with the rapid and sustained decrease observed in branchial receptors during transfers from SW to FW, suggests that corticosteroids play a role in promoting SW osmoregulation at least in part by inhibiting FW adaptation at the gill.

4.5. Hepatic corticosteroid receptor expression

In the liver, corticosteroid receptors are thought to have distinct functional roles; however, the distinction between downstream effects of each receptor is currently unclear. Some known downstream genomic effects of cortisol in the liver include energy substrate production, increasing cellular protection pathways, and reducing energy-demanding processes (Faught and Vijayan, 2016). In the current study, hepatic *gr1* expression initially increased while *gr2* and *mr* initially decreased during one-way transfers to both FW and SW. This finding suggests that *gr1*′s downstream effects may be specifically responsive to salinity stress while *gr2* and *mr* could be involved in other pathways that are non-essential to coping with salinity stress and therefore downregulated during salinity challenges. Nonetheless, the expression of these receptors in parallel controls (both FW and SW) also varied, suggesting that the transcriptional regulation of hepatic corticosteroid receptors could be more sensitive to other factors, including time of day and/ or nutritional status. Generally, hepatic *gr1, gr2*, and *mr* were less responsive to a TR than branchial transcripts, reinforcing the notion that the liver's responsiveness to cortisol is not as strongly affected by environmental salinity as the gill. In addition to considering genomic actions of cortisol through nuclear receptors, corticosteroid action in the tilapia liver has also been shown to be modulated through a membrane glucocorticoid receptor, suggesting rapid, non-genomic actions of cortisol in this organ (Johnstone et al., 2013). Compared with the gill, however, the lack of pronounced salinity-dependent changes in these three hepatic corticosteroid receptors agrees with the notion that hepatic and branchial corticosteroid receptors are controlled by unique mechanisms (Singer et al., 2007). Singer and colleagues suggested that, in rainbow trout (*Oncorhynchus mykiss*), hepatic corticosteroid receptors are responsive to circulating cortisol levels while branchial receptors respond directly to the external environment via direct osmosensing of signaling molecules such as osmotic stress transcription factor 1 (OSTF1). The direct hyperosmotic stimulation of branchial *ostf1* was also shown in Mozambique tilapia (Fiol et al., 2006), though it remains unclear whether there is a linkage between corticosteroid receptor transcription and the activation of other osmosensitive transcription factors. In the present study, the rapid changes in corticosteroid receptor expression in response to changes in salinity regime, especially the downregulation in FW and TF treatments during one-way and tidal transfers, respectively, suggest direct osmosensitivity of these transcripts. Moreover, plasma cortisol was not affected by transferring fish to FW or TF, further corroborating the muted responses of hepatic corticosteroid receptors relative to the robust changes observed in gill.

5. Conclusion

In summary, this study describes the responses of multiple endpoints of the HPI-axis to dynamic salinity changes in a model species that can naturally tolerate changes in environmental salinity that range between FW and SW. By employing both one-way transfers and TR paradigms, it

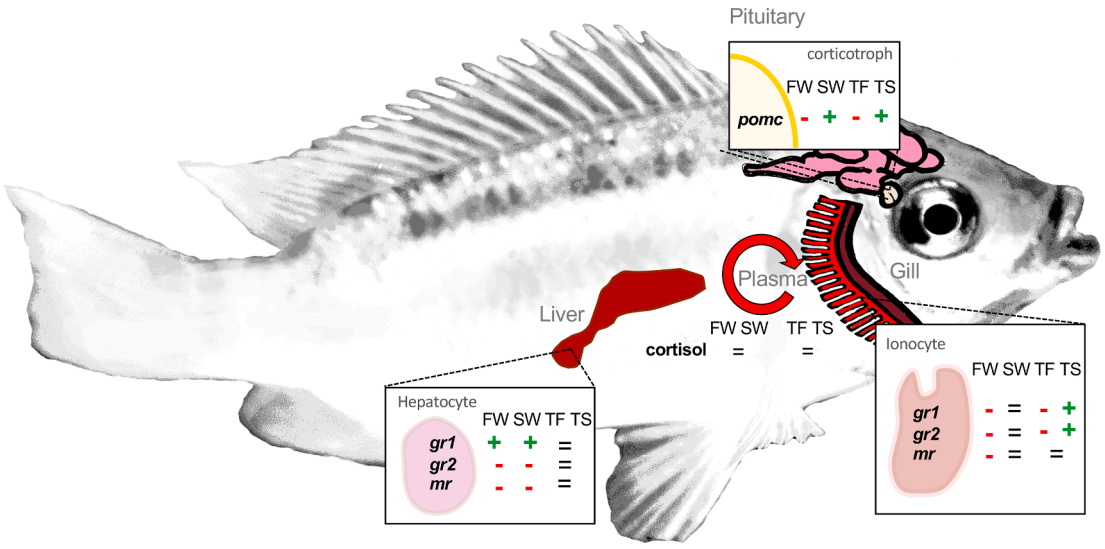

Fig. 5. Diagram summarizing the main responses of the targets measured in FW, SW and the two phases of the TR: TF and TS. For each target, symbols indicate increases (+), decreases (-), and no difference (=) between fish in steady-state FW and SW, and between TF and TS.

is possible to comprehensively examine how the HPI-axis responds to meet the demands of salinity challenges. Notably, the expression of branchial corticosteroid receptors were rapidly downregulated following transfers of fish from SW to FW, and in TF. Meanwhile, plasma cortisol remained largely unchanged, suggesting a shift from systemic to tissue level control of corticosteroid action during both sustained FW acclimation and in a TR. Together, these findings are summarized in Fig. 5. This increased responsiveness of peripheral endocrine control during rapid salinity challenges is similar to that described in other endocrine systems in this species, including Prl and Gh and their respective receptors as well as branchial deiodination of thyroid hormones (Moorman et al., 2016, Seale et al., 2019; Seale et al., 2021). While in this study we focused on branchial and hepatic transcriptional activation of corticosteroid nuclear receptors, the role of the HPI axis in regulating other osmoregulatory tissues such as the intestine and kidney, including the analysis of rapid, non-genomic corticosteroid actions in these tissues, shall further clarify the mineralocorticoid function of corticosteroids during both hyper- and hyposmotic stress. Overall, the findings of this study support the notion that, through the dynamic upregulation and downregulation of corticosteroid receptors in SW and FW, respectively, the HPI-axis facilitates acclimation of euryhaline fish to environments subject to dynamically changing salinities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2023.114340.

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