Aquaculture 519 (2020) 734766

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

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

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ARTICLE INFO

Keywords:
Tilapia
Growth
Growth hormone
Sex specific
Salinity
Tidal cycle
Photoperiod

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.

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 and Sheridan, 2018; Bern, 1983; Leung et al., 1991; Mancera and McCormick, 1998; McCormick, 1996; McLean and 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 and 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 and Takemura, 2006; Ayson et al., 2007; Bertucci et al., 2019; Bjornsson et al., 1995; Cleveland and 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; Sparks et al., 2003). Studies indicate that the accelerated growth achieved by tilapia treated with 17α-methyltestosterone (MT) (Kuwaye et al., 1993), a synthetic androgen, is due at least in part to the stimulation of growth factors (Riley et al., 2002b). Together, these results suggest that largely

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https://doi.org/10.1016/j.aquaculture.2019.734766
Received 29 August 2019; Received in revised form 2 November 2019; Accepted 21 November 2019
Available online 22 November 2019
0044-8486/

through activation of the GH/IGF system, male tilapia are optimized for somatic growth.

The use of tilapia species that are tolerant to wide excursions in salinity, such as the Mozambique tilapia, *Oreochromis mossambicus*, has provided a suitable model to investigate the interplay of sexual determination of growth and its modulation by environmental salinity. Regardless of sex, tilapia raised in seawater (SW) grow significantly faster than those in fresh water (FW) (Kuwaye et al., 1993; Morgan and Iwama, 1991; Riley et al., 2002b; Ron et al., 1995; Shepherd et al., 1997a; Sparks et al., 2003). Evidence suggests that faster growth of SW tilapia is tied, at least partly, to the activation of the GH/IGF system. Both circulating and pituitary GH increase following transfer from FW to SW, while GH release in vitro has been found to increase in response to increases in extracellular osmolality (Borski et al., 1994; Breves et al., 2010b; Helms et al., 1987; Pierce et al., 2007; Seale et al., 2006; Seale et al., 2002). Plasma IGF-1 is also higher in SW fish than in FW fish (Magdeldin et al., 2007). Nevertheless, evidence suggests that pituitary *gh* mRNA expression may be a better indicator of growth than circulating GH and IGF-1 levels (Riley et al., 2002b).

As a euryhaline species native to estuarine waters off the southeast coast of Africa (Trewavas, 1983), the Mozambique tilapia is capable of surviving in salinities equivalent to FW through double-strength SW (Fiess et al., 2007; Stickney, 1986), and salinities that dynamically change between FW and SW (Moorman et al., 2015). Recently, we have described the distinct osmoregulatory profile that Mozambique tilapia reared under tidally-changing salinities acquire 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 4% every 21–25 days until they were equivalent to 4% 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-5 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 B). 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 s 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.

2.4. 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 ($P < .05$) 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).

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., 2010a	AB075952
gh	Forward TTACATCATCAGCCCGATCG Reverse AGATCGACAGCAGCTTCAGGA	0.999	96.4	Magdeldin et al., 2007	AF033806

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

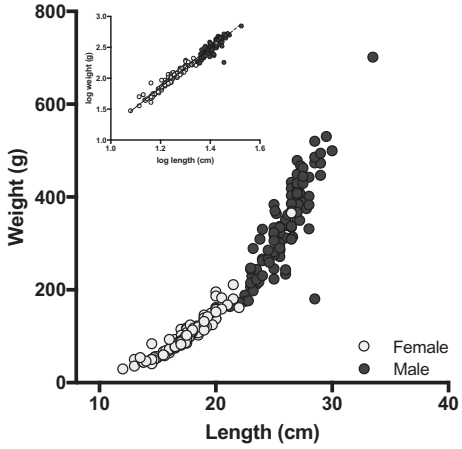

Fig. 1. Total length versus body weight in two year old male (dark circles) and female (light circles) Mozambique tilapia depicting sexual dimorphism. Inset represents log transformed length versus log transformed weight. The slope of the log transformed weight-length was not different between males and females.

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 post-hatch) Mozambique tilapia of both sexes every three hours (h) over the course of 24 h. We compared *gh* mRNA expression levels from male fish with those of females reared in FW, SW or TR and sampled during daylight and dark hours, in a three-way factorial design.

Pituitary *gh* mRNA levels from male and female tilapia reared in FW, SW and a TR were measured every 3 h throughout a 24 h sampling period that encompassed approximately 12 h of daylight (between 0945 and 1730, and 0545 and 1000) and approximately 12 h of dark (between 1730 and 0545) (Fig. 2A and B). Pituitary *gh* expression over time was generally higher in fish reared in SW and TR; this pattern was more evident in males (Fig. 2A) than in females (Fig. 2B). Moreover, *gh* expression in fish acclimated to all salinity regimens gradually rose during dark hours, with the onset of the rise occurring earlier in fish reared in SW or TR (2130) than those reared in FW (0100), especially in males (Fig. 2A). For statistical analysis, means were combined and parsed by sex (male and female), salinity (FW, SW and TR) and natural photoperiod (daylight and dark hours). A three-way ANOVA revealed single main effects of sex, salinity and sampling period ($P < .001$) and an interaction effect of salinity and sampling period ($P < .05$) (Fig. 3).

In fish sampled in daylight hours, pituitary mRNA expression of *gh* was 2.5-fold higher in males reared in SW and TR versus those reared in FW (Fig. 3). By contrast, *gh* mRNA expression in female fish sampled in daylight hours was only 1.5-fold higher in fish reared in SW and TR compared with those reared in FW. There were no sex differences in *gh* expression in fish reared in FW, regardless of the period in which they were sampled (Fig. 3). During daylight hours, sex differences in *gh* expression were observed in fish reared in SW and TR but not in FW. During dark hours, however, only fish reared in TR showed sex differences in *gh* expression. Males reared in all salinities increased *gh* expression during dark hours, whereas only females reared in SW and TR showed a similar pattern (Fig. 3).

4. Discussion

The objective of this experiment was to determine the effects of sex, salinity and sampling period on pituitary *gh* expression in adult Mozambique tilapia. This is the first study to describe a temporal profile in *gh* expression for adult male and female Mozambique tilapia reared for two years under three distinct salinity regimens, including cyclically changing salinity, which simulates some of the habitats to which this species is native. In light of the indication that pituitary *gh* expression is a reliable predictor of growth compared with other endpoints of the GH/IGF system (Moorman et al., 2016; Riley et al., 2002a) and recent findings showing that tilapia exposed to changing salinities grow faster through the activation of the GH/IGF system (Moorman et al., 2016), our study focused on examining the nuances of environmental regulation of pituitary *gh* in males and females of the same cohort. By comparing two-year old adult tilapia reared in steady-state FW and SW with fish reared under TR, our findings support the notion that salinity and time of day modulate sex-dependent patterns in the transcriptional regulation of *gh*. Specifically, the findings of this study indicate that transcript levels of *gh* in Mozambique tilapia acclimated to SW and TR are higher than those in FW. Moreover, the salinity-dependent induction of *gh* transcripts was more accentuated in males than in females, and elevated during dark hours compared with daylight hours.

Sexually dimorphic growth is a common phenomenon in fish. Based on their sex-dependent growth patterns, tilapia culture is frequently conducted with monosex populations, with males being favored due to their faster growth rates (Singh, 2013). Juvenile tilapia of undifferentiated sex can be induced to become phenotypic males by exposure to MT (Pandian and Sheela, 1995). Consequently, Mozambique tilapia treated with MT have been shown to grow faster than untreated controls (Kuwaye et al., 1993; Ron et al., 1995; Sparks et al., 2003). Hence, it was not surprising to find a clear sexually-dimorphic pattern in the distribution of weight and length of adult Mozambique tilapia of the same age (Fig. 1).

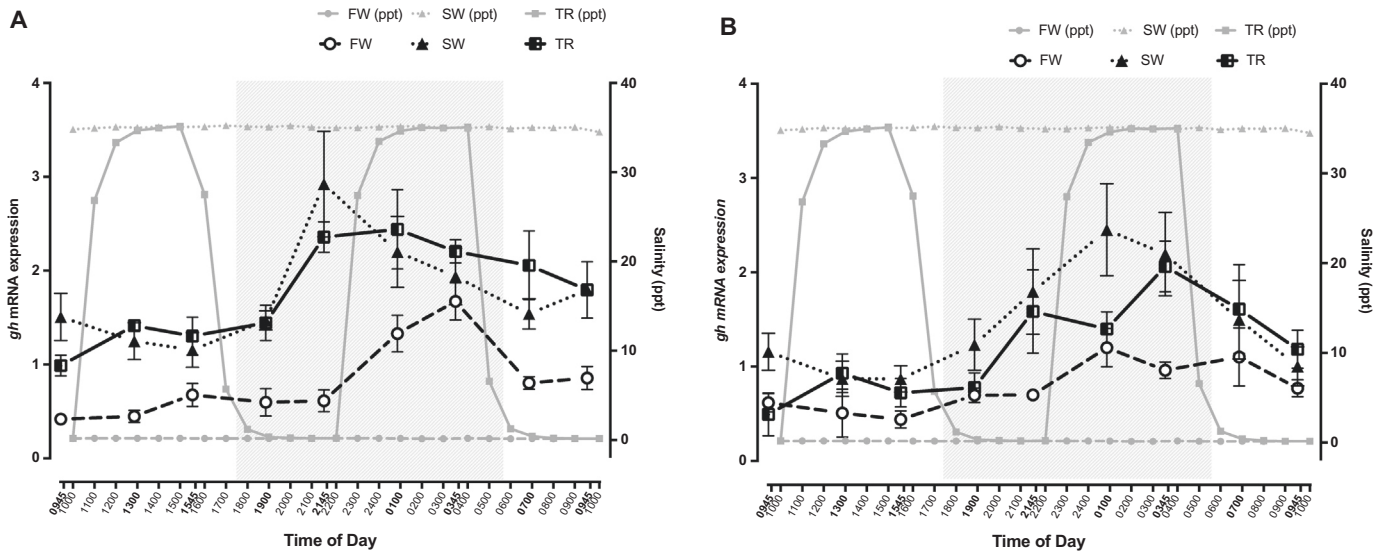

Fig. 2. Pituitary gene expression of *gh* in male (A) and female (B) Mozambique tilapia reared in FW (black dashed), SW (black dotted) and a TR (solid black) and sampled over 24 h. Data are normalized by *ef1α*, Values represent means ± S.E.M. (n = 3–5). Shading denotes dark hours. Black lines and symbols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

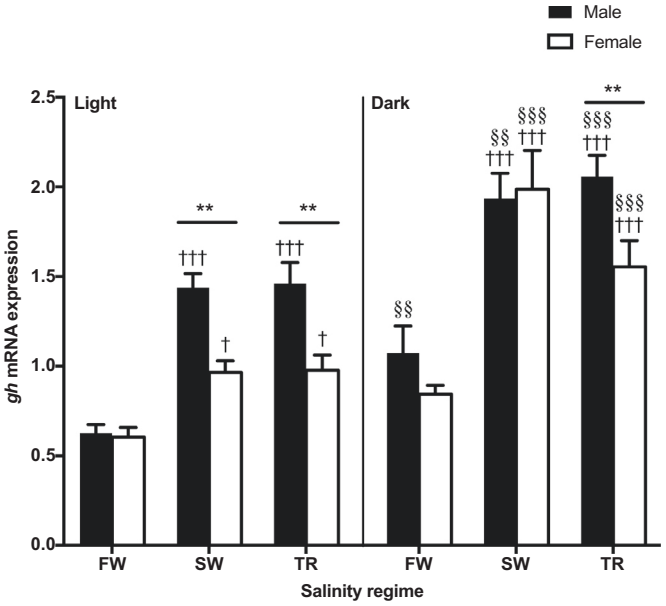

Fig. 3. Pituitary gene expression of *gh* in male (black bars) and female (white bars) Mozambique tilapia reared in FW, SW and TR, and sampled during light and dark hours. Data are normalized by *ef1α*. Values represent means ± S.E. M (n = 13). Sex, salinity and sampling period effects were analyzed by three-way ANOVA, followed by Fisher's protected LSD test when main or interaction effects were detected. **Significantly different from males at P < .01. †, ††† Significantly different from FW period at P < .05 and 0.001, respectively. §§, §§§ Significantly different from the daylight sampling period at P < .01 and 0.001, respectively.

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

It is well established that photoperiod influences growth in fish (Bjornsson et al., 2011; Boeuf and Bail, 1999). Extended light hours have been shown to increase growth rates in several species, including red sea bream (*Pagrus major*), gilthead sea bream (*Sparus aurata*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhyncus mykiss*) and Nile tilapia (*Oreochromis niloticus*) (Biswas et al., 2005; Johnsson and Bjornsson, 1994; Kissil et al., 2001; Oppedal et al., 1999; Rad et al., 2006). In salmonids, day length speeds up the parr-smolt transformation and associated growth, and increases plasma GH (Bjornsson et al., 1995; Bjornsson et al., 1989; McCormick et al., 1995). In crussian carp (*Carassius auratus*) *gh* expression in muscle was shown to oscillate, with peaks during dark hours in fish fasted for no longer than the duration of one light-dark cycle (Wu et al., 2018). In rabbitfish (*Siganus guttatus*), *gh* mRNA expression was significantly higher in the dark phase than in the light phase, suggesting a diurnal rhythm of expression (Ayson and Takemura, 2006). Consistent with the patterns observed in other teleosts, pituitary *gh* mRNA peaked during dark hours in Mozambique tilapia (Figs. 2 and 3). These nocturnal peaks in *gh* expression were further modulated with respect to sex and rearing salinity, indicating that multiple environmental factors are at play to produce a complex and nuanced pattern of pituitary *gh* regulation.

In this study, we have provided novel insights into the integrated

regulation of *gh* in Mozambique tilapia by sex, salinity regimen and sampling period. Together with our previous study, in which it was found that fish reared in TR for four months grew faster than those reared 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.

Declaration of Competing Interest

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

Acknowledgements

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. This work was funded in part by grants from the National Science Foundation (IOS-1755016), and the National Oceanic and Atmospheric Administration (NA18OAR4170347 to D.T.L. and A.P.S. and NA14OAR4170071, which is sponsored by the University of Hawai'i Sea Grant College Program projects R/SS-12 and R/SB-18 to A.P.S.), the National Institutes of Diabetes and Digestive and Kidney Diseases 1R21DK111775-01 from the National Institutes of Health to A.P.S., and the National Institute of Food and Agriculture Hatch no. HAW02051-H to A.P.S. The views expressed herein are those of the authors and do not necessarily reflect the views of the aforementioned granting agencies. University of Hawai'i Sea Grant publication number UNIHI-SEAGRANT-JC-14-66. We are grateful to Mr. Julian Leon for laboratory assistance and Dr. Lucia A. Seale and Ms. Amanda Reyes for sampling assistance.

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