Sex, salinity and sampling period dependent patterns of growth hormone mRNA expression in Mozambique tilapia.

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Abstract

Tilapias comprise the second most aquacultured finfish group in the world. Such popularity stems in part from their tolerance to a wide range of environmental conditions and their sexually dimorphic nature, where males grow larger than females. As in other vertebrates, growth in tilapia is regulated by the growth hormone/ insulin like growth factor (GH/IGF) system. Moreover, environmental salinity has previously been shown to directly modulate growth in tilapia. Less is known, however, regarding how salinity may modulate sexually dimorphic growth. Utilizing a species of tilapia of high salinity tolerance, the Mozambique tilapia, *Oreochromis mossambicus*, we compared *gh* expression from the pituitary of male and female adults reared in fresh water (FW), seawater (SW), and a tidal regime (TR) characterized by dynamically changing salinities between FW and SW every six hours, over a 24 h period. We found significant effects of sex, salinity regime and whether fish were sampled during daylight or dark hours. In both sexes, *gh* expression was greater in fish reared in SW and TR compared with those in FW, and greater in fish sampled during dark hours, compared with those sampled in daylight hours. Pituitary *gh* expression was greater in males than in females reared in SW and TR, but not in FW. These results provide insight on the sex-specific modulation of *gh* expression by environmental factors in Mozambique tilapia.

Keywords: Tilapia, growth, growth hormone, sex specific, salinity, tidal cycle, photoperiod

1. Introduction

Growth Hormone (GH) has been widely used as a biomarker for growth in species of relevance for aquaculture. Secreted by the pituitary gland, GH is involved in the regulation of most major physiological processes, such as growth, osmoregulation, metabolism, reproduction, development, immunity, and feeding behavior in fish (Bergan-Roller, Sheridan, 2018; Bern, 1983; Leung, et al., 1991; Mancera, McCormick, 1998; McCormick, 1996; McLean, Donaldson, 1993; Sakamoto, et al., 1997; Shepherd, et al., 2006; Shepherd, et al., 1997b; Yada, 2007). Growth in vertebrates is largely controlled through the coordinated activities of the growth hormone (GH)/insulin-like growth factor (IGF) axis. In the liver and muscle, GH stimulates cell division and differentiation among other functions, and initiates the production and release of IGF-I and IGF-II, which in turn stimulate a variety of growth-promoting actions in most tissues (Butler, Le Roith, 2001; Duan, et al., 2010; Le Roith, 2003; Wood, et al., 2005). Reflective of its key functions in the dynamic regulation of growth and metabolism, multiple endpoints of the GH/IGF system in various teleosts have been shown to be stimulated in response to a number of stimuli, including photoperiod, salinity, feeding, nutrient and sex steroid supplementation, and domestication (Ayson, Takemura, 2006; Ayson, et al., 2007; Bertucci, et al., 2019; Bjornsson, et al., 1995; Cleveland, Weber, 2015; Ma, et al., 2016; Magdeldin, et al., 2007; Peterson, et al., 2009; Tymchuk, et al., 2009; Velez, et al., 2016).

The tilapia, like many teleost fishes, exhibits a sexually dimorphic pattern of growth. This sexual dimorphism has led to a number of widespread strategies to produce and rear monosex populations (Singh, 2013). The basis for male tilapia growing faster and larger than females involves the differential actions of androgens and estrogens and their interplay with the GH/ IGF-1 axis (Kuwaye, et al., 1993; Riley, et al., 2002b; Ron, et al., 1995; Shepherd, et al., 1997b;

69 Sparks, et al., 2003). Studies indicate that the accelerated growth achieved by tilapia treated with
70 17α-methyltestosterone (MT) (Kuwaye, et al., 1993), a synthetic androgen, is due at least in part
71 to the stimulation of growth factors (Riley, et al., 2002b). Together, these results suggest that
72 largely through activation of the GH/IGF system, male tilapia are optimized for somatic growth.
73 The use of tilapia species that are tolerant to wide excursions in salinity, such as the
74 Mozambique tilapia, *Oreochromis mossambicus*, has provided a suitable model to investigate the
75 interplay of sexual determination of growth and its modulation by environmental salinity.
76 Regardless of sex, tilapia raised in seawater (SW) grow significantly faster than those in fresh
77 water (FW) (Kuwaye, et al., 1993; Morgan, Iwama, 1991; Riley, et al., 2002b; Ron, et al., 1995;
78 Shepherd, et al., 1997a; Sparks, et al., 2003). Evidence suggests that faster growth of SW tilapia
79 is tied, at least partly, to the activation of the GH/IGF system. Both circulating and pituitary GH
80 increase following transfer from FW to SW, while GH release *in vitro* has been found to increase
81 in response to increases in extracellular osmolality (Borski, et al., 1994; Breves, et al., 2010b;
82 Helms, et al., 1987; Pierce, et al., 2007; Seale, et al., 2006; Seale, et al., 2002). Plasma IGF-1 is
83 also higher in SW fish than in FW fish (Magdeldin, et al., 2007). Nevertheless, evidence suggests
84 that pituitary *gh* mRNA expression may be a better indicator of growth than circulating GH and
85 IGF-1 levels (Riley, et al., 2002b).
86 As a euryhaline species native to estuarine waters off the southeast coast of Africa
87 (Trewavas, 1983), the Mozambique tilapia is capable of surviving in salinities equivalent to FW
88 through double-strength SW (Fiess, et al., 2007; Stickney, 1986), and salinities that dynamically
89 change between FW and SW (Moorman, et al., 2015). Recently, we have described the distinct
90 osmoregulatory profile that Mozambique tilapia reared under tidally-changing salinities acquire
91 relative to fish reared in steady-state FW or SW since the yolk-sac fry stage (Moorman, et al.,

2015; Moorman, et al., 2014; Seale, et al., 2019) and after becoming adults (Pavlosky, et al., 2019). We have also found that rearing tilapia in water that varies in a tidal pattern between FW and SW increases growth by 4 months, while increasing GH levels in circulation, and pituitary *gh* mRNA expression (Moorman, et al., 2016). This same study also showed that, compared with GH and IGF-1 in circulation and *igf-1* and *gh receptor* (*ghr*) expression in muscle and liver, pituitary *gh* expression had the strongest positive correlation with body weight across all rearing salinities. It is unknown, however, whether the modulation of *gh* by rearing salinity regime varies with sex and natural photoperiod. By analyzing the interaction of natural factors otherwise known to individually modulate growth, we provide a new perspective on the nuanced and complex endocrine regulation of growth in fishes.

2. Materials and Methods

2.1 Fish rearing

Mozambique tilapia (*O. mossambicus*) yolk-sac larvae were collected from stocks maintained in outdoor FW (0.1 ± 0.1‰) tanks at the Hawai'i Institute of Marine Biology. The rearing protocol and sampling of fish for this study has been recently reported (Seale, et al., 2019). Briefly, 24 days post-collection, the yolk sacs were fully absorbed, and the fry were seeded to 700 L outdoor tanks filled with 140 L of FW, at a density of 120 fish per tank. Fry were fed ground trout chow pellets (Skretting, Tooele, UT) *ad libitum* daily. Water temperature was maintained at 27 ± 2 °C and fish were held under natural photoperiod. Two days after seeding, tanks were transitioned to brackish water (BW) of 10‰ by the addition of SW (34 ± 1‰; Kaneohe Bay, Oahu, HI). Five days after seeding, the salinity was further increased to 18 ± 2‰, and then eight days after seeding, two BW tanks were transitioned back to FW, two were

transitioned to SW, and the remaining four tanks put under a tidal regime (TR). Tanks subjected to the tidally changing salinity alternated between FW and SW every six hours, yielding a complete salinity transfer within two hours. The fish were maintained in either FW, SW or TR for two years prior to sampling. Following their initial transition to FW, SW or TR, fish were provided fixed rations of 18% mean body weight divided over two daily feedings. Rations were decreased by four percent every 21-25 days until they were equivalent to four percent mean body weight. The fish were reared under these conditions until the time of sampling. Fish were fasted during the 24 h sampling period; the final feeding of all treatment groups occurred immediately prior to the first sampling time point.

2.2 Treatments and sampling

Fish were sampled every three hours during a 24 h period, beginning at 0945 (nine forty-five AM) and ending at 0945 the following day. Five sampling periods occurred during daylight hours (0945, 1300, 1545, 0700 and 0945) and four during dark hours (1900, 2145, 0100, 0345). Four male and four female fish reared under the TR were sampled at the end of the FW and SW phases of the tidal cycle, as well as at the mid-point of each phase. For each time point at which TR fish were sampled, corresponding FW- and SW-control groups were also sampled. Fish were collected at each time point from across all of the replicate tanks for the FW, SW and TR treatments. There were two tanks each for FW and SW fish and four tanks for TR fish. Salinity was measured hourly in all tanks over the course of the 24 h sampling period (Fig. 2A and 2B). Salinity ranged between 0.1 and 0.2 ‰ in FW-control tanks, 34.5-35.2 ‰ in SW-control tanks, and 0.2-35.2‰ in TR tanks. At the time of sampling, fish were netted and lethally anesthetized with 2-phenoxyethanol (0.3 ml/l). After fish were weighed, blood was collected with a needle

and syringe coated with sodium heparin (200 U/ml, Sigma-Aldrich, St. Louis, MO). Plasma was separated by centrifugation and stored at -20°C until further analyses. Pituitaries were collected, frozen in liquid nitrogen, and stored at -80°C. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawai'i.

2.3 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from pituitary samples using TRI Reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). Using a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA), 30 ng of pituitary total RNA were reverse transcribed into cDNA. Quantitative real-time PCR (qRT-PCR) assays were set up as previously described (Pierce, et al., 2007), using the StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA). The PCR mixture (15 uL) contained Power SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of each primer, and 1 µl of cDNA (equivalent to 1.5 ng total RNA). PCR cycling parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The mRNA levels of reference and target genes were determined by a relative quantification standard curve. *Elongation factor 1α* (*ef1α*) was used as a reference gene to normalize the mRNA levels of target genes. Primer pairs employed and their efficiencies are listed in Table 1.

2.5 Statistical analyses

Statistical analysis of weight and length was conducted by linear regression of log-transformed data. Analyses of *gh* mRNA levels was conducted by three-way analysis of variance

(ANOVA) with sex, sampling period (daylight and dark hours) and salinity treatments (FW-controls, SW-controls, and TR fish) as main effects. Significant main and interaction effects (P<0.05) were followed up with Fisher's Protected Least Significant Difference (LSD) test. Data are expressed as means ± S.E.M. Statistical analyses were performed using Prism 8.0 software (GraphPad, La Jolla, CA).

3. Results

3.1 Sexually dimorphic growth

The relationship between total length and weight of male and female tilapia used in this study is shown in Fig. 1. All fish were sourced from the same cohort of yolk-sac fry. The inset depicts the natural log transformed total length versus weight of females and males, with linear regression equations of (Y= 3.23*X-2.03) and (Y= 3.25*X-2.08), respectively, and R^2= 0.93 and 0.79, respectively. While it is apparent that males had greater weight and length than females of the same age, the slopes of the log transformed length versus weight linear regressions were not significantly different between the two sexes. These data indicate that the length-weight relationship between male and female Mozambique tilapia are similar, despite their marked sexually dimorphic growth patterns.

3.2 Pituitary gh gene expression

To assess whether *gh* mRNA expression in male and female Mozambique tilapia may be differentially affected by salinity regime and natural photoperiod, we sampled adult (two years

184 post-hatch) Mozambique tilapia of both sexes every three hours (h) over the course of 24 h. We
185 compared *gh* mRNA expression levels from male fish with those of females reared in FW, SW
186 or TR and sampled during daylight and dark hours, in a three-way factorial design.
187 Pituitary g*h* mRNA levels from male and female tilapia reared in FW, SW and a TR were
188 measured every 3 h throughout a 24 h sampling period that encompassed approximately 12 h of
189 daylight (between 0945 and 1730, and 0545 and 1000) and approximately 12 h of dark (between
190 1730 and 0545) (Fig. 2A and 2B). Pituitary *gh* expression over time was generally higher in fish
191 reared in SW and TR; this pattern was more evident in males (Fig. 2A) than in females (Fig. 2B).
192 Moreover, *gh* expression in fish acclimated to all salinity regimens gradually rose during dark
193 hours, with the onset of the rise occurring earlier in fish reared in SW or TR (2130) than those
194 reared in FW (0100), especially in males (Fig. 2A). For statistical analysis, means were
195 combined and parsed by sex (male and female), salinity (FW, SW and TR) and natural
196 photoperiod (daylight and dark hours). A three-way ANOVA revealed single main effects of sex,
197 salinity and sampling period ($P < 0.001$) and an interaction effect of salinity and sampling period
198 ($P < 0.05$) (Fig. 3).
199 In fish sampled in daylight hours, pituitary mRNA expression of *gh* was 2.5-fold higher
200 in males reared in SW and TR versus those reared in FW (Fig. 3). By contrast, *gh* mRNA
201 expression in female fish sampled in daylight hours was only 1.5-fold higher in fish reared in
202 SW and TR compared with those reared in FW. There were no sex differences in *gh* expression
203 in fish reared in FW, regardless of the period in which they were sampled (Fig. 3). During
204 daylight hours, sex differences in *gh* expression were observed in fish reared in SW and TR but
205 not in FW. During dark hours, however, only fish reared in TR showed sex differences in *gh*

206 expression. Males reared in all salinities increased *gh* expression during dark hours, whereas
207 only females reared in SW and TR showed a similar pattern (Fig. 3).
208
209 **4. Discussion**
210 The objective of this experiment was to determine the effects of sex, salinity and
211 sampling period on pituitary *gh* expression in adult Mozambique tilapia. This is the first study to
212 describe a temporal profile in *gh* expression for adult male and female Mozambique tilapia
213 reared for two years under three distinct salinity regimens, including cyclically changing salinity,
214 which simulates some of the habitats to which this species is native. In light of the indication that
215 pituitary *gh* expression is a reliable predictor of growth compared with other endpoints of the
216 GH/IGF system (Moorman, et al., 2016; Riley, et al., 2002a) and recent findings showing that
217 tilapia exposed to changing salinities grow faster through the activation of the GH/IGF system
218 (Moorman, et al., 2016), our study focused on examining the nuances of environmental
219 regulation of pituitary *gh* in males and females of the same cohort. By comparing two-year old
220 adult tilapia reared in steady-state FW and SW with fish reared under TR, our findings support
221 the notion that salinity and time of day modulate sex-dependent patterns in the transcriptional
222 regulation of *gh*. Specifically, the findings of this study indicate that transcript levels of *gh* in
223 Mozambique tilapia acclimated to SW and TR are higher than those in FW. Moreover, the
224 salinity-dependent induction of *gh* transcripts was more accentuated in males than in females,
225 and elevated during dark hours compared with daylight hours.
226 Sexually dimorphic growth is a common phenomenon in fish. Based on their sex-
227 dependent growth patterns, tilapia culture is frequently conducted with monosex populations,
228 with males being favored due to their faster growth rates (Singh, 2013). Juvenile tilapia of

229 undifferentiated sex can be induced to become phenotypic males by exposure to MT (Pandian,
230 Sheela, 1995). Consequently, Mozambique tilapia treated with MT have been shown to grow
231 faster than untreated controls (Kuwaye, et al., 1993; Ron, et al., 1995; Sparks, et al., 2003).
232 Hence, it was not surprising to find a clear sexually-dimorphic pattern in the distribution of
233 weight and length of adult Mozambique tilapia of the same age (Fig. 1).

234 Environmental factors, such as salinity, play a major role in controlling growth in fishes
235 (Boeuf, Payan, 2001). Several studies have characterized the effects of salinity on growth and the
236 GH/IGF system in tilapia (Shepherd, et al., 2006). Regardless of sex, tilapia raised in SW grow
237 faster than those in FW (Kuwaye, et al., 1993; Morgan, et al., 1997; Riley, et al., 2002b; Ron, et
238 al., 1995; Shepherd, et al., 1997a; Sparks, et al., 2003). In tilapia reared under TR, growth rates
239 are even more pronounced than those of fish reared in SW (Moorman, et al., 2016). Circulating
240 GH and IGF-1, pituitary *gh* mRNA and hepatic *igf-1* mRNA are generally higher in SW fish than
241 in FW fish, though salinity-dependent patterns in circulating hormones are not as clear as their
242 transcripts (Breves, et al., 2010a; Breves, et al., 2010b; Magdeldin, et al., 2007; Moorman, et al.,
243 2016; Riley, et al., 2002b). In fish reared in TR, both plasma GH and pituitary *gh* mRNA were
244 elevated relative to levels observed in SW and FW fish (Moorman, et al., 2016). Despite ample
245 evidence indicating the activation of multiple endpoints of the GH/IGF system in conditions that
246 stimulate growth, the notion that *gh* mRNA is a suitable indicator of growth in tilapia (Riley, et
247 al., 2002b) was corroborated by Moorman and co-workers (Moorman, et al., 2016) who reported
248 that body weight correlated the strongest with *gh* mRNA compared with other GH/IGF system
249 endpoints. In the present study, *gh* mRNA levels were higher in fish acclimated to SW and TR
250 fish, compared with those in FW. Moreover, the salinity-induced elevation in *gh* mRNA was
251 more pronounced in males than in females. Inasmuch as *gh* levels are generally reflective of

252 body weight, our findings revealed a pattern consistent with that observed in the growth rates of
253 tilapia treated with MT in SW and FW, where MT-treated fish in SW grew faster than MT-
254 treated fish in FW and those that were untreated (Ron, et al., 1995; Sparks, et al., 2003).
255 It is well established that photoperiod influences growth in fish (Bjornsson, et al., 2011;
256 Boeuf, Bail, 1999). Extended light hours have been shown to increase growth rates in several
257 species, including red sea bream (*Pagrus major*), gilthead sea bream (*Sparus aurata*), Atlantic
258 salmon (*Salmo salar*), rainbow trout (*Oncorhyncus mykiss*) and Nile tilapia (*Oreochromis*
259 *niloticus*) (Biswas, et al., 2005; Johnsson, Bjornsson, 1994; Kissil, et al., 2001; Oppedal, et al.,
260 1999; Rad, et al., 2006). In salmonids, day length speeds up the parr-smolt transformation and
261 associated growth, and increases plasma GH (Bjornsson, et al., 1995; Bjornsson, et al., 1989;
262 McCormick, et al., 1995). In crussian carp (*Carassius auratus*) *gh* expression in muscle was
263 shown to oscillate, with peaks during dark hours in fish fasted for no longer than the duration of
264 one light-dark cycle (Wu, et al., 2018). In rabbitfish (*Siganus guttatus*), *gh* mRNA expression
265 was significantly higher in the dark phase than in the light phase, suggesting a diurnal rhythm of
266 expression (Ayson, Takemura, 2006). Consistent with the patterns observed in other teleosts,
267 pituitary *gh* mRNA peaked during dark hours in Mozambique tilapia (Figs. 2 and 3). These
268 nocturnal peaks in *gh* expression were further modulated with respect to sex and rearing salinity,
269 indicating that multiple environmental factors are at play to produce a complex and nuanced
270 pattern of pituitary *gh* regulation.
271 In this study, we have provided novel insights into the integrated regulation of *gh* in
272 Mozambique tilapia by sex, salinity regimen and sampling period. Together with our previous
273 study, in which it was found that fish reared in TR for four months grew faster than those reared
274 in steady-state FW or SW (Moorman, et al., 2016), our current findings may lead to applications

in aquaculture, where daylight and salinity can be adjusted to optimize sex-specific production practices. Moreover, the use of the TR rearing paradigm shall continue to bring forward novel physiological insights on the multi-factorial regulation of growth in tilapia and other euryhaline fish.

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Figure Legends:

Fig. 1. Total length versus body weight in two year old male (dark circles) and female (light circles) Mozambique tilapia depicting sexual dimorphism. Inset represents log transformed

297 length versus log transformed weight. The slope of the log transformed weight-length was not
298 different between males and females.

299 Fig. 2. Pituitary gene expression of *gh* in male (A) and female (B) Mozambique tilapia reared in
300 FW (black dashed), SW (black dotted) and a TR (solid black) and sampled over 24 h. Data are
301 normalized by *ef1α*, Values represent means ± S.E.M. (*n*= 3-5). Shading denotes dark hours.
302 Black lines and symbols denote pituitary *gh* expression (left y-axis). Grey lines and symbols
303 denote mean water salinity measured hourly in FW, SW, and TR tanks (right y-axis).
304

305 Fig. 3. Pituitary gene expression of *gh* in male (black bars) and female (white bars) Mozambique
306 tilapia reared in FW, SW and TR, and sampled during light and dark hours. Data are normalized
307 by *ef1α*. Values represent means ± S.E. M (n = 13). Sex, salinity and sampling period effects
308 were analyzed by three-way ANOVA, followed by Fisher's protected LSD test when main or
309 interaction effects were detected. **Significantly different from males at $P < 0.01$. †, ††† Significantly different from FW period at $P < 0.05$ and 0.001, respectively. §§, §§§ Significantly different from the daylight sampling period at $P < 0.01$ and 0.001, respectively.
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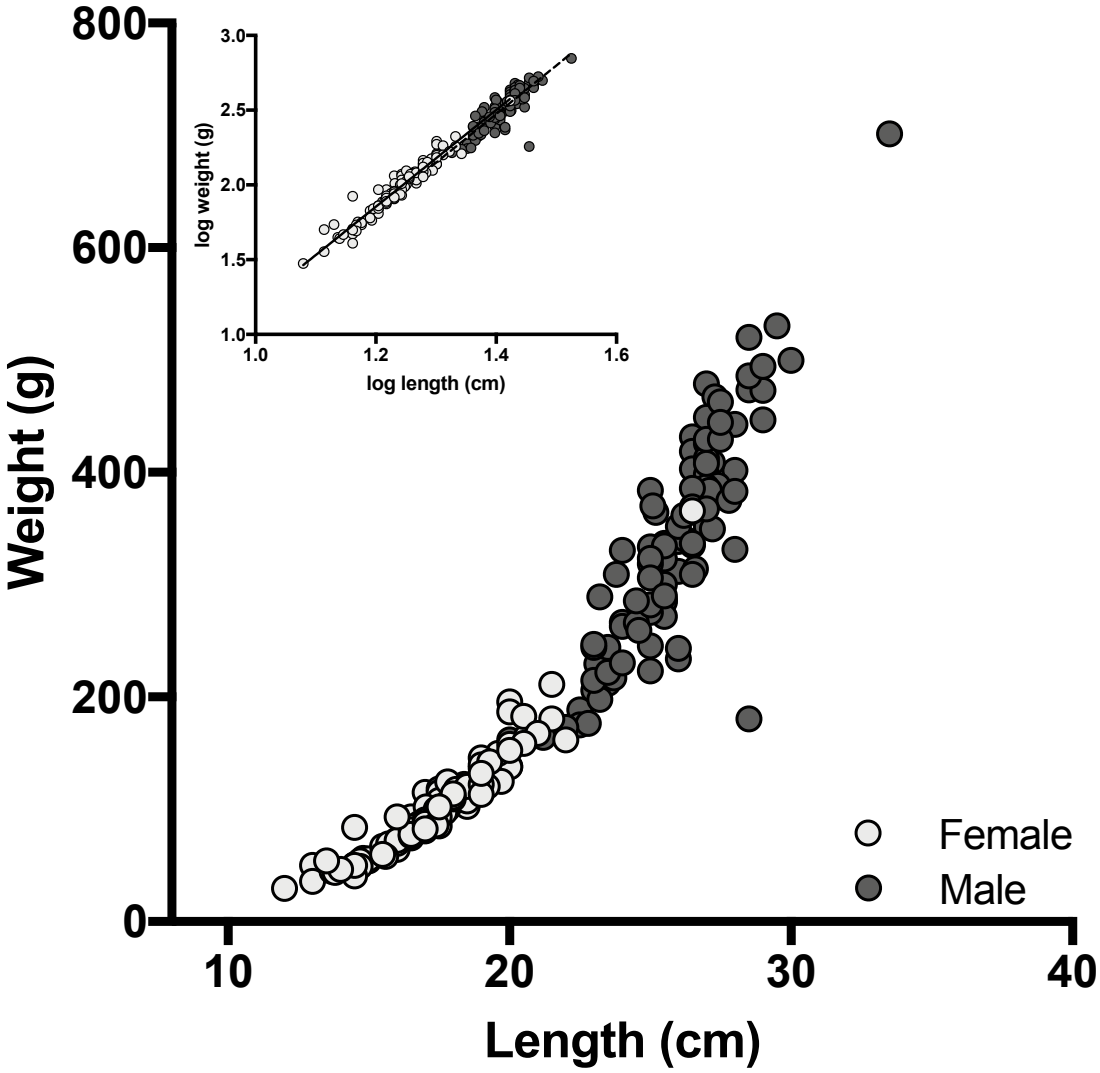
Table 1. Primers used for qPCR

Gene Name	Primer Sequence (5'-3')	R^2	% Efficiency	Reference	Accession Number
ef1α	Forward AGCAAGTACTACGTGACCATCATTG Reverse AGTCAGCCTGGGAGGTACCA	0.999	90.5	Breves et al., 2010	AB075952
gh	Forward TTACATCATCAGCCCGATCG Reverse AGATCGACAGCAGCTTCAGGA	0.999	96.4	Magdeldin et al., 2007	AF033806

516 EF1α: Elongation Factor 1α; GH: Growth Hormone.

517

Figure 1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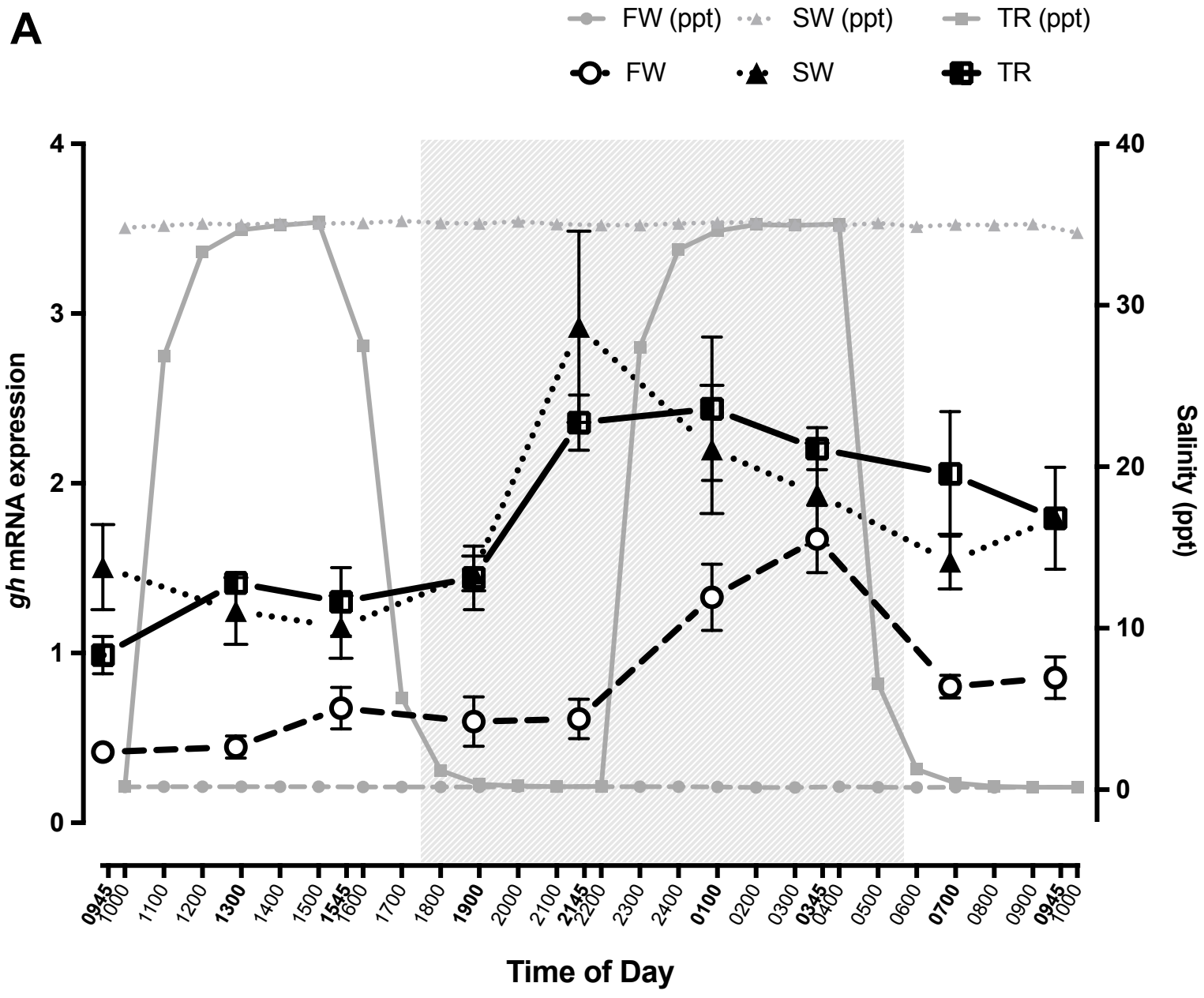
600
400
200
0
Weight (g)
10
20
30
40
Length (cm)
Female
Male
3.0
2.5
2.0
1.5
1.0
log weight (g)
1.0
1.2
1.4
1.6
log length (cm)

Figure 2


A
FW (ppt)
SW (ppt)
TR (ppt)
FW
SW
TR
gh mRNA expression
Salinity (ppt)
Time of Day
0945
1000
1100
1200
1300
1400
1500
1545
1600
1700
1800
1900
2000
2100
2145
2200
2300
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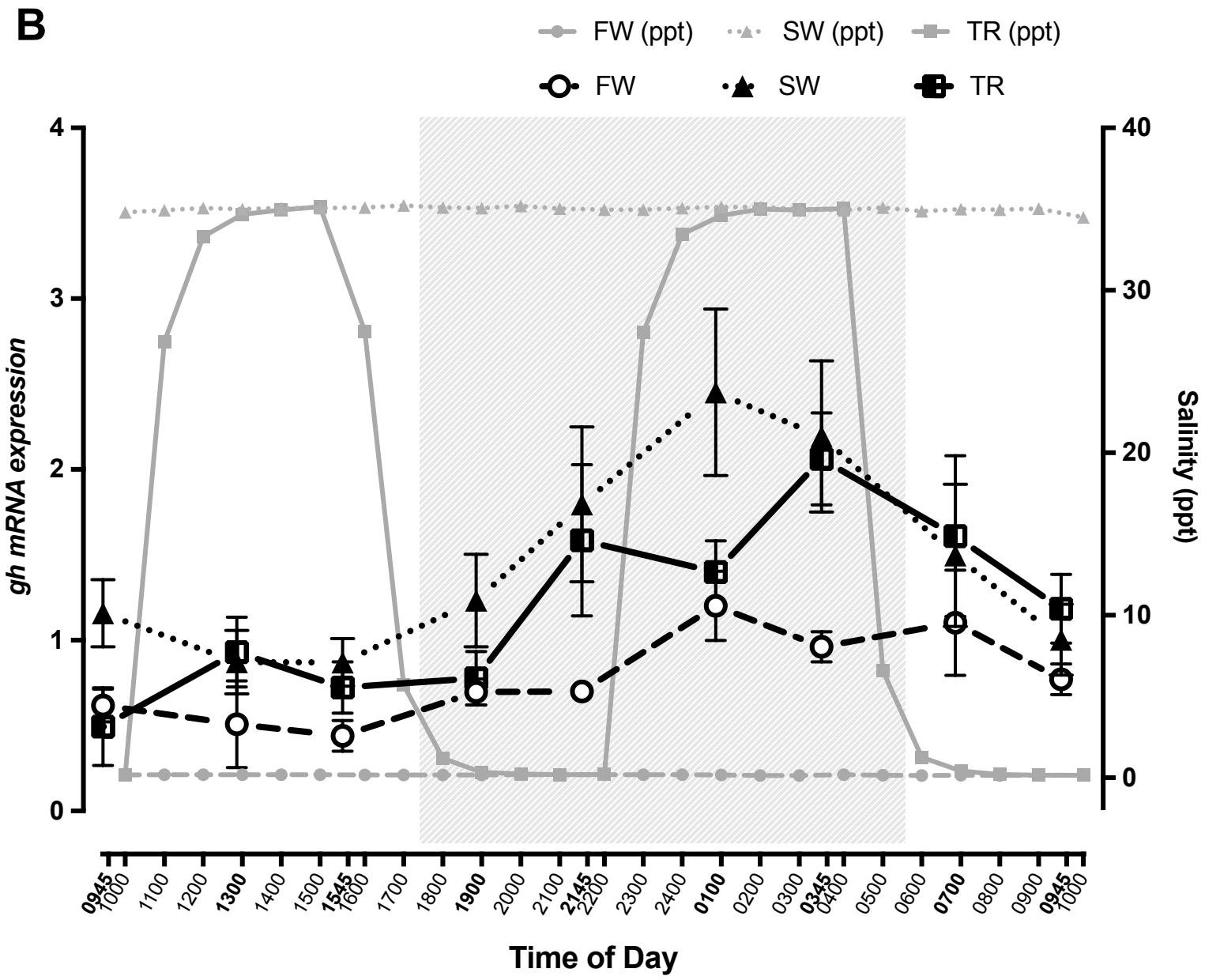
0700
0800
0900
0945
1000

Figure 2


B
FW (ppt)
SW (ppt)
TR (ppt)
FW
SW
TR
gh mRNA expression
Salinity (ppt)
Time of Day
0945
1000
1100
1200
1300
1400
1500
1545
1600
1700
1800
1900
2000
2100
2145
2200
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0100
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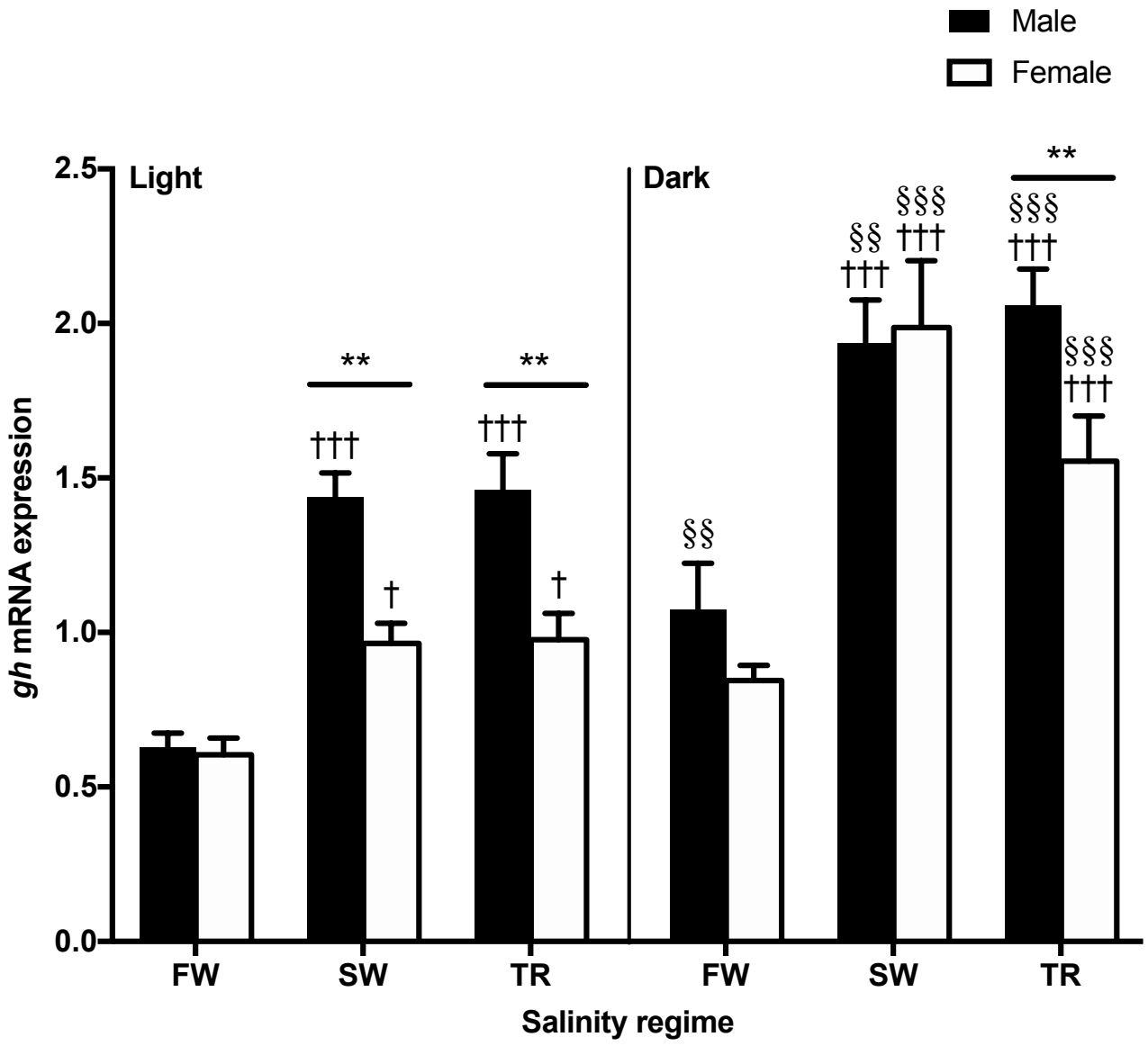
0700
0800
0900
0945
1000

Figure 3


Male
Female
Light
Dark
2.5
2.0
1.5
1.0
0.5
0.0
gh mRNA expression
**
**
**
†††
†
†††
†
§§
§§
†††
§§§
†††
§§§
†††
§§§
†††
FW
SW
TR
FW
SW
TR
Salinity regime