College of Tropical Agriculture and Human Resources
Department of Human Nutrition, Food and Animal Sciences

UNIVERSITY
of HAWAI'I®

MĀNOA

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Professor Michael Hedrick
Editor-in-Chief
Comparative Biochemistry and Physiology

Dear Professor Hedrick,

Thank you again for your kind consideration of our manuscript, "The effects of transfer from steady-state to tidally-changing salinities on plasma and branchial osmoregulatory variables in adult Mozambique tilapia" for publication in Comparative Biochemistry and Physiology Part A. We have revised our manuscript according to the comments raised by the reviewers.

We addressed the concerns from the reviewers about clarifying how salinity changed by the hour in tidal-regimen tanks by including an additional figure (now Fig. 1), and annotated each of the remaining graphs to indicate the exact number of fish sampled for each of the salinity conditions in each experiment. In addition to the revised manuscript, responses to reviewers' comments, and an updated file for the figures, we are also enclosing a marked version of the revised manuscript. This work has not been, and will not be, submitted for publication elsewhere until your journal has reached a decision on whether to publish the paper. We hope that you will find this manuscript suitable for publication in Comparative Biochemistry and Physiology.

Please find below a list of five suitable referees for this submission:

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Very truly yours,

Andre P. Seale
Research Professor

1 The effects of transfer from steady-state to tidally-changing salinities on plasma and branchial

2 osmoregulatory variables in adult Mozambique tilapia.

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33   **Abstract**

34

35   The Mozambique tilapia, *Oreochromis mossambicus*, is a teleost fish native to estuarine waters

36   that vary in salinity between fresh water (FW) and seawater (SW). The neuroendocrine system

37   plays a key role in salinity acclimation by directing ion uptake and extrusion in osmoregulatory

38   tissues such as gill. While most studies with *O. mossambicus* have focused on acclimation to

39   steady-state salinities, less is known about the ability of adult fish to acclimate to dynamically-

40   changing salinities. Plasma osmolality, prolactin (PRL) levels, and branchial gene expression of

41   PRL receptors (PRLR1 and PRLR2), Na^+/Cl^- and $Na^+/K^+/2Cl^-$ co-transporters (NCC and

42   NKCC), Na^+/K^+-ATPase (NKAα1a and NKAα1b), cystic fibrosis transmembrane conductance

43   regulator (CFTR), and aquaporin 3 (AQP3) were measured in fish reared in FW and SW steady-

44   state salinities, in a tidal regimen (TR) where salinities changed between FW and SW every 6 h,

45   and in fish transferred from FW or SW to TR. Regardless of rearing regimen, plasma osmolality

46   was higher in fish in SW than in FW fish, while plasma PRL was lower in fish in SW.

47   Furthermore, branchial gene expression of effectors of ion transport in TR fish showed greater

48   similarity to those in steady-state SW fish than in FW fish. By 7 days of transfer from steady-

49   state FW or SW to TR, plasma osmolality, plasma PRL and branchial expression of effectors of

50   ion transport were similar to those of fish reared in TR since larval stages. These findings

51   demonstrate the ability of adult tilapia reared in steady-state salinities to successfully acclimate

52   to dynamically-changing salinities. Moreover, the present findings suggest that early exposure to

53   salinity changes does not significantly improve survivability in future challenges to dynamically-

54   changing salinities.

55

56   Keywords: Ion transporter, Osmoregulation, Prolactin, Rearing salinity, Salinity transfer, Tidal

57   cycle, Tilapia

1. Introduction

Maintaining internal osmotic homeostasis is critical to life in many organisms, including vertebrates. Most vertebrates maintain plasma osmolality within a narrow physiological range, typically through exchange of ions and water between cells and the extracellular environment. In the Mozambique tilapia, *Oreochromis mossambicus*, as in other teleost fishes, plasma osmolality is maintained near one-third the osmolality of seawater (SW; McCormick, 2001). As a euryhaline species native to estuarine waters off the Southeast coast of Africa (Trewavas, 1983), the Mozambique tilapia is capable of recovering from major departures above or below their physiological range of plasma osmolality (between 305 and 443 mOsm/kg; Seale et al., 2013). This allows these fish to survive in external salinities equivalent to fresh water (FW) through double-strength SW (Fiess et al., 2007; Stickney, 1986). While the ability of Mozambique tilapia to tolerate steady-state environments of distinct salinities is well established, less is known about their osmoregulatory physiology in dynamically-changing salinities.

Fluctuations in salinity characterize some of the environments to which Mozambique tilapia are native, such as near shore estuaries. Recently, we have described the distinct osmoregulatory profile that tilapia reared under tidally-changing salinities acquire relative to fish reared in steady-sate FW or SW since the yolk-sac fry stage (up to 15 days post fertilization, until yolk is fully absorbed; Moorman et al., 2014; 2015). Here, we characterize whether the unique osmoregulatory profile of tidally-reared fish may be aqcuired by fully developed adult fish that have been reared in steady-state salinities for at least two years prior to a transfer to tidally-changing salinities without exposure to any salinity change during early development. Generally, tilapia and other teleosts in FW hyperosmoregulate to counteract a tendency to lose solutes to the environment and to become over-hydrated (McCormick, 2001). On the other hand, in SW they hypoosmoregulate to counteract a tendency to lose water to the environment and gain solutes (McCormick, 2001). Osmoregulation is conducted predominantly via gill, kidney and intestine, with gill as the site of direct contact with the external environment and major site of monovalent ion transport (Evans et al., 2005).

The pituitary hormone prolactin (PRL) is essential for hyperosmoregulation in fish in FW (Dharmamba et al., 1967; Manzon, 2002; Pickford and Phillips, 1959). Consistent with this action, plasma PRL in the Mozambique tilapia is inversely related to external osmolality (Seale et al., 2005), and PRL has been shown in FW to increase ion uptake and decrease water

89 permeability at the gill (Breves et al., 2014). There are two isoforms of PRL receptors reported

90 for Mozambique tilapia, PRLR1 and PRLR2 (Fiol et al., 2009). *In vitro*, receptors in the gill and

91 pituitary are differentially responsive to PRL and environmental osmolality: increases in

92 extracellular PRL stimulate *prlr1* expression (Inokuchi et al., 2015), whereas increased

93 extracellular osmolality stimulates *prlr2* expression (Inokuchi et al., 2015; Seale et al., 2012).

94 Hence, mounting evidence indicates the actions of PRL on osmoregulation are likely regulated

95 by both circulating levels of the hormone, and by the availability of its receptors.

96   Specialized ionocytes direct osmoregulation in the gill. These cells have been categorized

97 into FW and SW types based on their primary functions in ion uptake and extrusion, respectively

98 (Hiroi et al., 2005; Kaneko et al., 2008). Both FW and SW ionocytes express basolateral Na^+/K^+-

99 ATPase (NKA), an ion pump critical to establishing electrochemical gradients across the cell

100 membrane, which drives ion secretion and absorption (Hiroi et al., 2005). NKA comprises

101 multiple subunits, and two isoforms of NKA α sub-unit, α1a and α1b, have been described in

102 tilapia gill (Tipsmark et al., 2011). Branchial mRNA expression of *nkaα1a* is upregulated in

103 response to a fall in extracellular osmolality and to PRL, and is the prevalent isoform in FW type

104 ionocytes (Inokuchi et al., 2015; Tipsmark et al., 2011). On the other hand, branchial mRNA

105 expression of *nkaα1b* has been reported to increase when fish are transferred from FW to SW

106 (Tipsmark et al., 2011); recent results, however, were unable to fully corroborate this

107 relationship (Inokuchi et al., 2015; Moorman et al., 2014). The presence of Na^+/Cl^- cotransporter

108 (NCC) in the apical membrane is specific to FW ionocytes (Hiroi et al., 2005; Hiroi et al., 2008).

109 Transcription of *ncc* is directly regulated by PRL and a fall in extracellular osmolality (Breves et

110 al., 2010b; Inokuchi et al., 2015). Seawater ionocytes, on the other hand, are characterized by

111 presence of basolateral $Na^+/K^+/2Cl^-$ cotransporter (NKCC1a) and apical cystic fibrosis

112 transmembrane conductance regulator (CFTR, an ion channel responsible for Cl^- secretion by

113 ionocytes of teleost fish in SW; Hiroi et al., 2005). During acclimation to SW, CFTR is

114 trafficked into the apical membrane, while NKCC is translocated to the basolateral membrane of

115 ionocytes (Marshall et al., 2002). Expression of *nkcc1a* has been shown to be directly

116 osmosensitive, increasing with external osmolality (Inokuchi, et al., 2015). In euryhaline teleost

117 species, mRNA expression of *cftr* is elevated in SW-acclimated fish compared with FW-

118 acclimated fish (Moorman et al., 2014; Moorman et al., 2015; Tse et al., 2006). Additionally, *cftr*

119 expression increases when fish are moved from FW to SW, and decreases when subject to the

120 opposite transfer (Moorman et al., 2015; Scott and Schulte, 2005; Singer et al., 1998; Tse et al.,

121 2006). Importantly, it has been demonstrated that an increase in *cftr* expression in SW is linked

122 not only to the trafficking of CFTR to the apical membrane but to the actual secretion of Cl^-

123 (Marshall et al., 1999). Lastly, aquaporin 3 (AQP3), a basolaterally-located water channel,

124 occurs in both FW and SW ionocytes (Watanabe et al., 2005). In Mozambique tilapia and other

125 teleost species, branchial *aqp3* expression is elevated in FW-acclimated over SW-acclimated

126 animals (Cutler and Cramb, 2002; Jung et al., 2012; Lignot et al., 2002; Madsen et al., 2014;

127 Moorman et al., 2015; Tipsmark et al., 2011), and has recently been shown to increase in direct

128 response to PRL (Breves et al., 2016).

129 Much of the current understanding of osmoregulation in Mozambique tilapia, including

130 ionocyte morphology and function, is based on prior studies that are largely focused on fish

131 reared in steady-state FW or SW, or following one-way transfers between the two. Recently, we

132 described an experimental tidal regimen (TR) rearing paradigm in which Mozambique tilapia are

133 exposed to alternating six-hour phases of FW and SW, simulating salinity fluctuations found in

134 their native distribution (Moorman et al., 2014; Moorman et al., 2015). Those studies

135 characterized the osmoregulatory profile for fish reared in tidally-changing salinities from yolk-

136 sac fry to 4-month old fish. In our previous study it was concluded that developing tilapia

137 experiencing tidal-salinity oscillations could respond better to a future one-way transfer of

138 salinity from FW to SW, compared with fish reared in steady-state salinities (Moorman et al.,

139 2015). It is unknown, however, whether adult fish retain such physiological plasticity as

140 observed in juveniles. In anadromous species, individuals at different life stages often exhibit

141 distinct tolerances to environmental salinity (Jensen et al., 2015). Despite the remarkable

142 euryhalinity of the non-anadromous Mozambique tilapia, little is known on how osmoregulatory

143 capacity is established and maintained throughout their life history. Hence, we tested whether the

144 ability of adult fish to acclimate to TR required pre-exposure to both FW and SW during early

145 developmental stages and whether the key variables associated with osmoregulation paralleled

146 those of steady-state FW and SW fish. To address these questions, the following endpoints were

147 measured both in fish reared in FW, SW and TR for 2 years, and in those transferred from FW or

148 SW steady-states to TR for up to 1 week: 1) plasma osmolality; 2) circulating PRL levels; and 3)

149 branchial mRNA expression of PRL receptors and effectors of ion transport shown previously to

150 be responsive to changes in extracellular osmolality and /or PRL.

151

2. Materials and Methods

2.1 Experiment 1 – Salinity regime baseline

Male and female Mozambique tilapia were reared for two years from yolk-sac fry, under natural photoperiod, at the University of Hawai'i's Institute of Marine Biology (HIMB; Kaneohe, HI). Animals were kept in outdoor 700 L tanks supplied with either FW (0.1 ± 0.1‰) or SW (34 ± 1‰; Kaneohe Bay, Kaneohe, HI), or alternating FW and SW in 6-hour phases, simulating a tidally-changing salinity (TR), as previously described (Moorman et al., 2014). Physicochemical properties of the FW and SW employed have been recently reported elsewhere (Breves et al., 2017). Ninety-five % and 100 % changes in salinity were obtained by 2h and 3h, respectively, either from FW to SW or SW to FW (Fig. 1). Water temperature was kept at 25 ± 2°C. Fish were fed trout chow pellets (Skretting, Tooele, UT) once daily to satiation. At the time of sampling, fish weighed 191.6 g - 1.1 kg. Nine fish from each rearing salinity were sampled. Fish reared in TR were collected at the end of the FW and SW phases of the cycle.

2.2 Experiment 2 - Transfer from steady-state salinities to tidal regimen

Adult male and female Mozambique tilapia were collected from broodstock maintained at HIMB, and held under natural photoperiod in outdoor 700 L tanks supplied with FW or SW, as above. Ninety-six FW-acclimated fish were allocated randomly across four replicate FW tanks (24 fish per tank), and 96 SW-acclimated fish across four replicate SW tanks (24 fish per tank). Water temperature was kept at 25 ± 2°C. Fish were allowed an acclimation period of three weeks after seeding to the replicate tanks. Fish were fed trout chow pellets (Skretting, Tooele, UT) once daily to satiation. On Day 0 of the experiment, eight fish from each of the four FW and four SW tanks were sampled. Then, water supply to three of the FW and three of the SW tanks was adjusted to facilitate the following salinity transfers: FW to SW (one tank), FW to TR (two tanks), SW to FW (one tank), and SW to TR (two tanks). One FW tank and one SW tank were retained as parallel controls for the duration of the experiment. Fish transferred from FW to SW were first acclimated to 82-85% SW (29-30‰) over 48 h, and then the water supply was adjusted to full strength SW. From each of the eight experimental tanks, eight fish were sampled on Day 3 and Day 7. From the FW to TR and SW to TR tanks, fish from one tank were sampled at the end of the FW phase (TF) of the tidal cycle, and fish from the second tank were sampled at

182 the end of the SW phase (TS) of the tidal cycle. The same tanks were sampled at the end of the

183 same tidal phase for the entire experiment. Fish sampled over the seven-day period weighed 87-

184 570 g at the time of sampling.

185

186 *2.3 Sampling*

187   At the time of sampling, fish were netted and anesthetized with 2-phenoxyethanol (0.3 ml

188 l^{-1}; Sigma-Aldrich, St. Louis, MO). After fish were weighed, blood was collected from the

189 caudal vasculature with a needle and syringe coated with sodium heparin (200 U/ml, Sigma-

190 Aldrich, St. Louis, MO), and fish were euthanized by rapid decapitation. Plasma was separated

191 by centrifugation and stored at -20°C for further analysis. Gill filaments were collected from the

192 second gill arch on the left side of the fish, frozen in liquid nitrogen and stored at -80°C until

193 further analysis. All experiments and sampling were conducted in accordance with the principles

194 and procedures approved by the Institutional Animal Care and Use Committee, University of

195 Hawai'i.

196

197 *2.4 Quantitative real-time PCR (qRT-PCR)*

198   Total RNA was extracted from frozen gill samples using TRI Reagent according to the

199 manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Using the High Capacity

200 cDNA reverse transcription kit (Life Technologies, Carlsbad, CA), 5 µL of total RNA (400

201 ng/µL) was reverse transcribed into cDNA. Quantitative real-time PCRs (qRT-PCRs) were set up

202 as previously described (Pierce et al., 2007), using the StepOnePlus real-time PCR system

203 (Applied Biosystems). The mRNA levels of reference and target genes were determined by

204 absolute quantification. Standard curves for quantification were generated using serially diluted

205 target gene cDNA fragments of known concentration (standard cDNAs). Elongation factor 1α

206 (EF1α) was used as a reference gene to normalize the mRNA levels of target genes after it was

207 verified that *ef1α* mRNA expression did not vary across treatments. The PCR mixture (15 uL)

208 contained Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA), 200 nM of

209 forward and reverse primers as listed in Table 1, and 2 µl of standard cDNAs or cDNAs prepared

210 from experimental samples. Dilution of experimental cDNA ranged from 20- to 100-fold. PCR

211 cycling parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for

212 15 s and 60°C for 1 min. For both experiments, R^2 values and amplification efficiencies for

213 standard curves varied between 0.989-0.999, and 67.5-96.8%, respectively. Relative mRNA

214 abundance data are expressed as fold-change compared with FW:FW control Day 0 values, and

215 referred to as mRNA expression throughout the manuscript.

216

217 *2.5 Plasma osmolality and prolactin*

218 Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5100C;

219 Wescor, Logan, UT). Of the two isoforms of PRL, PRL_{177} and PRL_{188}, produced and released by

220 the pituitary of tilapia, PRL_{188} was measured in this study based on its robust responses to

221 changes in salinity (Seale et al., 2012), and is referred to as PRL throughout the text. Plasma

222 PRL was measured via homologous radioimmunoassay (RIA) as previously described (Ayson et

223 al., 1993; Yamaguchi et al., 2016).

224

225 *2.6 Statistical Analysis*

226 Data are expressed as means ± S.E.M. Main and interaction effects ($P < 0.05$) of salinity

227 (FW or SW) and rearing regimen (tidal or steady-state) in Experiment 1, and effects of salinity

228 treatment (eight experimental groups) and time (Day 0, 3 and 7) in Experiment 2 were analyzed

229 by two-way analysis of variance (ANOVA). Fisher's protected Least Significant Difference

230 (LSD) test was used to assess the effects of interactions when detected. Where applicable,

231 individual values were log-transformed prior to the analysis, to meet assumptions of normality

232 and equal variance. Statistical calculations were performed using a statistical software program,

233 Prism 6.0 (GraphPad, La Jolla, CA).

234

235 **3. Results**

236 *3.1 Experiment 1- plasma osmolality and prolactin*

237 Plasma osmolality and PRL were compared between fish in FW and SW, reared under

238 steady-state and tidal regimens. A two-way ANOVA revealed an effect of salinity on plasma

239 osmolality, which was elevated in SW fish compared with FW fish; there was no effect of

240 rearing regimen (Fig. 2A). Effects of salinity and rearing regimen on plasma PRL were observed:

241 PRL levels were higher in FW fish than in SW fish, and in tidal fish compared with steady-state

242 fish (Fig. 2B).

243

244 *3.2 Experiment 1 - Branchial gene expression of PRL receptors, ion and water transporters and*

245 *ion ATPases*

246   The branchial gene mRNA expression of PRL receptors, ion and water transporters and

247 ion ATPases was compared between fish in FW and SW, and reared under steady-state and tidal

248 regimens. A two-way ANOVA revealed an interaction effect of salinity and rearing regimen on

249 branchial mRNA expression of *prlr1*; while expression of *prlr1* in steady-state was higher in fish

250 in FW than those in SW, in a tidal regimen, expression was higher in fish in SW (Fig. 3A). By

251 contrast, a single effect of salinity was detected on *prlr2* mRNA expression, which was elevated

252 in SW regardless of rearing regimen (Fig. 3B). Single and interaction effects of both salinity and

253 rearing regimen were observed on *ncc* mRNA expression; *ncc* expression in steady-state FW fish

254 was nearly 100-fold higher than that of SW, TF and TS fish, which were mutually similar (Fig.

255 3C). Single and interaction effects of salinity and rearing regimen were also observed in

256 branchial *nkcc1a* mRNA expression; unlike *ncc* mRNA expression, however, expression was

257 higher in steady-state SW fish than in FW fish, and highest in SW fish reared in a tidal regimen

258 (Fig. 3D). Salinity, rearing regimen and interaction effects were observed on branchial mRNA

259 expression of *nkaα1a*; expression in steady-state FW fish was nearly 10-fold higher compared

260 with SW fish, and similar to that of fish reared in a tidal regimen (Fig. 3E). With a single effect

261 of salinity, *nkaα1b* mRNA expression was higher in fish in SW compared with those in FW (Fig.

262 3F). Salinity, rearing regimen and interaction effects were observed on *cftr* mRNA expression;

263 expression in both steady-state and tidal SW fish exceeded that of FW fish reared under the same

264 regimens (Fig. 3G). By contrast, salinity and interaction effects on branchial *aqp3* mRNA

265 expression indicated higher expression in steady-state fish in FW compared with those in SW.

266 While expression was similar across both phases of the tidal cycle, it was higher in FW than in

267 SW steady-state fish (Fig. 3H).

268

269 *3.3 Experiment 2 - plasma osmolality and prolactin*

270   Plasma osmolality and PRL were compared between fish reared in FW or SW and

271 transferred to steady-state or tidally-changing salinities over a 7-day period. A two-way ANOVA

272 revealed salinity and interaction effects on plasma osmolality (Fig. 4A). By Day 3, plasma

273 osmolality increased in fish transferred from FW to SW (FW:SW) and decreased in fish

274 transferred from SW to FW (SW:FW) when compared with both parallel (FW:FW and SW:SW,

275 respectively) and time 0 controls. Plasma osmolality was elevated in fish in TS compared with

276 those in TF following transfers from either FW or SW to tidal salinities by Day 3 (FW:TF vs.

277 FW:TS and SW:TF vs.. SW:TS, respectively).

278   Salinity, time, and interaction effects were observed on plasma PRL (Fig. 4B). In FW

279 controls (FW:FW) and SW controls (SW:SW), PRL levels remained steady and similar

280 throughout the experiment. Plasma PRL was lower in fish transferred from FW to SW (FW:SW),

281 than in FW controls (FW:FW) by Day 7. On the other hand, in fish transferred from SW to FW

282 (SW:FW) PRL was significantly elevated compared to SW controls (SW:SW) by Day 3. By Day

283 7, plasma PRL in fish in FW:SW, FW:TF and FW:TS groups were similar to those in SW:TF

284 and SW:TS groups, which remained unchanged from SW controls throughout the experiment.

285

286 *3.4 Experiment 2 - Branchial gene expression of PRL receptors, ion and water transporters and*

287 *ion ATPases*

288   The branchial gene expression of PRL receptors, ion and water transporters and ion

289 ATPases were compared between fish reared in FW or SW and transferred to steady-state or

290 tidally-changing salinities over a 7-day period. A two-way ANOVA indicated interaction effects

291 of salinity and time for all genes analyzed (Fig. 5 A-H.) Branchial *prlr1* mRNA expression

292 decreased following transfer from FW to SW by Day 3, and increased following transfer from

293 SW to FW by Day 7 relative to time-matched steady-state controls (FW:SW vs. FW:FW and

294 SW:FW vs. SW:SW, respectively; Fig. 5A). Expression of *prlr1* was elevated in fish in TF

295 compared with those in TS following transfers from either FW or SW to tidal salinities by Day 7

296 (FW:TF vs. FW:TS and SW:TF vs. SW:TS, respectively). By contrast, branchial *prlr2* mRNA

297 expression increased and decreased following transfers from FW to SW and SW to FW,

298 respectively, by Day 3 (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively); this pattern,

299 however, was not sustained through Day 7 (Fig. 5B). Moreover, by Day 7, expression of *prlr2*

300 was elevated in fish in TS compared with those in TF following transfers from either FW or SW

301 to tidal salinities (FW:TS vs. FW:TF and SW:TS vs. SW:TF, respectively).

302   Branchial *ncc* mRNA expression decreased following transfer from FW to SW, and

303 increased following SW to FW transfer by Day 3, as compared with time-matched, steady-state

304 controls (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5C). Also by Day 3,

305 *ncc* expression in TF fish was elevated over TS fish transferred from SW, but in those transferred

306 from FW, *ncc* expression in TS fish was elevated over TF fish, converging by Day 7 (SW:TS vs.

307 SW:TF and FW:TS vs. FW:TF, respectively). Conversely, branchial *nkcc1a* mRNA expression

308 increased after FW to SW transfer, and decreased after SW to FW transfer by Day 3, compared

309 with steady-state controls (Fig. 5D). Following transfer from FW, *nkcc1a* mRNA expression in

310 TS fish was nearly double that of TF fish on Day 3; this difference, however, was no longer

311 observed by Day 7 (FW:TF vs. FW:TS). By contrast, following transfer from SW, there was no

312 difference in expression on Day 3 between TF and TS fish; on Day 7, however, expression in TF

313 fish exceeded that of TS fish (SW:TF vs. SW:TS).

314   Branchial mRNA expression of *nkaα1a* decreased and increased following transfer from

315 FW to SW and SW to FW, respectively, compared with time-matched, steady-state controls

316 (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5E). These differences were

317 observed by Day 3 and were further enhanced by Day 7. Although there was a difference in

318 *nkaα1a* mRNA expression between TF and TS in fish transferred from FW by Day 3, by Day 7

319 there was no difference in expression between TF and TS-sampled fish, regardless of transfer

320 from FW or SW. There was no difference in branchial *nkaα1b* mRNA expression following

321 transfer from FW to SW or vice versa, compared to time-matched, steady-state controls (Fig.

322 5F). The same pattern was observed for fish transferred from FW and sampled during TF and

323 TS. In fish transferred from SW, expression in TF fish was higher than in TS fish by Day 3, but

324 this pattern was not sustained by Day 7.

325   Branchial mRNA expression of *aqp3* was decreased in fish transferred from FW to SW,

326 and increased in fish subject to the opposite transfer by Day 3, as compared with steady-state

327 controls (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5G). In TF fish

328 transferred from FW, *aqp3* mRNA expression remained unchanged, whereas in TS fish

329 expression decreased by Day 3, with TF and TS expression at similar levels by Day 7 (FW:TF

330 and FW:TS; Fig. 5G). Following transfer from SW, expression in TF and TS fish increased over

331 the 7-day period, reaching mutually similar levels by Day 3, but with TF exceeding TS by Day 7

332 (SW:TF and SW:TS; Fig. 5G). Branchial *cftr* mRNA expression increased in fish transferred

333 from FW to SW and decreased in those transferred from SW to FW by Day 3, compared to

334 steady-state controls (FW:SW vs. FW:FW and SW:FW vs. SW:SW, respectively; Fig. 5H).

335 Branchial *cftr* expression in TF fish was lower than in TS fish by Day 3 regardless of transfer

336 from FW or SW; this difference was absent by Day 7 (FW:TF vs. FW:TS and SW:TF vs.

337 SW:TS, respectively; Fig. 5H).

338

339 **4. Discussion**

340

341   The objective of this experiment was to determine the capacity of adult fish, reared in

342 steady-state FW or SW, to acclimate to TR, by characterizing plasma osmolality, PRL, and

343 branchial gene expression of PRLRs, ion transporters, and ion ATPases. This is the first study to

344 both describe an osmoregulatory profile for adult Mozambique tilapia reared for two years under

345 cyclically changing salinity, which is similar to the species' native habitat, and to investigate in

346 adult fish the osmoregulatory effects of transfer from FW and SW steady-state rearing conditions

347 to a tidal environment. In light of recent findings suggesting that tilapia exposed to changing

348 salinities during early stages of larval development may better respond to subsequent salinity

349 challenges (Moorman et al., 2015), we tested the central notion of whether there is an adaptive

350 advantage of rearing fish in changing salinities from the yolk-sac fry stage. By comparing 2-year

351 old adult tilapia reared in steady-state FW and SW with fish reared under TR, our findings

352 support the notion that the physiological experience of dynamically-changing salinities during

353 early life history does not significantly improve survivability or osmoregulatory responses

354 compared with fish that were exposed to TR for the first time as adults.

355   Specifically, the findings of this study were: 1) adult Mozambique tilapia acclimated to

356 TR maintain a distinct osmoregulatory profile, which neither coincides fully with that of FW-

357 nor SW-acclimated counterparts; 2) fish reared since yolk-sac fry for 2 years in steady-state

358 salinities (either FW or SW) can rapidly acclimate to a tidal regimen, a finding that previously

359 had only been observed in young fish (4 months of age); 3) by 7 days post-transfer, the

360 osmoregulatory profile of fish reared in steady-state salinity and transferred to TR is similar to

361 that of fish reared in TR since yolk-sac fry.

362   Upon conducting the salinity transfer experiments of this study, we found that adult

363 tilapia reared under both steady-state FW and SW could withstand a direct transfer to TR, with

364 100% survival by 7 days. Specifically, FW fish transferred to TR suffered no mortalities despite

365 their initial exposure to full-strength SW within 2 h of the first TS phase. This was suggestive of

366 an ability of 2-year-old adult fish to survive exposure to dynamic salinity changes, regardless of

367 acclimation history. It is well established that FW-acclimated tilapia cannot survive direct

368 transfer to SW, but are able to survive when first transferred to an intermediate salinity (Breves

369 et al., 2010a; Moorman et al., 2015; Seale et al., 2012; Seale et al., 2002; Yada et al., 1994).

370 Consistent with these findings, the present protocol involved transfer to 80% SW for 48h, then to

371 full strength SW. The current observations indicate that transition of FW fish to cyclically-

372 changing salinity is less challenging than to steady-state SW.

373   In Mozambique tilapia, plasma osmolality is higher in fish acclimated to steady-state SW

374 than in those acclimated to FW (Grau and Borski, 1994; Seale et al., 2002, Seale et al., 2006).

375 Moreover, the inverse relation between plasma osmolality and PRL release has been well

376 established (Grau et al., 1981; Nagahama et al, 1975; Seale et al., 2002; Seale et al., 2006; Seale

377 et al., 2012). This relation is consistent with the potent hyperosmoregulatory action of PRL in

378 gill and other osmoregulatory epithelia (Manzon, 2002). Consistent with previous reports, in

379 Experiment 1 plasma osmolality was higher in fish in SW than those in FW, whether fish were

380 kept in a steady-state or tidal regimen (Moorman et al., 2014; Moorman et al., 2015; Seale et al.,

381 2006; Seale et al., 2002; Yada et al., 1994). In the same experiment, plasma PRL was higher in

382 fish in FW compared with those in SW, regardless of rearing regimen, which is also consistent

383 with the expected release of PRL in response to a reduction in plasma osmolality. The similarity

384 in plasma PRL levels in TF and TS fish observed in Experiments 1 and 2 was consistent with

385 previous reports (Moorman et al., 2014; Moorman et al., 2015), suggesting that the fish reared in

386 or transferred to a tidal cycle are not as physiologically dependent on osmotically-driven

387 variations in circulating PRL as fish that are acclimated to steady-state salinities.

388   Environmental salinity has been shown to modulate the actions of PRL not only by

389 regulating its release from the pituitary, but also by directing the expression of its receptors in

390 osmoregulatory epithelia (Breves et al., 2011; Inokuchi et al., 2015). Additionally, mRNA

391 expression of *prlr1* in gill is stimulated in a dose-dependent manner by PRL (Inokuchi et al.,

392 2015). In Experiment 1 and in three of the four comparisons in Experiment 2, *prlr1* expression in

393 FW steady-state fish was elevated over that in SW fish. Elevated branchial expression of *prlr1* in

394 FW relative to SW is consistent with previous reports where fish were sampled in either FW or

395 SW steady-states, or in FW and SW phases of a tidal regimen, or following transfer from SW to

396 FW (Breves et al., 2011; Fiol et al., 2009; Moorman et al., 2014; Moorman et al., 2015).

397 Moreover, branchial *prlr1* expression in TR fish varied between fish in TF and TS. Moorman

398 and colleagues (2014) suggested that the differential regulation of branchial *prlr1* expression
399 between the two phases of TR may be attributable to direct regulation of transcription at the
400 tissue level by environmental salinity. It is also possible that nuances in *prlr1* expression in TR-
401 acclimated fish are associated with variables other than salinity and age, such as sex or size,
402 which may be elucidated with additional studies using this tidal paradigm.

403 Branchial mRNA expression of *prlr2* has also been reported to vary with extracellular
404 osmolality, in vivo and in vitro; unlike *prlr1*, however, its expression increases in hyperosmotic
405 conditions (Fiol et al., 2009; Inokuchi et al., 2015; Seale et al., 2012). In the present study,
406 branchial *prlr2* expression was higher in fish in SW than those in FW in both tidal and steady-
407 state rearing regimens, whether they had been exposed to TR for 2 years or 7 days. This finding
408 is consistent with our recent results employing 4-month old tilapia (Moorman et al., 2014 and
409 2015), suggesting that tight regulation of PRLR2 by salinity is independent of acclimation
410 history. Moreover, binding of PRL to PRLR2 may not elicit the same hyperosmoregulatory
411 response as binding to PRLR1 (Fiol et al., 2009). It has been postulated that increased *prlr2*
412 expression in hyperosmotic conditions may facilitate acclimation of tilapia to SW (Seale et al.,
413 2012; Moorman et al., 2014; Inokuchi et al., 2015, Yamaguchi et al., 2018). The molecular
414 mechanism underlying this outcome may be associated with PRL binding either the regular
415 length or short form of PRLR2. While the former has been hypothesized to activate a different
416 pathway than PRLR1 upon binding PRL, the latter is thought to reduce the formation of
417 functional receptors, thereby preventing PRL's actions (Fiol et al., 2009). In the present study,
418 primers that detect regular length *prlr2* were employed. It is tenable, therefore, that salinity
419 driven changes in *prlr2* in tidally-acclimated fish facilitate the attenuation of PRL's effects by
420 diverting downstream signaling from hyperosmoregulatory outcomes. Moreover, the observed
421 dynamic changes in *prlr2* transcription with environmental salinity, regardless of rearing
422 regimen, strongly suggest that this isoform is highly osmosensitive.

423 In previous studies of salinity acclimation in euryhaline teleosts, including the
424 Mozambique tilapia, it has been shown that NCC and NKAα1a are involved in ion uptake in gill
425 and highly expressed in FW, whereas NKCC1a, CFTR and NKAα1b are involved in ion
426 extrusion and predominantly expressed in SW (Hiroi et al., 2005; Hiroi et al., 2008; Kaneko et
427 al., 2008; Tipsmark et al., 2011). In tilapia, AQP3 has been implicated in FW-acclimation as it is
428 highly expressed in response to both hyposmotic stimuli and PRL (Breves et al., 2016). The

429   overall similarity in branchial *ncc*, *nkcc1a*, and *cftr* mRNA expression between SW and TR fish

430   in both Experiments 1 and 2 was consistent with a previous report on TR-acclimated, 4-month

431   old fish (Moorman et al., 2014), as was the finding that branchial *aqp3* expression in TR fish was

432   intermediate to levels in FW and SW controls. The intermediate expression of *aqp3* in TR is

433   likely a reflection of the shifting need for water transport in a dynamically-changing

434   environment. By contrast, the mRNA expression of ion transporters, *ncc*, *nkcc1a* and *cftr* in

435   dynamically-changing environments were either strongly suppressed (*ncc*) or elevated (*nkcc1a*

436   *and cftr*). The expression patterns of these three ion transporters in TR follows those observed in

437   SW-type ionocytes (Breves et al., 2010b; Inokuchi et al., 2015). Moreover, the strong

438   suppression of *ncc* in fish reared in TR or transferred to TR, is consistent with the previously

439   reported immunohistochemistry results indicating suppression of NCC protein in the apical

440   region of branchial ionocytes of tilapia reared in TR (Moorman et al., 2014). Conversely, in the

441   same study, signal intensities for NKCC and CFTR were consistently high in TR fish. Together,

442   the mRNA results of this and other studies suggest that transcriptional regulation of these three

443   key ion transporters in steady-state and dynamically-changing conditions are paralleled by

444   changes in protein abundance. In both experiments, branchial mRNA expression of *nkaα1a* was

445   higher in FW fish than in SW fish. This pattern is consistent with that previously reported in fish

446   acclimated to steady-state salinities (Tipsmark et al., 2011, Moorman et al., 2014), although in

447   the current study, the *nkaα1a* expression pattern in TR was variable; in some instances similar to

448   that of FW fish, and in others, intermediate to that of FW and SW controls. Likewise, branchial

449   expression of *nkaα1b* observed in TR-acclimated fish (Experiment 1) was consistent with

450   previous studies reporting up-regulation in response to increased extracellular osmolality

451   (Inokuchi et al., 2015; Tipsmark et al., 2011). Overall, these findings suggest that the

452   osmoregulatory profile of adult fish reared in TR for two years is more similar to that of SW fish

453   than that of FW fish.

454   By Day 7 of transfer from steady-state salinities to TR, patterns in plasma variables and

455   branchial gene expression of PRLRs, ion transporters, and AQP3 were largely similar across

456   both phases of the tidal cycle, regardless of whether fish were initially reared in FW or SW.

457   Moreover, by Day 7, these osmoregulatory parameters were largely similar to those in the TR-

458   acclimated fish sampled in Experiment 1. Together, these observations indicate that tilapia

459   retain, even after being reared in a steady-state salinity for 2 years, the remarkable

460 osmoregulatory capacity to overcome fluctuations in environmental salinity, regardless of the

461 salinity in which they were reared.

462   In the current study, we have provided novel insights into osmoregulation of tilapia under

463 TR rearing conditions at a life stage not previously examined under this paradigm. It is worth

464 mentioning that in our previous study, fish reared in TR for 4-months grew faster than those

465 reared in steady-state FW or SW (Moorman, et al., 2016). Such finding may lead to applications

466 in aquaculture production, and bears particular importance to tilapia in general, which rank 2nd as

467 the most widely aquacultured fish in the world (FAO, 2015). The use of the TR rearing paradigm

468 can foster the elucidation of novel and comprehensive physiological insights, including

469 providing a potential means to develop optimal rearing conditions for Mozambique tilapia and

470 other euryhaline fish.

471

482

483 **Figure Legends:**

484 Fig. 1. Salinity (ppt) changes in a tank subjected to a tidal regimen between 10AM and 10PM.

485 Fig. 2. Effects of rearing condition on plasma osmolality (A) and plasma prolactin (B) in fish

486 sampled in steady-state FW and SW, and at the end of the FW and SW phase of the tidal cycle.

487 Values are expressed as means ± S.E.M. (n = 7-11). Numbers within bars represent sample sizes

488   for each group. Effects of salinity and regimen were analyzed by two-way ANOVA

489   ([*,**]Significant at $P < 0.05$ and 0.01, respectively).

490

491   Fig. 3. Effects of rearing condition on branchial mRNA expression of *prlr1* (A), *prlr2* (B), *ncc*

492   (C), *nkcc1a* (D), *nkaα1a* (E), *nkaα1b* (F), *cftr* (G) and *aqp3* (H) in fish sampled in steady-state

493   FW and SW, and at the end of the FW and SW phase of the tidal cycle. Values are expressed as

494   means ± S.E.M. (n = 6-12). Numbers within bars represent sample sizes for each group. Effects

495   of salinity and regimen were analyzed by two-way ANOVA ([*,**,***]Significant at $P < 0.05$, 0.01

496   and 0.001, respectively). Interaction effects were followed up by Fisher's LSD test. Means not

497   sharing the same letter are significantly different at $P < 0.05$.

498

499   Fig. 4. Plasma osmolality (A), and plasma PRL (B) in fish sampled in FW, SW, and following

500   transfer from FW or SW to FW, SW or to TR. TR fish were sampled at the end of the either FW

501   or SW phases of the tidal cycle (TF or TS, respectively). Values are expressed as means ±

502   S.E.M. (n = 6-8). Numbers within bars represent sample sizes for each group. Effects of salinity

503   and time were analyzed by two-way ANOVA ([*,**,***]Significant at $P < 0.05$, 0.01, and 0.001,

504   respectively). Within each time point, means not sharing the same letter are significantly

505   different at $P < 0.05$. Daggers indicate difference from Day 0 within salinity treatments

506   ([†,††,†††]Significant at $P < 0.05$, 0.01 and 0.001, respectively; Fisher's LSD test).

507

508   Fig. 5. Branchial mRNA expression of *prlr1* (A), *prlr2* (B), *ncc* (C), *nkcc1a* (D), *nkaα1a* (E),

509   *nkaα1b* (F), *cftr* (G) and *aqp3* (H) in fish FW, SW, and following transfer from FW or SW to

510   FW, SW or to TR. TR fish were sampled at the end of the either FW or SW phases of the tidal

511   cycle (TF or TS, respectively). Values are expressed as means ± S.E.M. (n = 7-8). Numbers

512   within bars represent sample sizes for each group. Effects of salinity and time were analyzed by

513   two-way ANOVA ([*,**,***]Significant at $P < 0.05$, 0.01 and 0.001, respectively). Within each time

514   point, means not sharing the same letter are significantly different at $P < 0.05$. Daggers indicate

515   difference from Day 0 within salinity treatments ([†,††,†††]Significant at $P < 0.05$, 0.01 and 0.001,

516   respectively; Fisher's LSD test).

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Table 1. Primers used for qPCR.

Gene name	Primer sequence (5'-3')		Reference
ef1α	Forward	AGCAAGTACTACGTGACCATCATTG	Breves et al, 2010b
	Reverse	AGTCAGCCTGGGAGGTACCA	
prlr1	Forward	TGGGTCAGCTACAACATCACTGT	Pierce et al., 2007
	Reverse	GGATGGGGCTTGACAATGTAGA	
prlr2	Forward	GCCCTTGGGAATACATCTTCAG	Breves et al., 2010b
	Reverse	GTGCATAGGGCTTCACAATGTC	
ncc	Forward	CCGAAAGGCACCCTAATGG	Inokuchi et al., 2008
	Reverse	CTACACTTGCACCAGAAGTGACAA	
nkcc1a	Forward	GGAGGCAAGATCAACAGGATTG	Inokuchi et al., 2008
	Reverse	AATGTCCGAAAAGTCTATCCTGAACT	
nkaα1a	Forward	AACTGATTTGGTCCCTGCAA	Tipsmark et al., 2011
	Reverse	ATGCATTTCTGGGCTGTCTC	
nkaα1b	Forward	GGAGCGTGTGCTTCATCACT	Tipsmark et al., 2011
	Reverse	ATCCATGCTTTGTGGGGTTA	
cftr	Forward	CATGCTCTTCACCGTGTTCT	Moorman et al., 2014
	Reverse	GCCACAATAATGCCAATCTG	
aqp3	Forward	CATGTACTATGATGCTTTGTTGCTC	Watanabe et al., 2005
	Reverse	CAAAGAAACCATTGACAAGTGTGA	

ef1α: elongation factor 1α; *prlr1*: prolactin receptor 1; *prlr2*: prolactin receptor 2; *ncc*: Na^+/Cl^- cotransporter; *nkcc1a*: $Na^+/K^+/2Cl^-$ cotransporter; *nkaα1a*: Na^+/K^+-ATPase α sub-unit isoform 1a; *nkaα1b*: Na^+/K^+-ATPase α sub-unit isoform 1b; *cftr*: cystic fibrosis transmembrane conductance regulator; *aqp3*: aquaporin 3.
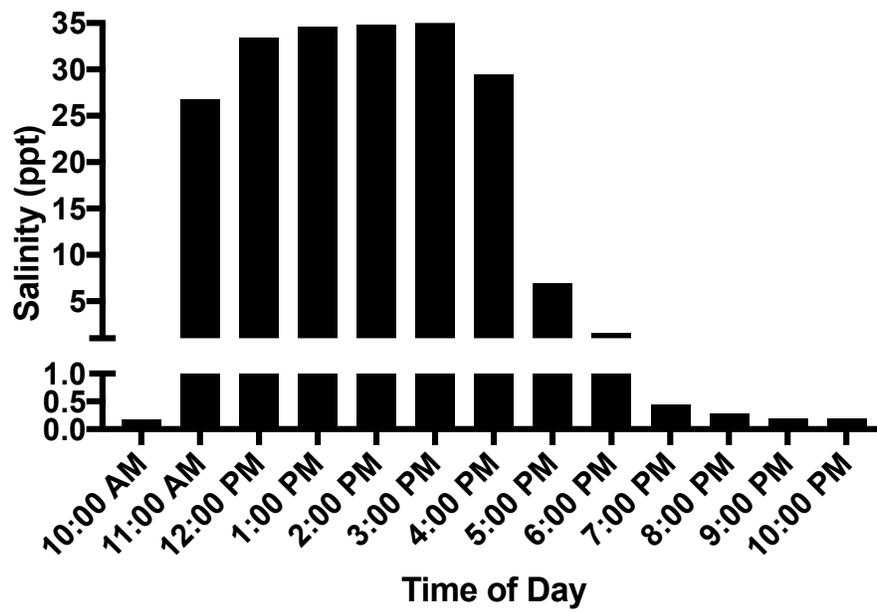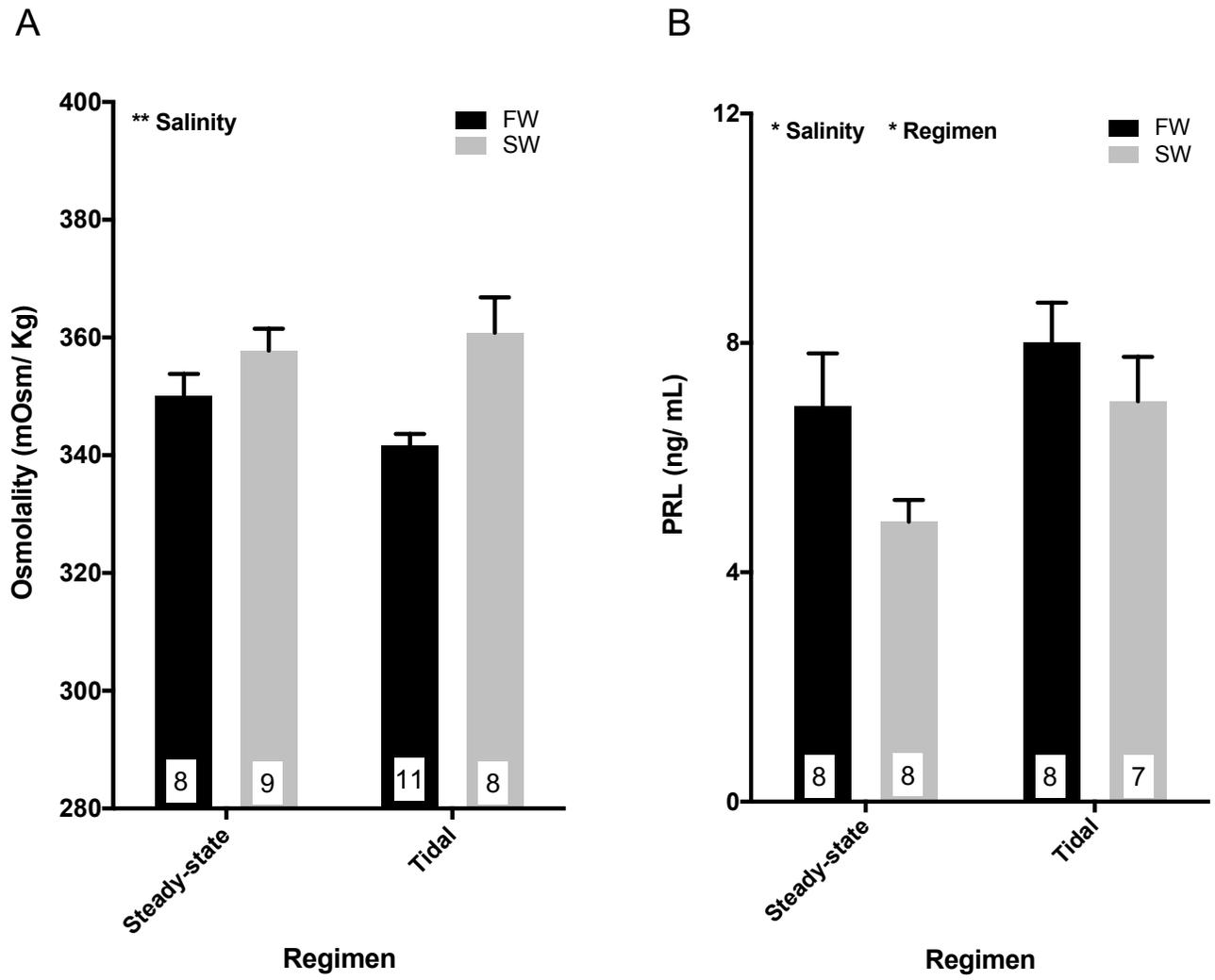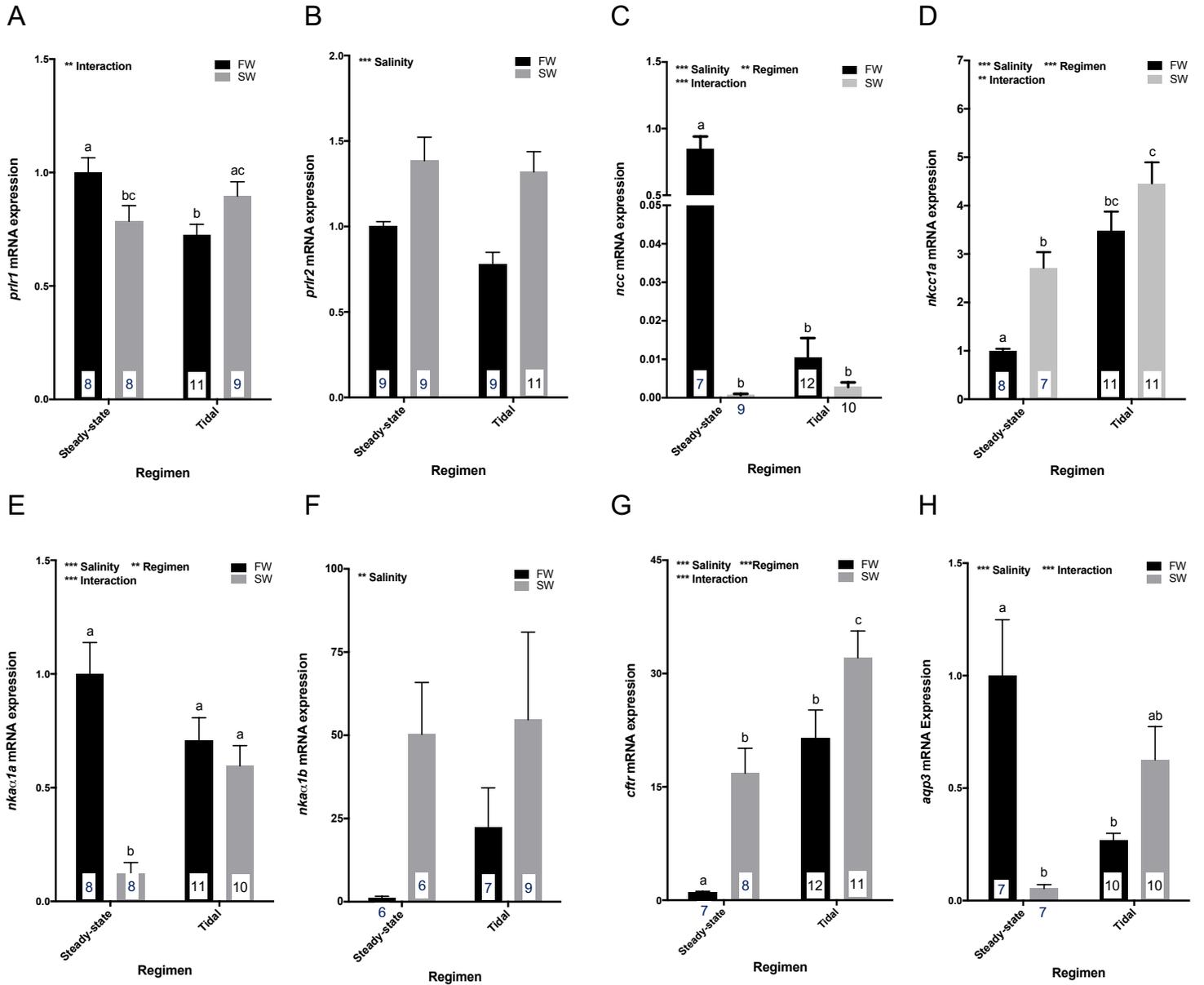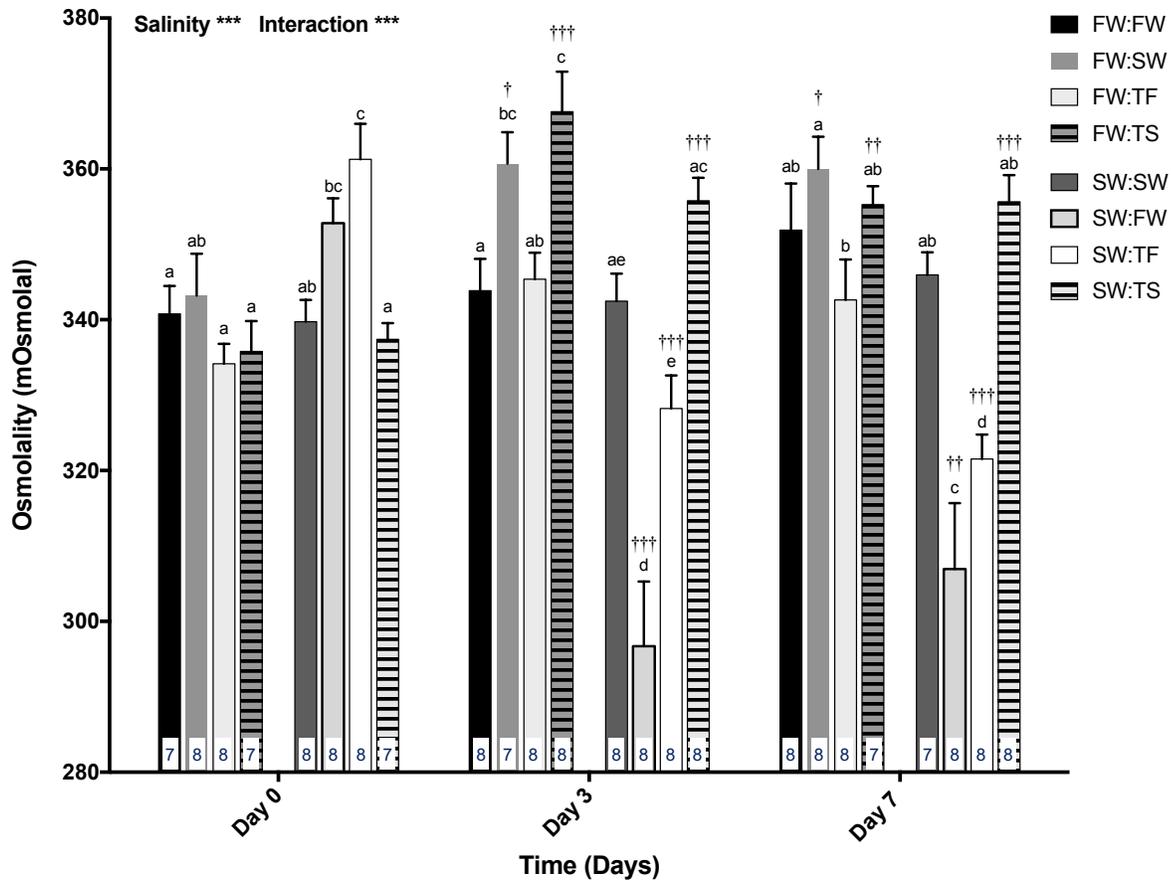
Fig. 1

Fig. 2

A

B

Fig. 3

Fig. 4

A

B

Fig. 5

A

B

E

Salinity ***   Time***   Interaction ***

nkaα1a mRNA expression

Legend:
- FW:FW
- FW:SW
- FW:TF
- FW:TS
- SW:SW
- SW:FW
- SW:TF
- SW:TS

Time (Days)

F

Interaction *

nkaα1b mRNA expression

Legend:
- FW:FW
- FW:SW
- FW:TF
- FW:TS
- SW:SW
- SW:FW
- SW:TF
- SW:TS

Time (Days)
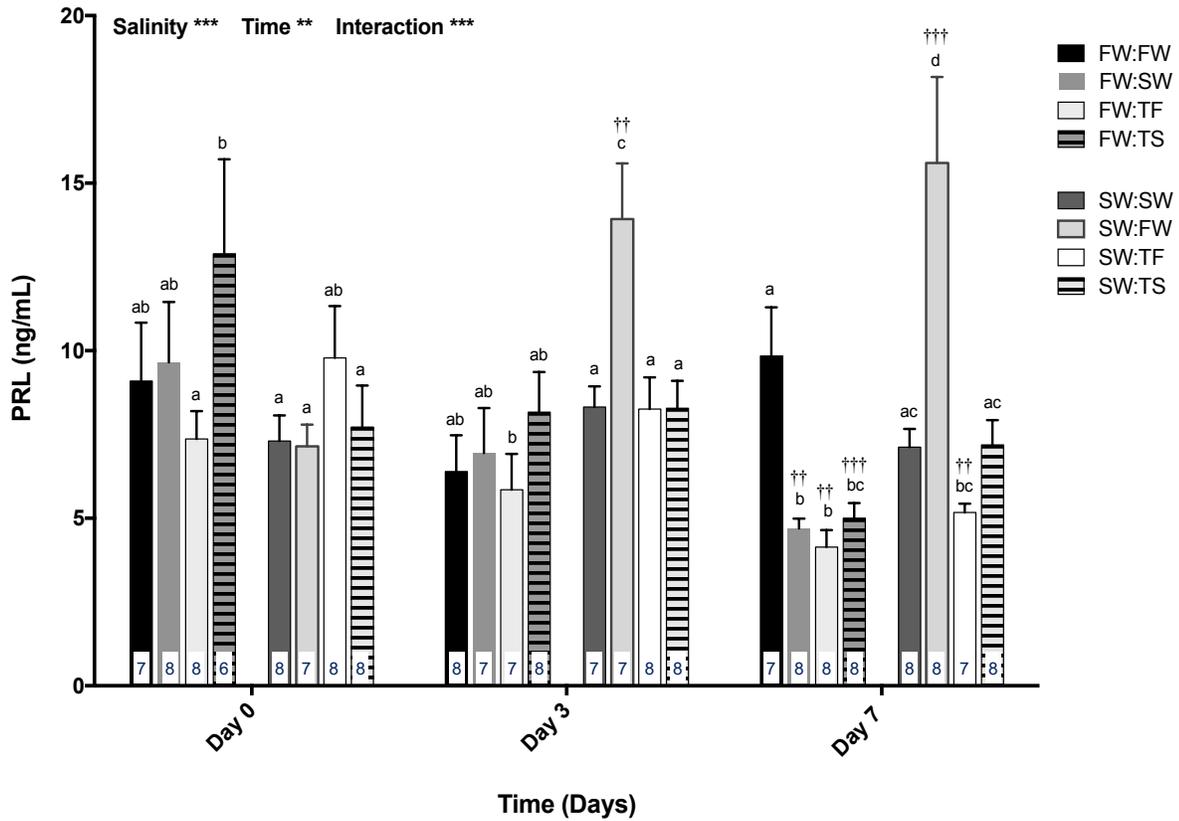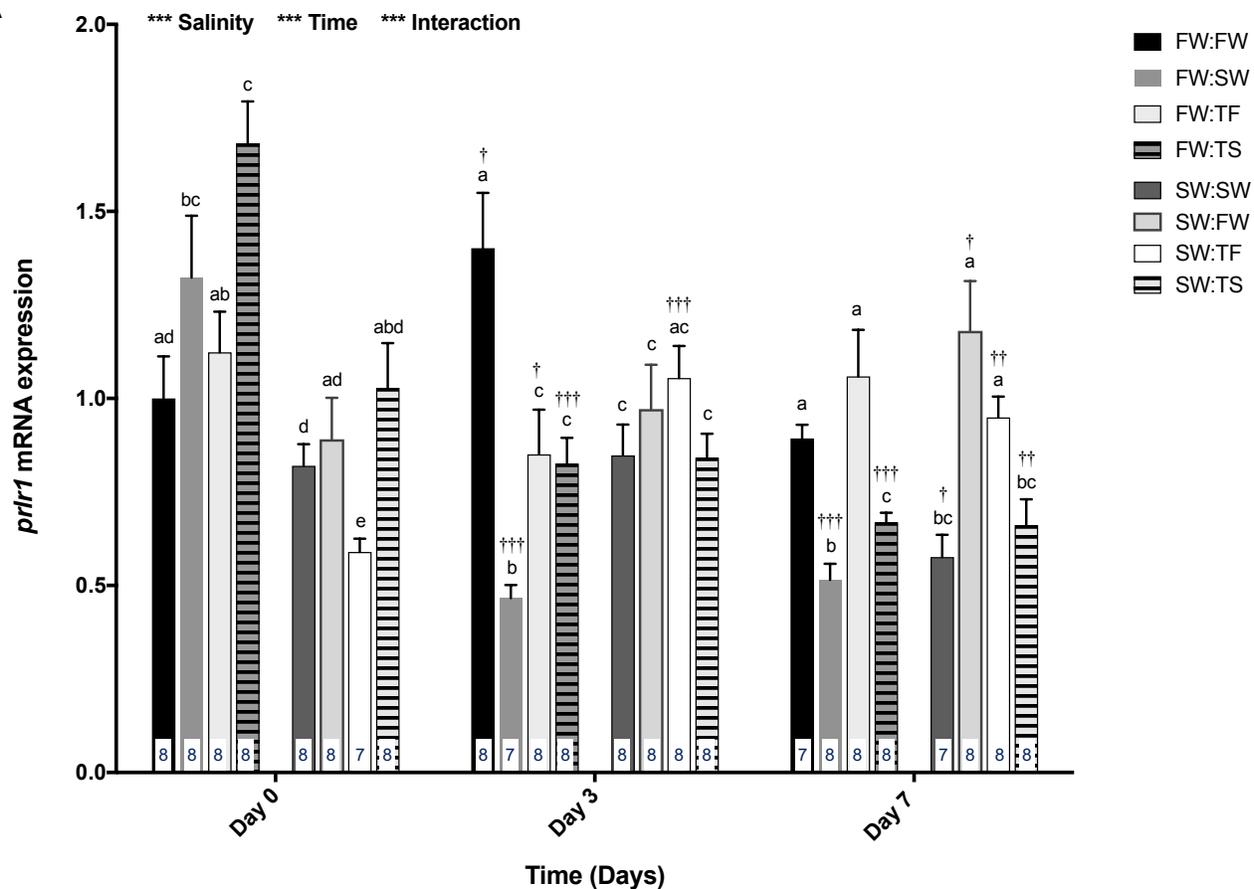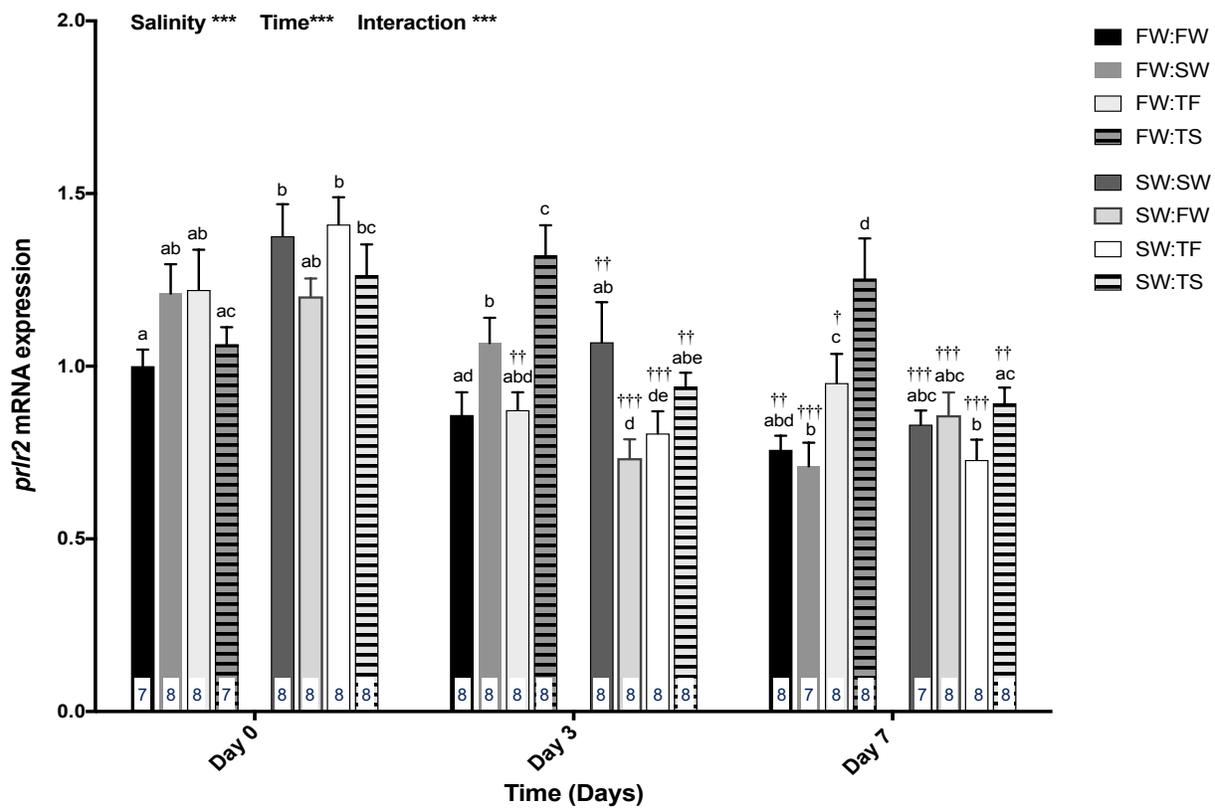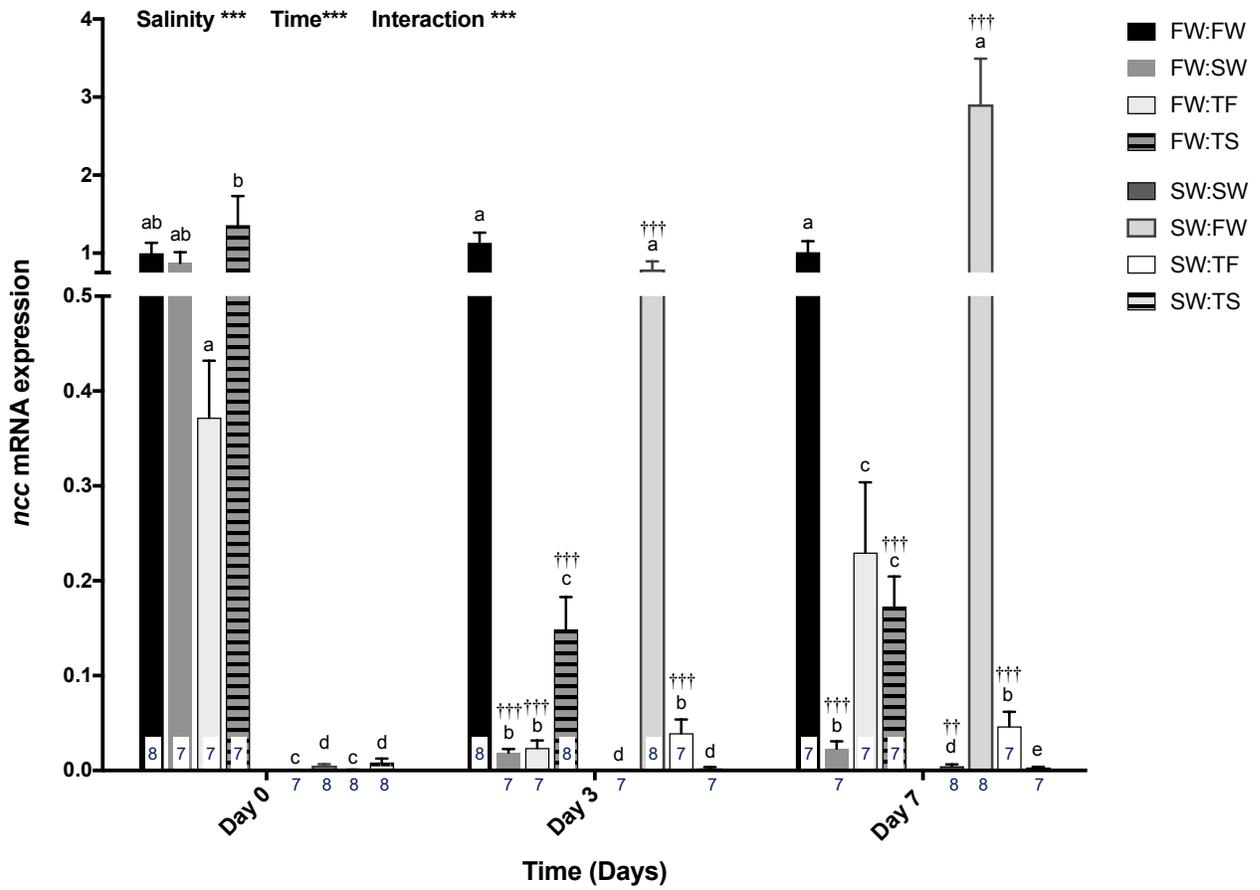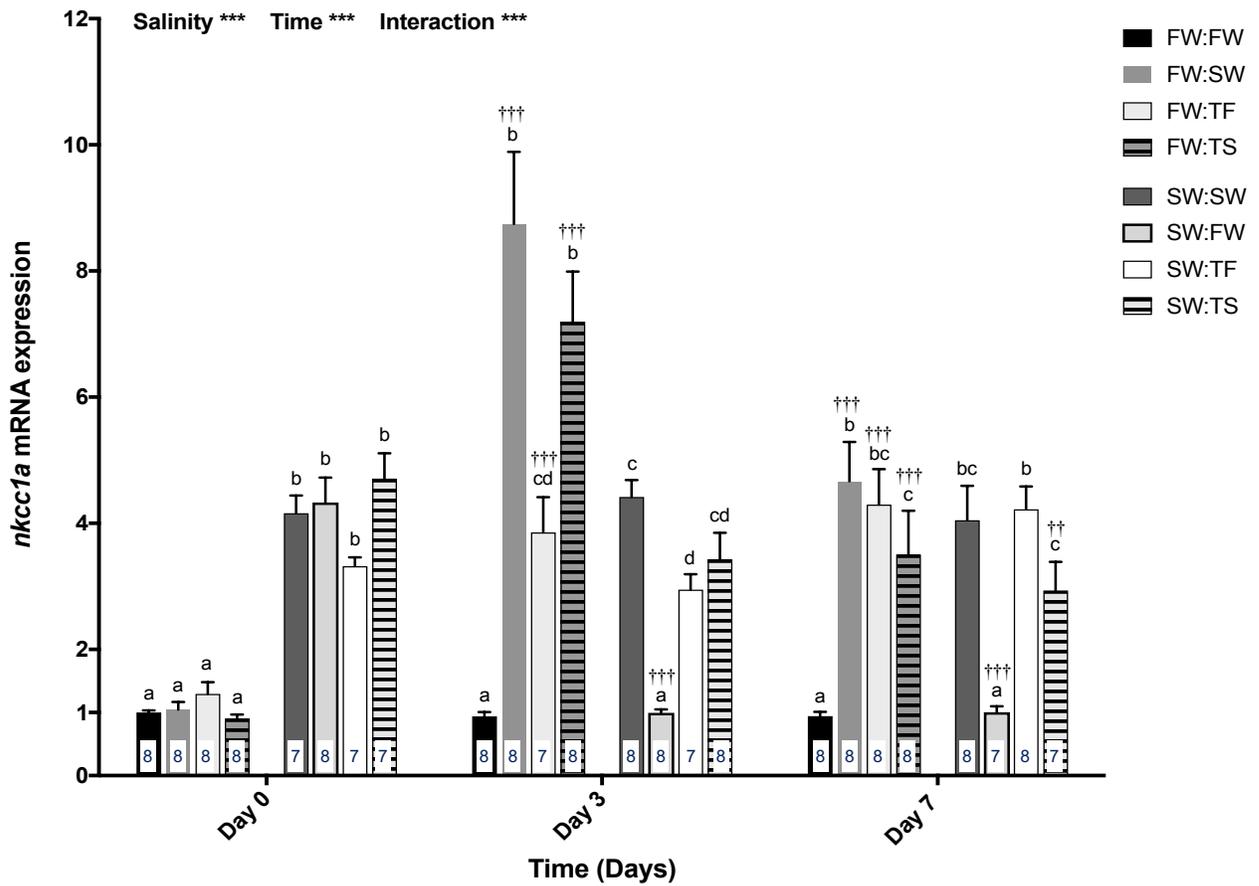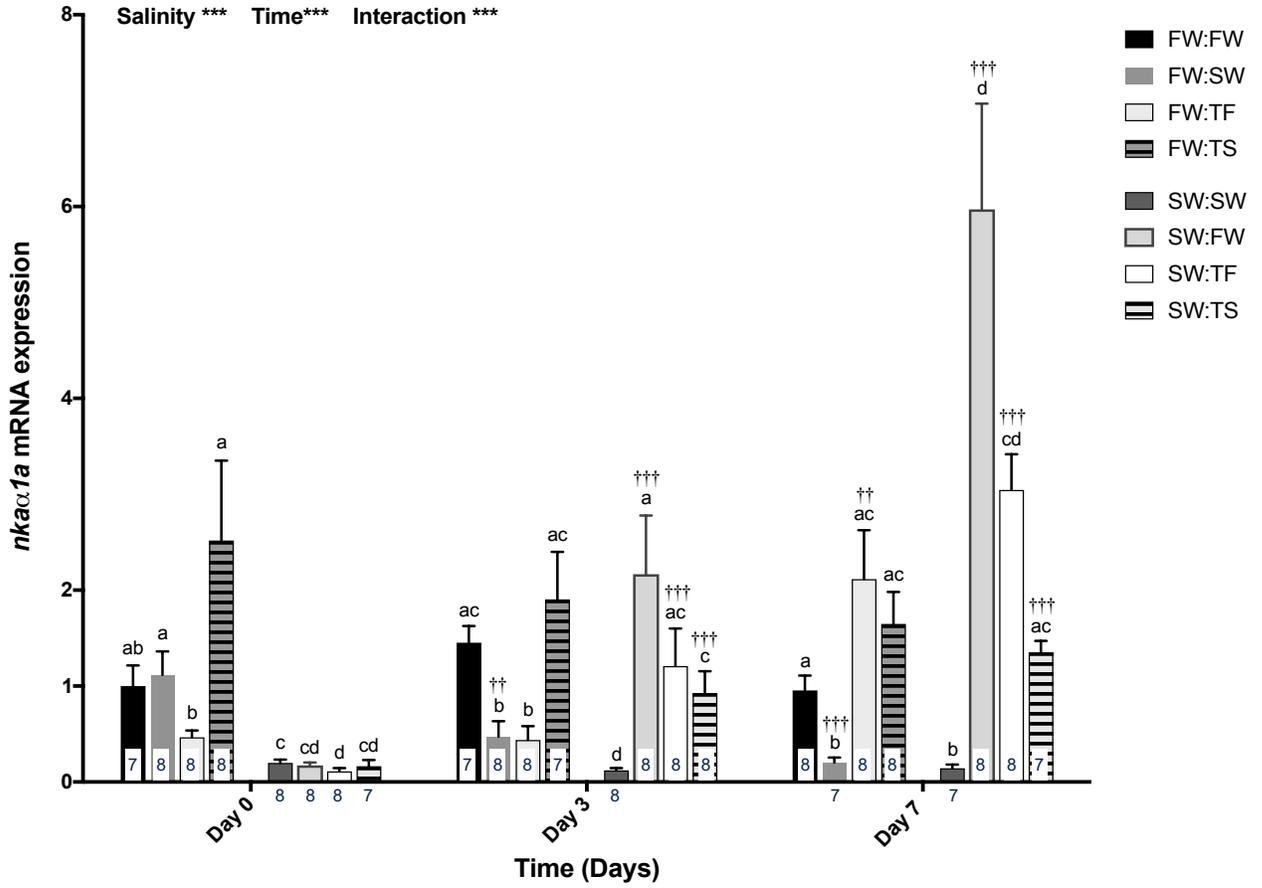
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Responses to Reviewers

Reviewer 1

This manuscript examines the impact of freshwater, seawater and a simulation of tidally driven changes in salinity on a number of osmoregulatory parameters (plasma osmolality, prolactin and mRNA levels of prolactin receptor and gill ion transporters) changes in Mozamb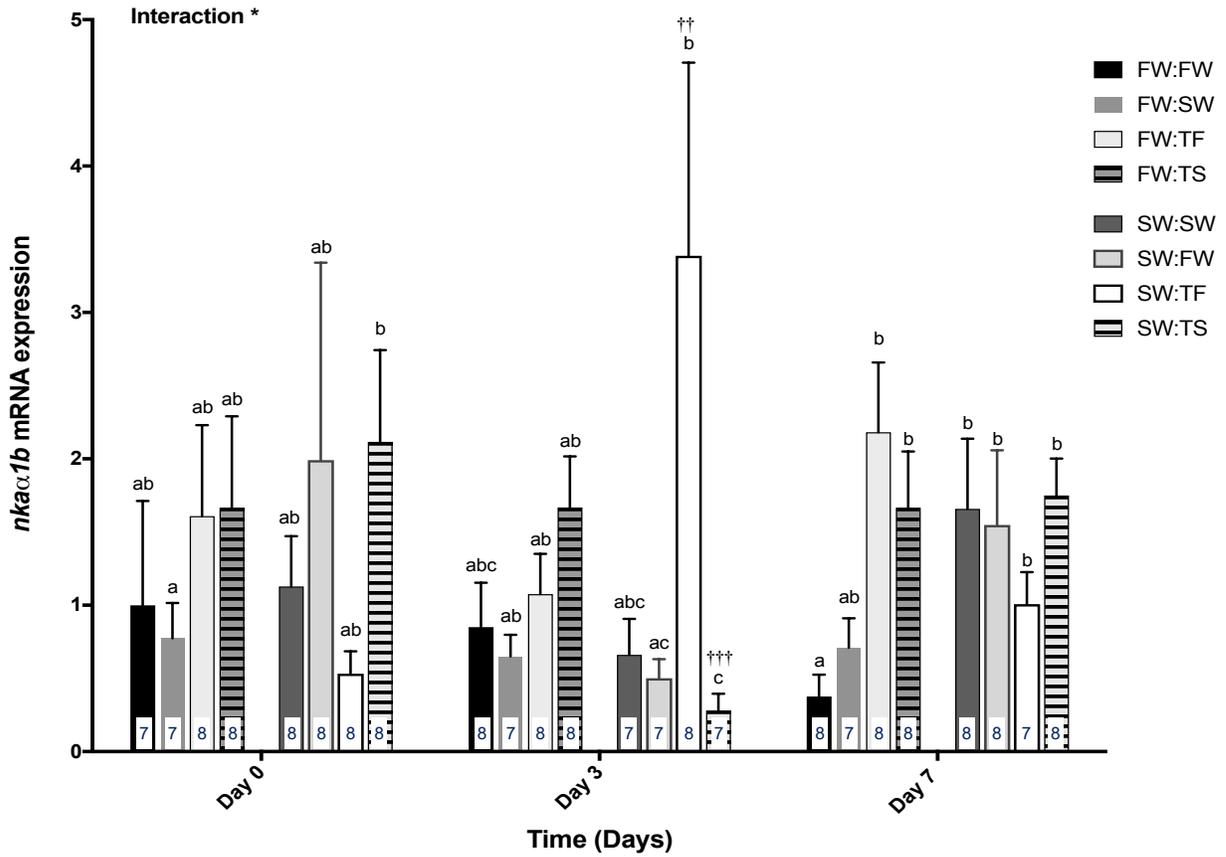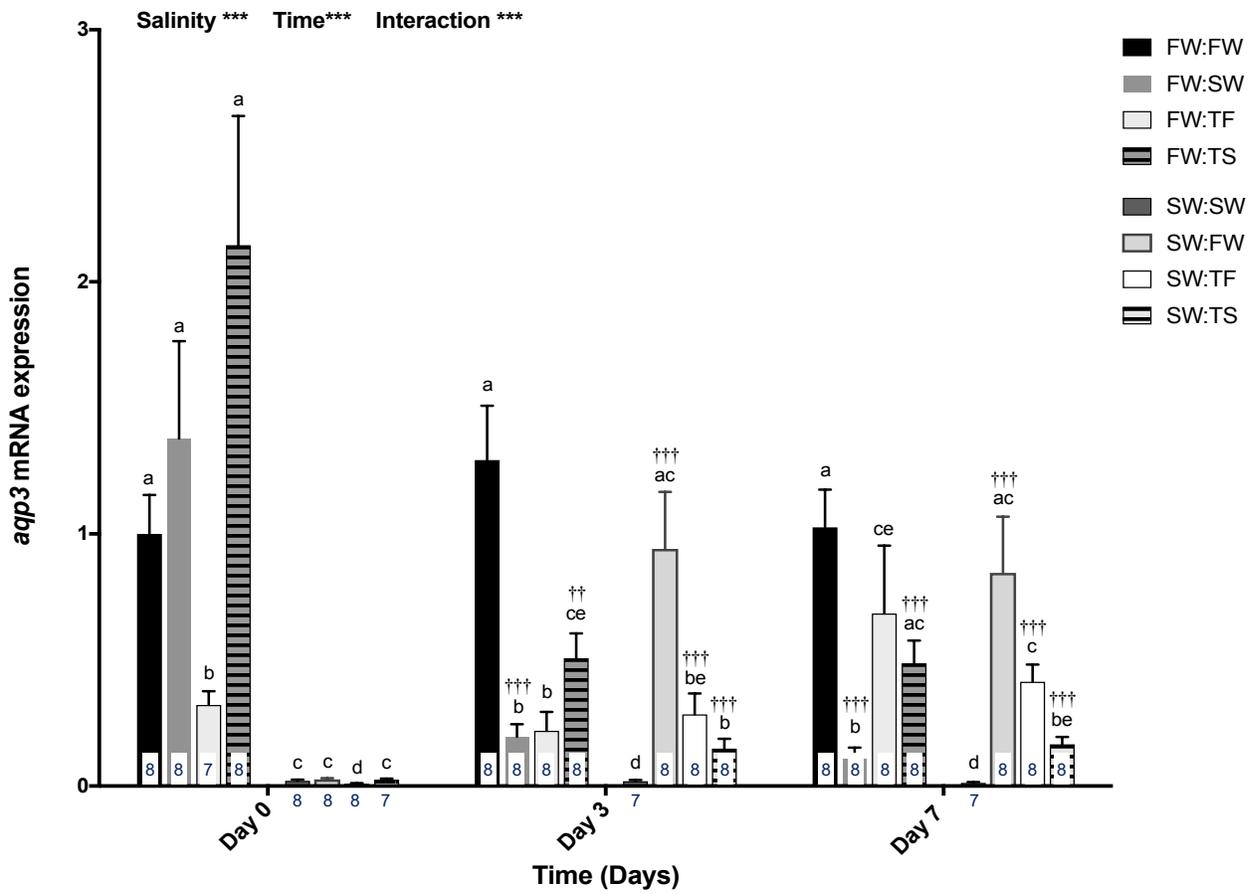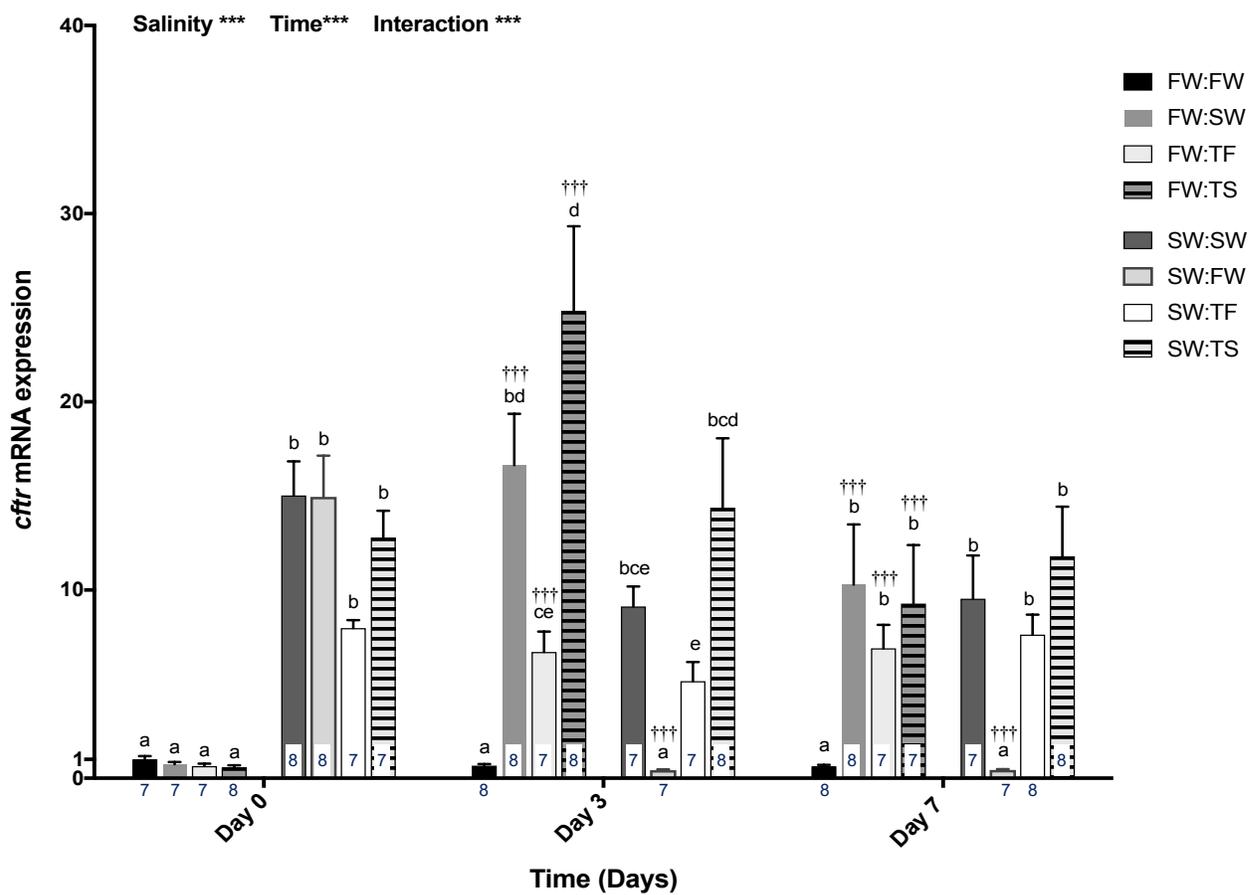ique tilapia. These parameters include plasma osmolality, prolactin and gill mRNA levels of prolactin receptor and gill ion transporters. The physiological responses to cyclical salinity change are of interest, but what little work has been done is with this species.

 Response: We thank you for your time and thoughtful considerations for improving this manuscript.

Major comments:

Comment #1:
The introduction primarily consists of general background on osmoregulatory mechanisms and their hormonal control. There is very little justifying why the examination of adults should be any different from the previously published results of Moorman et al. 2015 who examined the same parameters under similar conditions in 4-month old juveniles. In addition, the goals of the paper are poorly stated; at present, they simply state the results will be similar to those of fry [actually 4 month old juveniles]. A more complete explanation of rational and objective with explicit predictions of results is required.

Response: We appreciate the reviewer's remarks. Following the recommendations to provide stronger justification for the examination of adults in the current work and a complete rationale with structured objectives and hypotheses we have extensively revised and added text to the Introduction section as follows.

    Line 68: "While the ability of Mozambique tilapia to tolerate steady-state environments of distinct salinities is well established, less is known about their osmoregulatory physiology in dynamically-changing salinities.
    Fluctuations in salinity characterize some of the environments to which Mozambique tilapia are native, such as near shore estuaries. Recently, we have described the distinct osmoregulatory profile that tilapia reared under tidally-changing salinities acquire relative to fish reared in steady-sate FW or SW since the yolk-sac fry stage (up to 15 days post fertilization, until yolk is fully absorbed; Moorman et al., 2014; 2015). Here, we characterize whether the unique osmoregulatory profile of tidally-reared fish may be aqcuired by fully developed adult fish that have been reared in steady-state salinities for at least two years prior to a transfer to tidally-changing salinities without exposure to any salinity change during early development. Generally, tilapia and other teleosts in FW hyperosmoregulate to counteract a tendency to lose solutes to the environment and to become over-hydrated (McCormick, 2001)."

Line 136: "In our previous study it was concluded that developing tilapia experiencing tidal-salinity oscillations could respond better to a future one-way transfer of salinity from FW to SW, compared with fish reared in steady-state salinities (Moorman et al., 2015). It is unknown, however, whether adult fish retain such physiological plasticity as observed in juveniles. In anadromous species, individuals at different life stages often exhibit distinct tolerances to environmental salinity (Jensen et al., 2015). Despite the remarkable euryhalinity of the non-anadromous Mozambique tilapia, little is known on how osmoregulatory capacity is established and maintained throughout their life history. Hence, we tested whether the ability of adult fish to acclimate to TR required pre-exposure to both FW and SW during early developmental stages and whether the key variables associated with osmoregulation paralleled those of steady-state FW and SW fish. To address these questions, the following endpoints were measured both in fish reared in FW, SW and TR for 2 years, and in those transferred from FW or SW steady-states to TR for up to 1 week:…"

Comment #2:
It is not clear from the present paper (or any of the previous papers using this approach) how the salinity changed during the tidal simulation.

Response: The salinity changed gradually from FW to SW and vice-versa with 95% and complete salinity changes by 2 and 3 h, respectively, of the initiation of each cycle. This information is now included under "Materials and Methods".

Line 160: The following sentence was added: "Ninety-five % and 100 % changes in salinity were obtained by 2h and 3h, respectively, of change from FW to SW or SW to FW (Fig. 1)."

Comment #3:
Understanding how salinity changed during both addition of FW and SW would be of great value, as they are unlikely to be symmetrical and important to anyone wishing to repeat the results or simulate these conditions. I strongly recommend inclusion of a figure showing an example of salinity changed during tidal treatments.

Response: A Figure (1) has now been included in the manuscript to represent the hourly tracking of salinity during a full tidal cycle between 10AM to 10PM.

Comment #4:
The methods also state that temperature was maintained at 25 + 2 C, a relatively broad variation that could have significant physiological impacts. Did temperature change as a function of salinity?

Response: Although thermostats set at 26C were used in each tank to minimize temperature variations, they were subject to decreases in temperature overnight as they were set outdoors. All tanks were subject to similar environmental temperature effects. Temperatures were 1.2-1.5 C warmer in SW compared with FW in tidal tanks. In a previous experiment, where tilapia were maintained at 20C, 28C and 35C for 24h, there was no significant difference in plasma osmolality, Na^+/K^+ ATPase or plasma glucose across temperature treatments in FW-acclimated fish (Fiess et al., 2007).

Comment #5:
As with the introduction, the discussion should examine how these results differ (or not) from Moorman et al. 2015, and what is the relevance of these differences.

Response: Thank you for pointing out this shortcoming in our discussion. Following the recommendations to provide a stronger parallel with Moorman et al., 2015 and highlight the relevance of the differences, we specifically tested the findings against a hypothetical model described in Moorman et al 2015, and now included in the introduction. To that extent, we made modifications to the Discussion section as follows:

Line 347: "In light of recent findings suggesting that tilapia exposed to changing salinities during early stages of larval development may better respond to subsequent salinity challenges (Moorman et al., 2015), we tested the central notion of whether there is an adaptive advantage of rearing fish in changing salinities from the yolk-sac fry stage. By comparing 2-year old adult tilapia reared in steady-state FW and SW with fish reared under TR, our findings support the notion that the physiological experience of dynamically-changing salinities during early life history does not significantly improve survivability or osmoregulatory responses compared with fish that were exposed to TR for the first time as adults ."

Comment #6:
Line 347: The statement that "plasma PRL is was higher in FW compared to SW regardless of regimen" is not supported by the data. There are several time points in figure 3B where they are the same.

Response: The data from Experiment 1 supports the statement as there were salinity effects on both osmolality and PRL. The statement has been adjusted to specifically reflect the experiment being discussed:

Line 378: Consistent with previous reports, in Experiment 1 plasma osmolality was higher in fish in SW than those in FW, whether fish were kept in a steady-state or tidal regimen (Moorman et al., 2014; Moorman et al., 2015; Seale et al., 2006; Seale et al., 2002; Yada et al., 1994). In the same experiment, plasma PRL was higher in fish in FW compared with those in SW, regardless of rearing regimen, which is also consistent with the expected release of PRL in response to a reduction in plasma osmolality.

Comment #7:

Line 358   The statement that prlr1 expression in FW steady-state was elevated over that in SW fish is not quite accurate. My interpretation of their figure is that this pattern was not established until day 7. There is relatively little current discussion of the time course of changes in any of the parameters, but in some cases these are quite important.

Response: With the exception of the steady-state FW vs SW comparison on Day 0 of experiment 2 (Fig. 5A), which despite the tendency for upregulation in FW was not significantly different, all other instances (4 total) where prlr1 expression was compared between FW and SW

steady-state fish (Experiment 1, Fig 3.A and Experiment 2, Fig. 5A, Day 0 FW:SW vs SW:FW; Day 3 FW:FW vs SW:SW; Day 7 FW:FW vs SW:SW), FW acclimated steady-state fish had greater expression than SW counterparts.

To maintain the accuracy of the statement as aptly pointed out by the reviewer, we have rephrased the statement as follows:

Line 392: **"**In Experiment 1 and in three of the four comparisons in Experiment 2, *prlr1* expression in FW steady-state fish was elevated over that in SW fish."

Comment #8:

The manuscript would have been greatly improved if protein levels of the major ion transporters had been examined. Methods for these measurements are available, and this would have allowed the mRNA levels to be placed in a physiological context. At present, it is unclear whether the observed changes in mRNA levels are just signal alterations due to altered salinity patterns, and may have little relevance to protein abundances.

Response: Thank you for this important observation. While the measurement of protein levels of major ion transporters would have undoubtedly added important information to this manuscript, the authors feel that it would not have added a novel interpretation to the conclusions inasmuch as such assessment has already been conducted in a previous experiment (Moorman, 2014). By tracking the immunofluorescence of NCC, NKCC, NKA and CFTR, the authors found that while tidal fish had a pattern of protein abundance that was intermediate to that of steady state FW and SW fishes, changes in immunosignal between both phases of the tidal cycle were not observed. This is a similar outcome as observed with circulating PRL protein in TF vs TS fish. Hence, we focused our approach on detecting changes in gene expression patterns in steady-state and tidal fish, at least in part to unveil potential differences between FW and SW phases of the tidal cycle. We have now included a summary of this perspective in the Discussion as follows:

Line 437: "Moreover, the strong suppression of *ncc* in fish reared in TR or transferred to TR, is consistent with the previously reported immunohistochemistry results indicating suppression of NCC protein in the apical region of branchial ionocytes of tilapia reared in TR (Moorman et al., 2014). Conversely, in the same study, signal intensities for NKCC and CFTR were consistently high in TR fish. Together, the mRNA results of this and other studies suggest that transcriptional regulation of these three key ion transporters in steady-state and dynamically-changing conditions are paralleled by changes in protein abundance."

Minor comments:

Comment #1:

The term fry is used in several places, but without consistency, sometimes referring to "yolk sac fry" and sometimes to 4 month old juveniles. This term should either be defined or not used.

Response: The definition of yolk-sac fry is now clarified as follows. In other instances throughout the text, the term fry was replaced by "yolk-sac fry" where applicable.

Line 74: "…yolk-sac fry stage (up to 15 days post fertilization, until yolk is fully absorbed)."

Comment #2:

Line 213 "regimen" is too vague and should be changed to "tidal regimen" here and elsewhere in the manuscript.

Response: In some cases, "regimen" can refer to either steady state or tidal, such as when defining main effects of a 2-way ANOVA (salinity and regimen); in others, it refers specifically to the tidal regimen. Use of the term was double-checked throughout the manuscript and changed accordingly.

Comment #3:
Line 363   it is not clear what is meant by "expression varied between both phases of the tidal cycle"

Response: The sentence has been rewritten as follows:
Line 397: "…expression varied between fish in TF and TS."

Reviewer 2

General
This manuscript describes a large multifaceted experimental series to resolve whether rearing of tilapia in fluctuating salinity has any effect on their later facility to acclimate to salinities. The TF and TS treatments particularly offer interesting insights into the physiology of tilapia in fluctuating salinity. The experiment is carefully planned and executed and the results are clearly presented and the analysis is conservative and sound. In the figures, sample size ranges are provided (e.g. n = 7-11), whereas it would be more informative to add the exact sample sizes for the individual treatments (i.e. full disclosure). The manuscript is overall well-written. The discussion is rather short and to the point, but some further discussion of the results is warranted, particularly linking the results to a hypothetical model. The authors have not discussed some of the results, instead coming to general and not very interesting conclusions. The important result in Figure 2C is not put forward strongly enough. The fact that oscillating salinity almost completely suppresses ncc expression is interesting and ncc regulation is introduced (line 97) but not discussed later except a brief listing on line 390. Are the results consistent with previous findings?
There is room for the authors to test the results (Figure 3 and 4) against a hypothetical model and conclude whether the model is sound. The notion that developing fish experiencing tidal salinity oscillations could respond better to salinity challenge,

compared to fish raised in steady-state salinity, implies some sort of unspecified epigenetic advantage. The results do not support this model, but the conclusion connected to this is unstated. The conclusion: "The present findings do not support a model wherein physiological experience significantly improves survivability in future salinity challenges." could (should) be added to the abstract.
There are numerous minor suggestions for increasing readability.

Response:
We are glad to hear that this reviewer finds the manuscript well-written, carefully planned and executed, and appreciate the comments for improving the interpretations and implications of findings. The suggestion for linking with a hypothetical model related to existence or not of a developmental advantage in tidal fish is particularly useful for better elaborating and addressing a specific hypothesis. Following these comments, we have made a number of changes to improve the discussion and interpretation of results, with some changes to the proposed wording. We drew stronger connections between current ncc findings and our previously reported immunohistochemistry results. We also reframed the introduction and compared previous studies where fish were reared in tidally-changing salinities during early development and whether this early exposure is required for success in future salinity challenges. The conclusion along with other additions to the discussion were added as follows:

Line 52: "Moreover, the present findings suggest that early exposure to salinity changes does not significantly improve survivability in future challenges to dynamically-changing salinities."

Line 347: "In light of recent findings suggesting that tilapia exposed to changing salinities during early stages of larval development may better respond to subsequent salinity challenges (Moorman et al., 2015), we tested the central notion of whether there is an adaptive advantage of rearing fish in changing salinities from the yolk-sac fry stage. By comparing 2-year old adult tilapia reared in steady-state FW and SW with fish reared under TR, our findings support the notion that the physiological experience of dynamically-changing salinities during early life history does not significantly improve survivability or osmoregulatory responses compared with fish that were exposed to TR for the first time as adults."

Line 437: "Moreover, the strong suppression of *ncc* in fish reared in TR or transferred to TR, is consistent with the previously reported immunohistochemistry results indicating suppression of NCC protein in the apical region of branchial ionocytes of tilapia reared in TR (Moorman et al., 2014). Conversely, in the same study, signal intensities for NKCC and CFTR were consistently high in TR fish. Together, the mRNA results of this and other studies suggest that transcriptional regulation of these three key ion transporters in steady-state and dynamically-changing conditions are paralleled by changes in protein abundance."

Specific recommendations for revision
Maj
none

Minor

Comment #1: Line 2 "parameters" are fixed and constant aspects. I think you mean variables.
Response: The term "parameters" was changed to "variables".

Comment #2: Line 68 and elsewhere: Cf. means to compare and should be cf.. Even if it is being used here as to mean "confer", it is expected of a reference that the reader should do this and thus asking the reader to confer is redundant. Please delete all cases.
Response: The term "Cf." was removed as suggested.

Comment #3: Line 77-78 Awkward. How about "There are two isoforms of PRL receptors reported for Mozambique tilapia, PRLR1 and PRLR2 (Fiol..."
Response: Modified as suggested.

Comment #4: Line 89 No hyphen required in "subunit"
Response: Modified as suggested.

Comment #5: Line 100: CFTR is an anion channel that will transport different anions in different local circumstances. Better would be "CFTR, an anion channel responsible for Cl-secretion by ionocytes of teleost fish in SW," It would be appropriate to point out that during acclimation to SW, CFTR is trafficked into the apical membrane, while NKCC traffics to the basolateral membrane of ionocytes (Marshall et al. J exp Biol 205:1265-1273, 2002)
Response: Modified as suggested. Sentences were modified and added as follows:
Line 110: "Seawater ionocytes, on the other hand, are characterized by presence of basolateral $Na^+/K^+/2Cl^-$ cotransporter (NKCC1a) and apical cystic fibrosis transmembrane conductance regulator (CFTR, an ion channel responsible for Cl^- secretion by ionocytes of teleost fish in SW; Hiroi et al., 2005). During acclimation to SW, CFTR is trafficked into the apical membrane, while NKCC is translocated to the basolateral membrane of ionocytes (Marshall et al., 2002)."

Comment #6: Line 105 The increase in cftr expression also parallels the development of Cl-Transport by ionocytes (Marshall et al. J exp Biol 202:1535-1544 1999). It is important to point out the tight linkage between gene expression, protein trafficking to its functional locus and its actual operation in ion transport.
Response: The following sentence was added:
Line 121: "Importantly, it has been demonstrated that an increase in *cftr* expression in SW is linked not only to the trafficking of CFTR to the apical membrane but to the actual secretion of Cl^- (Marshall et al., 1999)."

Comment #7: Line 133 It is pertinent for readers to know the water hardness or calcium content if they are to be able to repeat the experiment. Please provide information or a reference to the freshwater chemistry.
Response: A reference to both FW and SW chemistries was added as follows:
Line 159: Physicochemical properties of the FW and SW employed have been recently reported elsewhere (Breves et al., 2017).

Comment #8: Line 145 and elsewhere "twenty-four" Generally, integers greater than 10 can be

included as the numerals "24" (although line 144 is correct with "Ninety-six", but only as the first word of a sentence). Please replace word numbers with numerals.
Response: Modified as suggested.

Comment #9: Line 185 It is unclear how much cDNA was added; could the authors provide a range of amounts/concentrations (1-3 μg) of cDNA and the accuracy to which this was determined (e.g. +/- 1%) in the qPCR mix?

Response: A total of 2 ug of total RNA was used to produce cDNA for experimental samples. cDNA was diluted in a range that varied between 20- to 100-fold depending on the relative abundance of each gene. Adjustments to the methods description were provided as follows:

Line 199: "Using the High Capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA), 5 μL of total RNA (400 ng/μL) was reverse transcribed into cDNA."

Line 210: "Dilution of experimental cDNA ranged from 20- to 100-fold."

Comment #10: Line 188 "fold-change from FW values" Unclear. Was the reference group the FW-FW transfer controls? If so, then "fold-change compared to FW-FW control Day 0 values" would be more descriptive.
Response: Modified as suggested.

Comment #11: Line 190 and 209 and 242 "Plasma parameters" should be "Plasma variables" or, to be abundantly clear: "Plasma osmolality and PRL".
Response: Modified as suggested.

Comment #12: Line 222 "branchial mRNA expression". Strictly speaking, what was measured was "relative mRNA abundance", the product of gene expression, not gene expression itself. Suggest replacing "mRNA expression" with "mRNA abundance".
Response: To ensure that the term "gene expression" employed here refers to "relative mRNA abundance", we have clarified the use of this nomenclature once under Materials and Methods as follows:
Line 213: "Relative mRNA abundance data are expressed as fold-change compared with FW:FW control Day 0 values, and referred to as mRNA expression throughout the manuscript."

Comment #13: Line 269 "FW:TF vs FW:TS and SW:TF vs. SW:TS" Be consistent with this abbreviation: "vs." (this journal prefers no italics)
Response: All instances of "vs" were replaced with "vs."

Comment #14: Line 290 "and further enhanced" add "were" for parallelism.
Response: Modified as suggested.

Comment #15: Line 307 Starting a sentence with "cftr" is awkward. How about "Branchial cftr" ion ATPases
Response: Modified as suggested.

Comment #16: Line 317 and elsewhere "and pumps". Unclear. "ion ATPases" or "NAK isoforms" would be more clear.
Response: Where applicable, "pumps" was replaced with "ion ATPases".

Comment #17: Line 325 ", which previously" Unclear. Better: "a finding that previously"
Response: Modified as suggested.

Comment #18: Line 336 Consider mentioning that "Consistent with these findings, the present protocol involved transfer to 80% SW for 48h, then to full strength SW."
Response: Modified as suggested.

Comment #19: Line 348 "expected response of PRL" More clear to say "expected release of PRL in response"
Response: Modified as suggested.

Comment #20: Line 363 "Based on our previous observation of plasma PRL levels not varying between fish in TF and TS, it is unlikely..." The finding does not jibe with the present work. The present results show that FW:TF has lower PRL than both SW:TF and SW:TS groups at Day 3 and FW:TF has lower PRL than SW:TS at Day 7 (Fig 3B). Thus it appears TF could have a lower plasma PRL than TS, possibly because of PRL usage/metabolism during the FW part of the cycle. I suggest deletion of the present sentence because it adds little and addition of an interpretation of the present results (Fig 3B).
Response: The sentence was deleted as suggested.

Comment #21: Line 372 No italics for in vitro and in vivo
Response: Italics removed for "in vitro" and "in vivo".

Comment #22: Line 381-2 How does PRLR2 rise in sw? Explanation A: Regular length PRLR2 activates a different pathway distinct from the PRLR1 pathway and could prevent inappropriate PRL action in SW by diverting circulating PRL into this alternate pathway. If so, the increase in PRLR2 in SW makes sense. Explanation B: Alternatively, the hypothesis has been (Fiol et al.) that PRLR2 (short form) helps prevent inappropriate PRL action in SW by reducing functional receptor formation. If so, an increase in PRLR2 (short form) in SW also makes sense. The question is whether your selected primers include or exclude the PRLR2 (short form). It seems the primer set would exclude mRNA from the short form of the gene (search of primer GCCCTTGGGAATACATCTTCAG on ACG61366.1, short form), so explanation A seems the right interpretation. Either way, the point is worthy of (some) discussion.
Response: Thank you for this important observation which is worthy of further discussion. To reflect the proposed explanations we have added the following:
Line 551: "The molecular mechanism underlying this outcome may be associated with PRL binding either the regular length or short form of PRLR2. While the former has been hypothesized to activate a different pathway than PRLR1 upon binding PRL, the latter is thought to reduce the formation of functional receptors, thereby preventing PRL's actions (Fiol et al., 2009). In the present study, primers that detect regular length *prlr2* were employed. It is tenable, therefore, that salinity driven changes in *prlr2* in tidally-acclimated fish facilitate the attenuation of PRL's effects by diverting downstream signaling from hyperosmoregulatory outcomes."

Comment #23: Figure 4b is not discussed completely, particularly the strongly significant time effect that shows prlr2 expression diminishes during acclimation in most (7 of 8) of the transfer groups at Day 7. Is this possibly a recovery from the stress of transfer?
Response: It is possible that there is a stress effect involved, however, both FW and SW steady state controls also decreased prlr2 expression by day 7 suggesting that there may be another factor(s) involved other than salinity transfer alone.

Comment #24: Line 390-393 "This is likely a reflection..." the expression patterns of ncc, nkcc1a and cftr are not discussed. "This" refers only to aqp1 expression. What about the need for ion transporters in a dynamically-changing environment? How is it that ncc is suppressed almost 100% and the other two are augmented? Particularly, do

the present results confirm the previous work of other labs (Breves et al 2010 and Inokuchi et al 2015)?

Response: The sentence was replaced and further discussion on the expression of ion-transporters in TR was added as follows:

Line 574: "The intermediate expression of *aqp3* in TR is likely a reflection of the shifting need for water transport in a dynamically-changing environment. By contrast, the mRNA expression of ion transporters, *ncc*, *nkcc1a* and *cftr* in dynamically-changing environments were either strongly suppressed (*ncc*) or elevated (*nkcc1a and cftr*). The expression patterns of these three ion transporters in TR follows those observed in SW-type ionocytes (Breves et al., 2010b; Inokuchi et al., 2015).

Comment #25: Line 403 "...than that of FW fish..." (insert that)
Response: Modified as suggested.

Comment #26: Line 407 "parameters" should be variables
Response: Modified as suggested.

Comment #27: Line 439 "n=7-11" Also in other figures 1-4. This reader would strongly prefer to see the sample size of each group as a numeral below or in each histogram to reveal which group had what sample size. The power of this inclusion is to allow the reader to consider whether a large standard error in a given group is the result of smaller sample size or whether it is real physiologically-derived variability, e.g. in the highly variable PRL titer when fish are transferred to FW. It also allows the reader to convert the SEM back to SD if they prefer.
Response: Thank you for this suggestion to increase the power of interpretation of results. On Figures 2-5, we have positioned numerals within histograms, or below, where space was unavailable, to indicate the sample size of each group.

Comment #28: Line 447 "by by" delete one.
Response: Modified as suggested.

Comment #29: References: A few typos: A year in parentheses, initials lacking periods and ions lacking superscripts.
Response: The references were revised for typos. Three new references were added in response to comments above.