

Sex-specific responses to growth hormone and luteinizing hormone in a model teleost, the Mozambique tilapia

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ARTICLE INFO

Keywords:

Gonadotropins
Fish
Growth
Liver
Muscle
Receptors
Reproduction
Sexual dimorphism

ABSTRACT

Across the vertebrate lineage, sexual dimorphism in body size is a common phenomenon that results from trade-offs between growth and reproduction. To address how key hormones that regulate growth and reproduction interact in teleost fishes, we studied Mozambique tilapia (*Oreochromis mossambicus*) to determine whether the activities of luteinizing hormone (Lh) are modulated by growth hormone (Gh), and conversely, whether targets of Gh are affected by the presence of Lh. In particular, we examined how gonadal morphology and specific gene transcripts responded to ovine GH (oGH) and/or LH (oLH) in hypophysectomized male and female tilapia. Hypophysectomized females exhibited a diminished gonadosomatic index (GSI) concomitant with ovarian follicular atresia. The combination of oGH and oLH restored GSI and ovarian morphology to conditions observed in sham-operated controls. A similar pattern was observed for GSI in males. In control fish, gonadal *gh receptor* (*ghr2*) and *estrogen receptor β* (*erβ*) expression was higher in females versus males. A combination of oGH and oLH restored *erβ* and *arβ* in females. In males, testicular *insulin-like growth factor 3* (*igf3*) expression was reduced following hypophysectomy and subsequently restored to control levels by either oGH or oLH. By contrast, the combination of both hormones was required to recover ovarian *igf3* expression in females. In muscle, *ghr2* expression was more responsive to oGH in males versus females. In the liver of hypophysectomized males, *igf2* expression was diminished by both oGH and oLH; there was no effect of hypophysectomy, oGH, or oLH on *igf2* expression in females. Collectively, our results indicate that gene transcripts associated with growth and reproduction exhibit sex-specific responses to oGH and oLH. These responses reflect, at least in part, how hormones mediate trade-offs between growth and reproduction, and thus sexual dimorphism, in teleost fishes.

1. Introduction

Sexual dimorphism with respect to body size is prevalent among animal taxa from crustaceans to mammals, including fishes. In bony fishes, this pattern is observed across a wide range of species, such as garfish (*Belone belone*; Samsun et al., 2006), mackerel (*Scomber scombrus*; Eltink, 1987), several groundfish (Hanson and Courtenay, 1997; Jiménez et al., 1998), and tilapia (Chervinski, 1965). Sexual dimorphism results from differences in sexual maturation, growth, and life span between sexes, in addition to other selective pressures (Fairbairn et al., 2007; Hüsey et al., 2012; Ralls and Mesnick, 2009; Stamps, 1993). Moreover, growth and reproductive processes interact in many vertebrates and evidence suggests that differences in the allocation of energy towards somatic growth versus reproductive capacity may underlie sex-specific growth rates (Cox, 2006; Rennie et al., 2008; Taylor and

DeNardo, 2005).

Like many other teleost fishes, tilapia exhibit sexually dimorphic growth patterns, with males growing faster than females. In Mozambique tilapia (*Oreochromis mossambicus*), these differences in growth rate were previously attributed to the direct actions of androgens and estrogens as well as their modulation of the growth hormone (Gh)/insulin-like growth factor 1 (IgF1) axis (Kuwaye et al., 1993; Riley et al., 2002; Seale et al., 2020; Sparks et al., 2003). Different growth rates between sexes may also result from the timing of gonadal development and sexual maturation (Bhatta et al., 2012b). In males, growth rates decreased only after complete sexual maturation, while in females, growth decreased prior to sexual maturation. Moreover, the removal of gonads retarded growth while ectopic transplantation of gonadal tissue restored normal body growth of Mozambique tilapia (Bhatta et al., 2012a), suggesting that gonadal hormones impact growth in both sexes.

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<https://doi.org/10.1016/j.ygcen.2022.114119>

Received 9 June 2022; Received in revised form 21 August 2022; Accepted 22 August 2022

Available online 24 August 2022

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The endocrine intermediaries between growth and reproduction, however, require elucidation. In salmonids, somatic growth is reduced during sexual maturation when lipids are mobilized from visceral adipose tissue and muscle (Aussanasuwannakul et al., 2011; Manor et al., 2012; Nassour and Leger, 1989). During the sexual maturation of rainbow trout (*Oncorhynchus mykiss*), nutrition and energy are redirected away from somatic growth to support ovarian development by increasing muscle protein turnover (Cleveland and Weber, 2011, 2016; Weber et al., 2022). It remains unclear, however, how the interaction between growth and reproductive development is regulated by hormonal signals within males and females.

The endocrine system of fishes regulates a range of physiological processes, including growth and reproduction. Secreted by the pituitary gland, Gh regulates various physiological processes including somatic growth (Duan, 1997) through its receptors (Ghr). Two *ghrs* subtypes were found in Mozambique tilapia, *ghr1* and *ghr2* (Kajimura et al., 2004; Pierce et al., 2007), with *ghr2* encoding the primary Gh receptor (Kajimura et al., 2004; Pierce et al., 2007, 2012). Gh regulates growth and development directly through the Ghr2 and indirectly through the stimulation of Igfs (Butler and Le Roith, 2001; Duan, 1998; Duan et al., 2010; Le Roith et al., 2001; Reindl and Sheridan, 2012). Also secreted by the pituitary gland, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) regulate gametogenesis and the production of gonadal hormones in males and females (Levavi-Sivan et al., 2010; Schulz et al., 2001; Swanson et al., 2003). In teleosts, it was established that the two gonadotropins, Gth I and Gth II are orthologues of tetrapod Fsh and Lh, respectively (Li and Ford, 1998; Quérat et al., 2000; Suzuki et al., 1988a–c). Lh and Fsh operate through their cognate receptors in Leydig cells and Sertoli cells in males, and follicular cells in females (Lubzens et al., 2017; Schulz et al., 2001), to stimulate the production of androgens and estrogens, respectively (Kagawa et al., 1998; Okada et al., 1994; Planas et al., 2000; Planas and Swanson, 1995; Swanson et al., 2003). Gonadal steroids further regulate gametogenesis and sexual maturation (Forsgren and Young, 2012; Miura and Miura, 2003; Monson et al., 2017; Nagahama, 1994; Nagahama et al., 1994; Schulz et al., 2001) with androgen and estrogen receptors (*ar* and *er*) mediating the gonadal responsiveness to sex steroid hormones (Gross and Yee, 2002; Park et al., 2007). Gh may also directly modulate reproduction by exerting both gonadotropin-dependent and -independent actions in both males and females (Hull and Harvey, 2014). Hence, it is necessary to examine the effects of hormones that regulate growth and reproduction on Ghr2, Igfs, and sex steroid receptors to understand the endocrine mechanisms that may underlie sexually dimorphic growth.

The combination of hypophysectomy with hormone replacement is a classic experimental approach to identify the endocrine effects of pituitary hormones. Through this approach, we previously described the effects of Gh on hepatic leptin levels (Douroso et al., 2016), plasma Igfs and hepatic *ghr2*, *igf1*, and *igf2* expression (Breves et al., 2014), and intestinal *ghr2* expression (Petro-Sakuma et al., 2020). Given their well-described patterns of sexually dimorphic growth, we used hypophysectomized Mozambique tilapia as a model to examine whether the activities of Lh are modulated by Gh, and conversely, whether targets of Gh are affected by the presence of Lh. We administered ovine GH and ovine LH alone or in combination before examining the morphology of gonads and gene expression patterns of *ghr2*, *igfs*, *ers*, and *ars* in gonad, muscle, and liver.

2. Materials and methods

2.1. Animals and rearing conditions

Male and female Mozambique tilapia (*O. mossambicus*) with mean ($\pm$ S.E.M.) body weights (BW) of 92.6 ± 3.1 and 93.0 ± 3.0 g, respectively, were obtained from stocks maintained at the Hawai'i Institute of Marine Biology. Given the sexually-dimorphic nature of this species, the mean BW was kept similar between males and females to control for any

influence of BW on sex-specific responses. Fish were maintained outdoors with a continuous flow of fresh water (FW; municipal water) under natural photoperiod and fed a commercial diet (Silver Cup Trout Chow, Nelson & Sons Inc., Murray, UT). Water temperatures were maintained between 24 and 26 °C. All housing and experimental procedures were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee of the University of Hawai'i.

2.2. Hypophysectomy and hormone replacement

Hypophysectomy was performed by the transorbital technique developed by Nishioka (1994). Mozambique tilapia were anesthetized by immersion in buffered tricaine methanesulfonate (100 mg/l, Argent Chemical Laboratories, Redmond, WA) and 2-phenoxyethanol (2-PE; 0.3 ml/l, Sigma-Aldrich, St Louis, MO) in FW. After removal of the right eye and underlying tissue, a hole was drilled through the neurocranium and the pituitary was aspirated with a modified Pasteur pipette. The orbit was then packed with microfibrillar collagen hemostat (Ethicon, Somerville, NJ) and fish were allowed to recover for 5 d in brackish water (12 ‰) composed of seawater diluted with FW. Following recovery, fish were transferred to re-circulating experimental aquaria containing aerated brackish water and treated with kanamycin sulfate (National Fish Pharmaceuticals, Tucson, AZ). Sham operations were carried out in the same manner but without aspiration of the pituitary.

To characterize the effects of GH and LH on gonadosomatic index (GSI; (gonad weight/BW)*100), and gonadal, muscle, and hepatic gene expression, hypophysectomized fish ($n = 8$) were administered ovine GH (oGH; 5 µg/g BW, National Hormone and Peptide Program) and ovine LH (oLH; 5 µg/g BW, National Hormone and Peptide Program) alone or in combination via intraperitoneal injections over the course of 5 d. oGH and oLH were delivered in saline vehicle (0.9 % NaCl; 1.0 µl/g BW). Forty-eight hours after the initial injection, second and third injections were administered 48 h apart. Twenty-four hours after the third injection, fish were netted, lethally anesthetized in 2-PE, and weighed prior to the removal of the gonads for calculation of GSI. Muscle, liver, and gonad samples were collected, immediately snap-frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. A portion of the gonads was fixed in 4 % paraformaldehyde and processed for histological analyses. Two additional groups, hypophysectomized and sham-operated fish (control), were injected with saline vehicle only. Fish were not fed for the duration of the recovery and post-injection periods. At sampling, the completeness of hypophysectomy was confirmed by post-mortem inspection of the hypothalamic region.

2.3. Histological analysis

Fixed ovary and testis fragments were dehydrated in a series of graded ethanol, cleared with xylene, and embedded in paraffin wax. The embedded gonadal fragments were cut into 5 µm sections and stained with hematoxylin-eosin for histological analysis. Gonadal sections were examined using a light microscope (Olympus BX43; Olympus Corp., Center Valley, PA) equipped with a digital camera (Infinity 3s; Lumenera, Ottawa, ON).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from gonad, muscle, and liver using TRI Reagent (MRC, Cincinnati, OH) according to the manufacturer's protocols. The concentration and purity of extracted RNA were assessed using a microvolume spectrophotometer (NanoDrop One, Thermo Fisher Scientific, Waltham, MA). Total RNA (300–500 ng) was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The mRNA levels of reference and target genes were determined by the relative quantification method using a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The qRT-

PCR reaction mix (15 µl) contained Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 200 nM of forward and reverse primers, and 1 µl cDNA. Dilution of experimental cDNA from gonad, muscle, and liver ranged from 10- to 50-fold. PCR cycling parameters were: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All qRT-PCR primers have been previously described and are listed with PCR efficiencies in Table 1. The geometric mean ($\sqrt[3]{x_1 \times x_2 \times x_3}$; where x = quantity of each reference gene) of *elongation factor 1α* (*ef1α*), *β-actin*, and *18s* levels was used to normalize target genes levels (Celino-Brady et al., 2019).

2.5. Statistical analysis

Group comparisons were performed by two-way ANOVA followed by Fisher's protected LSD test when significant main or interaction effects were detected. In order to meet assumptions of normality (assessed by Shapiro-Wilk test), individual values were log-transformed when necessary prior to statistical analysis. Pearson correlation coefficients were used to describe the relationship between GSI and *igf3* levels. Statistical calculations were performed using Prism 9.0 (GraphPad, La Jolla, CA). Significance for all tests was set at $P < 0.05$.

3. Results

3.1. Effects of hypophysectomy and hormonal treatment on GSI and gonadal morphology

Overall, there was a strong effect of sex on GSI. GSI was higher in females than in males, and was reduced in females following hypophysectomy. While neither oGH nor oLH when administered alone could restore GSI, the combined administration of both hormones restored GSI to values similar to sham-operated controls. On the other hand, although not significant ($P = 0.49$), there was a tendency for GSI in males to decrease following hypophysectomy and a tendency for the combined administration of oGH and oLH to restore GSI (Fig. 1).

Histological analysis indicated that sham-operated fish exhibited normal gonad morphology. Males displayed maturing testes with spermatozoa, and in females, ovaries with vitellogenic oocytes were visible (Figs. 2 and 3). Except for vacuolization, hypertrophy of interstitial tissue, and death of a few germ cells, there were no profound histological

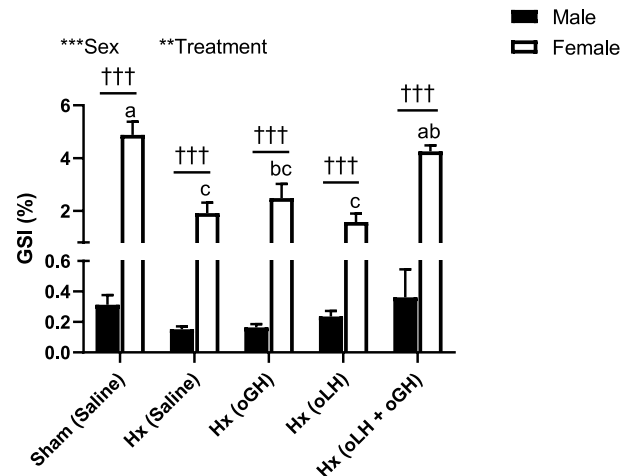


Fig. 1. Effects of hypophysectomy (Hx), and ovine growth hormone (oGH) and ovine luteinizing hormone (oLH) or their combination (oLH + oGH) on gonadosomatic index (GSI) in male and female Mozambique tilapia. Following Hx, Mozambique tilapia received three intraperitoneal injections of oGH alone, oLH alone (5 µg/g body weight), or their combination over 5 days. Sham-operated and Hx fish received saline injections. Values represent means $\pm$ S.E.M. ($n = 7-9$). Data were analyzed by two-way ANOVA followed by Fisher's protected LSD test when significant main or interaction effects were detected ($**P < 0.01$, $***P < 0.001$). Means not sharing the same letter are statistically significant at $P < 0.05$. Male and female treatment means not sharing the same uppercase and lowercase letters, respectively, are significantly different. ††† indicates significant difference between sexes at $P < 0.001$.

changes between the testes from control and hypophysectomized groups (Fig. 2A and B). Testis morphology of fish treated with oGH, oLH, or both hormones in combination did not markedly differ from control fish (Fig. 2). In females, however, hypophysectomy was followed by extensive atresia of ovarian follicles in 57 % of the fish (Fig. 3A and B). The administration of oGH and oLH alone had no effect on hypophysectomy-induced atresia (Fig. 3C and D); the administration of oGH with oLH restored normal ovarian morphology in most of the fish (Fig. 3E).

Table 1

List of primers used in qPCR assays.

Gene	Primer Sequence (5'-3')	R ²	Eff. %	Amplicon size (bp)	Accession no.	Reference
<i>18s</i>	F: GCTACCACATCCAAGGAAGGC R: TTCGTCACTACCTCCCGAGT	0.989	87.4	69	AF497908	Magdeldin et al., 2007
<i>ef1α</i>	F: AGCAAGTACTACGTGACCATTTG R: AGTCAGCCTGGGAGGTACCA	0.999	96.7	85	AB075952	Breves et al., 2010
<i>β-actin</i>	F: CTCTTCCAGCCTTCCTTCCT R: ACAGGTCTTACGGATGTCG	0.992	81.1	100	FN673689	Tipsmark et al., 2011
<i>ghr2</i>	F: CACACCTCGATCTGGACATATTACA R: CGGTTGGACAATGTCATTAAACA	0.995	94.4	102	EF452496	Pierce et al., 2007
<i>igf1</i>	F: CTGCTTCCAAAGCTGTGAGCT R: GATCGAGAAATCTTGGGAGTCTTG	0.991	85.9	75	AF033796	Kajimura et al., 2004
<i>igf2</i>	F: GCTTTTATTTCAGTAGGCCAACCA R: CACAGCTACAGAAAGACATCCTCTA	0.990	119.6	90	AH006117*	Davis et al., 2008
<i>igf3</i>	F: CAGACACTCCAGGTGCTGTGTG R: CAAGCCTTTACGTAAATAGATTCC	0.993	82.2	168	NM_001279636.1	Li et al., 2012
<i>era</i>	F: GGCTCAGCAGCAGTCAAGAA R: TGCTTGAGGTCTGTAAGT	0.989	77.6	302	AM284390	Park et al., 2007
<i>erβ</i>	F: ACCTTCCGGCAGCAGTACAC R: TCCAACATCTCCAGCAACAG	0.994	95.6	149	AM284391	Park et al., 2007
<i>ara</i>	F: GTCCCTGCTCAGCATCTAC R: TCACTCCATCCATGACAGC	0.976	92.8	221	AB045211	Park et al., 2007
<i>arβ</i>	F: CAGCCTCAATGAATTGGGAGA R: ATCCCAAGGCCAACACCATC	0.997	97.5	146	AB045212	Park et al., 2007

*Primer sequences were designed from the nucleotide sequences associated with the provided accession numbers. This exact accession number, however, is not available anymore in NCBI, and the revised versions do not contain the forward primer. Hence, XM_025908435.1 was used to deduce the amplicon size.

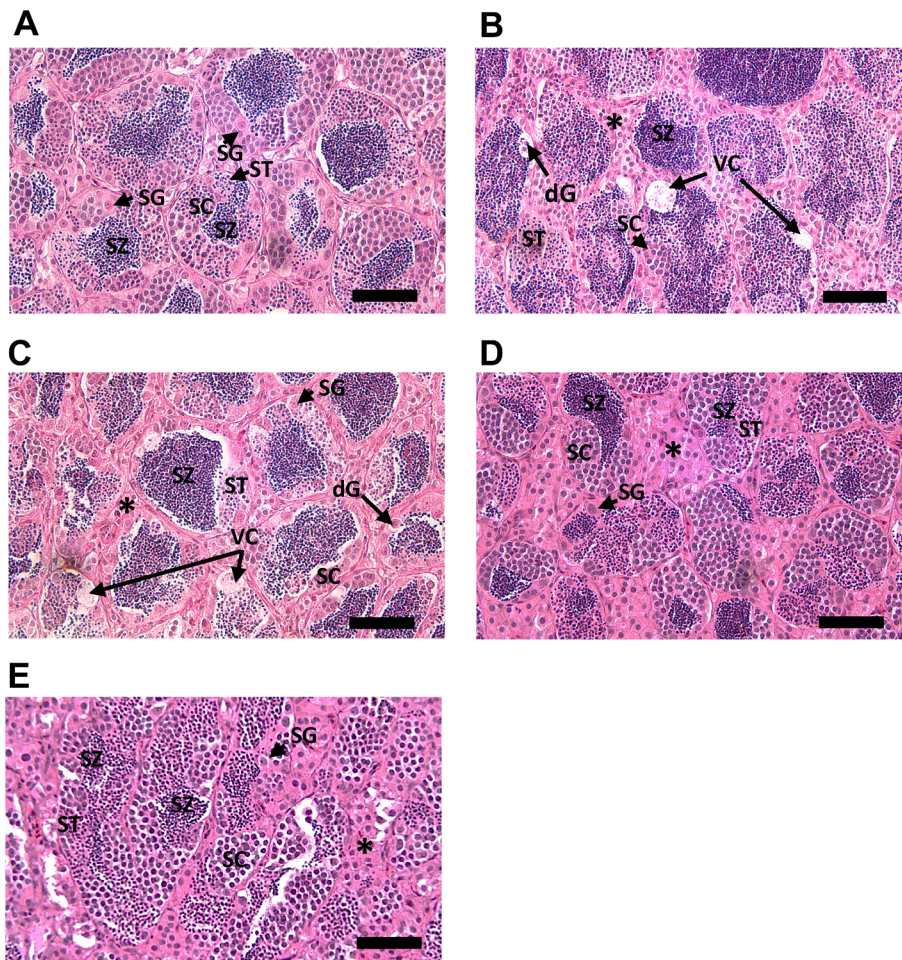


Fig. 2. Representative micrographs of testis from sham-operated control (A), hypophysectomized (Hx) males (B), or Hx males injected with ovine growth hormone (oGH) (C), luteinizing hormone (oLH) (D), or the combination of oLH and oGH (E). Following Hx, Mozambique tilapia received three intraperitoneal injections of oGH alone, oLH alone (5 μg/g body weight), or their combination over 5 days. Sham-operated and Hx fish received saline injections. SG, spermatogonia; SC, spermatocyte; ST, spermatid; SZ, spermatozoa; dG, dead germ cell/s; VC, vacuole; asterisk, enlarged interstitial tissue. Scale bar = 50 μm.

3.2. Effects of hypophysectomy and hormonal treatment on gonadal *ghr2* and *igf*s

In control groups, gonadal *ghr2* levels were higher in females than in males. Ovarian *ghr2* levels were reduced following hypophysectomy and restored by oLH or the combination of oLH and oGH; when administered alone, oGH only partially restored *ghr2* levels. By contrast, hypophysectomy did not affect *ghr2* levels in testis; administration of oLH alone or in combination with oGH reduced *ghr2* levels (Fig. 4A).

In controls, *igf1* levels were higher in the testis compared with ovary. Testicular *igf1* was reduced following hypophysectomy and restored by oGH alone or in combination with oLH. When administered alone, oLH had no effect on testicular *igf1* expression. In females, there were no effects of hypophysectomy or hormones on ovarian *igf1* levels (Fig. 4B). Ovarian *igf2* was reduced in hypophysectomized females but there were no clear effects of oGH or oLH administration. In males, while testicular *igf2* was unaffected by hypophysectomy, *igf2* expression was stimulated by oGH both alone or in combination with oLH (Fig. 4C). Gonadal *igf3* levels were higher in males than in females. Among gonadal *igf*s, *igf3* was markedly reduced following hypophysectomy in both males and females. In testis, *igf3* levels were restored by both oGH and oLH alone or in combination. In females, *igf3* expression was only restored by the combination of both hormones (Fig. 4D). *igf3* expression in both males and females was significantly correlated with GSI ($r^2 = 0.32$ and $r^2 = 0.29$, respectively).

3.3. Effects of hypophysectomy and hormonal treatment on gonadal *ar* and *er*

The expression of gonadal *era* was higher in males than females. Testicular *era* levels were reduced following hypophysectomy; oGH and oLH alone, or in combination, restored or increased *era* levels beyond controls. Ovarian *era* was also reduced following hypophysectomy; neither oGH nor oLH had an effect on *era* (Fig. 5A). Unlike *era*, gonadal *erb* expression was higher in females versus males. The co-administration of oGH and oLH stimulated ovarian *erb* after hypophysectomy. There were no clear responses to hypophysectomy or hormone treatments in the testis (Fig. 5B).

There was no response of gonadal *ara* to any treatments in either males or females (Fig. 5C). On the other hand, *arb* was higher in the testis compared with ovaries. Ovarian *arb* decreased following hypophysectomy and was stimulated by oLH alone or in combination with oGH. The combined treatment of oLH with oGH increased *arb* to levels observed in controls. By contrast, there were no effects of oGH or oLH on *arb* in the testis (Fig. 5D).

3.4. Effects of hypophysectomy and hormonal treatment on muscle *ghr2* and *igf*s

In both sexes, muscle *ghr2* expression decreased following hypophysectomy. The response of *ghr2* to hormonal treatments followed a similar pattern in both sexes; oGH alone or combination with oLH stimulated *ghr2* levels. In males, however, *ghr2* was more responsive to oGH (Fig. 6A). The responses of *igf1* to all treatments were similar between males and females. Hypophysectomy was followed by a reduction

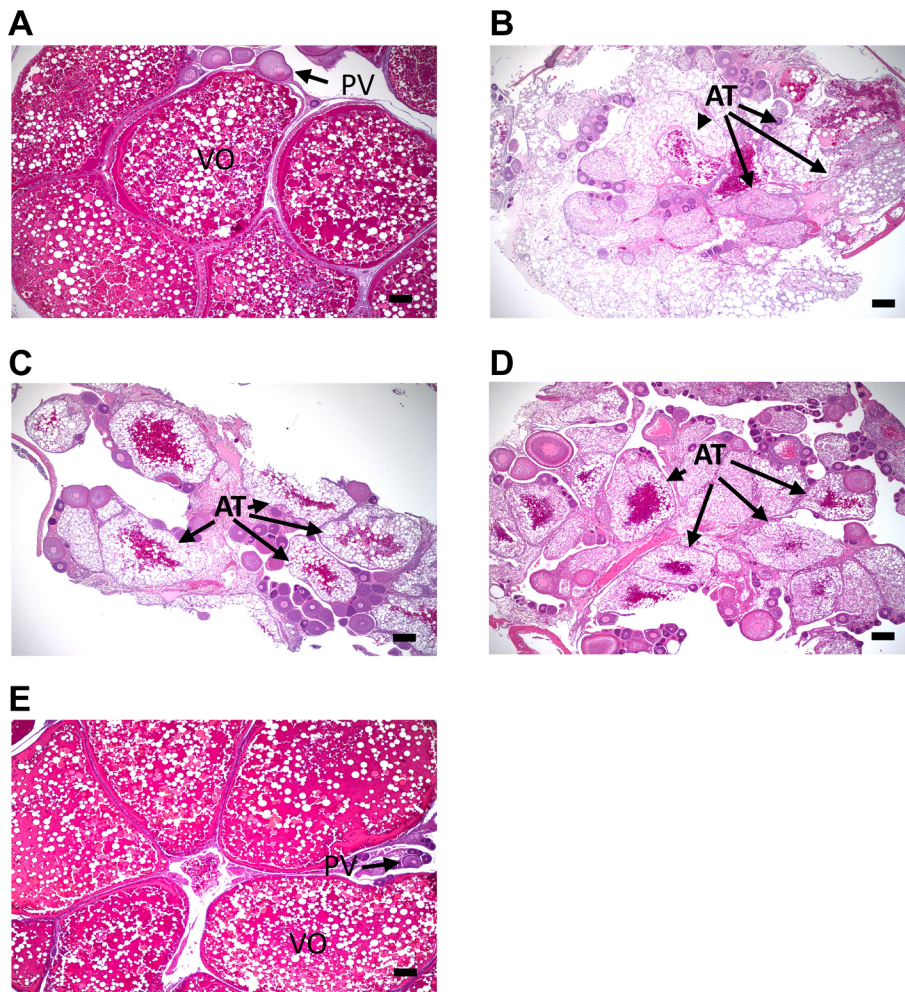


Fig. 3. Representative micrographs of ovary from sham-operated control (A), hypophysectomized (Hx) females (B), or Hx females injected with ovine growth hormone (oGH) (C), luteinizing hormone (oLH) (D), or the combination of oLH and oGH (E). Following Hx, Mozambique tilapia received three intraperitoneal injections of oGH alone, oLH alone (5 µg/g body weight), or their combination over 5 days. Sham-operated and Hx fish received saline injections. PV, previtellogenic follicle; VO, vitellogenic follicle; AT, atretic follicle. Scale bar = 200 µm.

in *igf1* that was restored by oGH alone or combined with oLH. oLH did not affect *igf1* (Fig. 6B). In males, an increase in *igf2* expression occurred following hypophysectomy; *igf2* was further elevated by oGH alone or in combination with oLH. oLH alone, however, slightly diminished *igf2* in hypophysectomized fish. In females, *igf2* did not respond to hypophysectomy but was stimulated by oGH or oLH alone and in combination. Moreover, *igf2* was more responsive to oLH in females than in males (Fig. 6C).

3.5. Effects of hypophysectomy and hormonal treatment on hepatic *ghr2* and *igfs*

In the liver, *ghr2* levels were higher in males than in females. In males, there were no changes in *ghr2* following hypophysectomy; oGH had the tendency to increase hepatic *ghr2* levels while treatment with oLH alone or in combination with oGH diminished *ghr2* when compared with fish treated with oGH alone. In females, *ghr2* expression was reduced following hypophysectomy; oGH and oLH could not fully restore *ghr2* expression to control levels (Fig. 7A). There were no changes in *igf1* expression in either sex following hypophysectomy. The administration of oLH alone or in combination with oGH reduced *igf1* in males. In females, oGH diminished *igf1* expression compared with saline-injected hypophysectomized fish (Fig. 7B). *igf2* expression was markedly increased following hypophysectomy in males; oGH and oLH alone or in combination diminished *igf2* levels. There were no changes in *igf2* expression following hypophysectomy and hormone administration in females (Fig. 7C).

4. Discussion

While the manifestations of sexually dimorphic growth have been addressed in terms of energetic costs or reproductive strategy (Cox, 2006; Fairbairn et al., 2007; Henderson et al., 2003; Rennie et al., 2008; Taylor and DeNardo, 2005) the endocrine mechanisms underlying these phenomena have not been fully resolved. In this study, we identified sex-specific responses to oGH and oLH in hypophysectomized Mozambique tilapia by endocrine/paracrine regulators of growth and reproduction. We found that following hypophysectomy the combined administration of oGH and oLH restored GSI and ovarian morphology. Further examination of ovarian and testicular gene targets revealed sex-specific responses by *ghr2*, *igfs*, and steroid hormone receptors. Sex-specific responses of *ghr2* and *igfs* were also observed in muscle and liver.

The importance of Gh and Lh to gonadal function was underscored by the restoration of GSI following their combined administration to hypophysectomized females. By contrast, hypophysectomy had no effect on male GSI. These results were consistent with histological observations, where hypophysectomy resulted in atresia in ovarian follicles and vacuolization and hypertrophy of interstitial cells in the testis. The reduced GSI in hypophysectomized females and the tendency for GSI to decrease in males could be attributed to the morphological changes and degeneration of gonadal cells in both sexes. Ovarian atresia is a common phenomenon in teleosts under both natural and experimental conditions (Guraya, 1986; Saidapur, 1978). Atretic ovarian follicles are frequently associated with changes in hormone levels (Nagahama, 1983, 1994; Nagahama et al., 1995). In fish, impairment of endocrine function through the reduction of pituitary Fsh, and plasma E₂ and Igf1, is

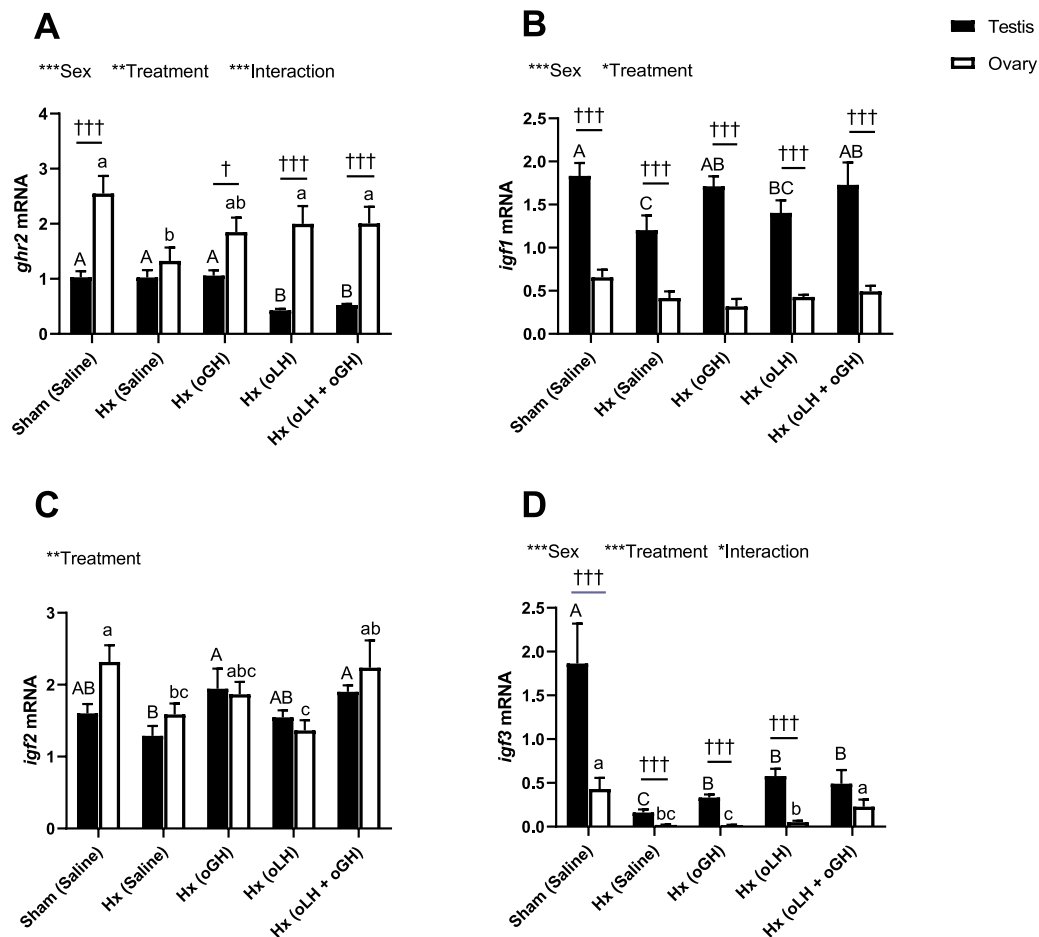


Fig. 4. Effects of hypophysectomy (Hx), and injections of ovine growth hormone (oGH) and ovine luteinizing hormone (oLH) or their combination (oLH + oGH) following Hx on gonadal *ghr2* (A), *igf1* (B), *igf2* (C), and *igf3* (D) mRNA levels in male and female Mozambique tilapia. Values represent means $\pm$ S.E.M. ($n = 4-9$). Data were analyzed by two-way ANOVA followed by Fisher's protected LSD test when significant main or interaction effects were detected (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Means not sharing the same letter are statistically significant at $P < 0.05$. Male and female treatment means not sharing the same uppercase and lowercase letters, respectively, are significantly different. †, †† indicates significant difference between sexes at $P < 0.05$, and $P < 0.001$, respectively.

associated with the induction of follicular atresia (Guraya, 1986; Yamamoto et al., 2011). Consistent with these studies, our results show that both Gh and Lh are necessary for complete ovarian development. In males, like in females, gametogenesis is regulated by gonadotropins, Gth I and Gth II, orthologues of tetrapod Fsh and Lh (Levavi-Sivan et al., 2010; Schulz et al., 2001). Miura et al. (1991a, b) demonstrated the function of gonadotropins through the induction of complete spermatogenesis by human chorionic gonadotropin, an analog of Lh, *in vitro*.

In addition to its role in regulating growth, Gh also modulates reproduction. For example, Gh stimulated spermatogonial proliferation in Japanese eel (*Anguilla japonica*) (Miura et al., 2011). Despite only a tendency for hypophysectomy to reduce GSI and for hormonal administration to restore GSI, hypophysectomy elicited morphological changes in the testis. The more pronounced effect of hypophysectomy, oGH, and oLH in females may stem from the greater requirement for these hormones in ovarian development at later stages and the more energy that is allocated to gonadal development in females compared with males.

Gh plays an important role in the regulation of Ghr2 and Igfs in a variety of organs including gonads (Breves et al., 2014; Hull and Harvey, 2014; Petro-Sakuma et al., 2020; Pierce et al., 2011, 2012). Furthermore, Lh also regulates circulating and gonadal Igf1 (Bradley et al., 2006; Chandrashekar and Bartke, 2003). Our results indicate that oGH and oLH modulated gonadal *ghr2* and *igf* levels in both males and females. The Gh/Igf1 axis is not only central to vertebrate growth, but is

also implicated in the control of reproduction (Duan, 1997; 1998). Inasmuch as sexual differentiation requires germ cell proliferation and gonadal tissue growth (Nakamura et al., 1998), growth factors are critical regulators of reproductive processes. In particular, Igfs exert actions on gonad development and differentiation (Duan et al., 1997; Lu et al., 2005; Wood et al., 2005).

Furthermore, gonadal steroid hormones modulate the Gh/Igf1 axis, a process that is important for the initiation of puberty and sexually dimorphic growth (Leung et al., 2004; Meinhardt and Ho, 2006). So far, there are limited studies comparing the expression of Gh/Igf axis genes between testis and ovary in fish. In Mozambique tilapia and fathead minnow (*Pimephales promelas*), *ghr2* expression is greater in ovary versus testis (Davis et al., 2008; Filby ad Tyler, 2007). These previous findings coincide with our results that show higher *ghr2* expression in control ovaries compared with testes. There were also differences in the sensitivity of gonadal *ghr2* to both hypophysectomy and hormonal treatments between sexes. In Nile tilapia (*O. niloticus*), *ghr2* transcripts were highest in the ovary during sexual maturation, while in the testis, *ghr2* expression was highest during sexual recrudescence and declined during maturation and regression (Ma et al., 2007). These findings concur with our results and explain the lower gonadal *ghr2* and its lack of response to oGH in males.

In contrast to *ghr2*, higher levels of *igf1* transcripts were found in the testis compared with the ovary. Furthermore, although Igf1 is implicated in both male and female gametogenesis, not much is known about

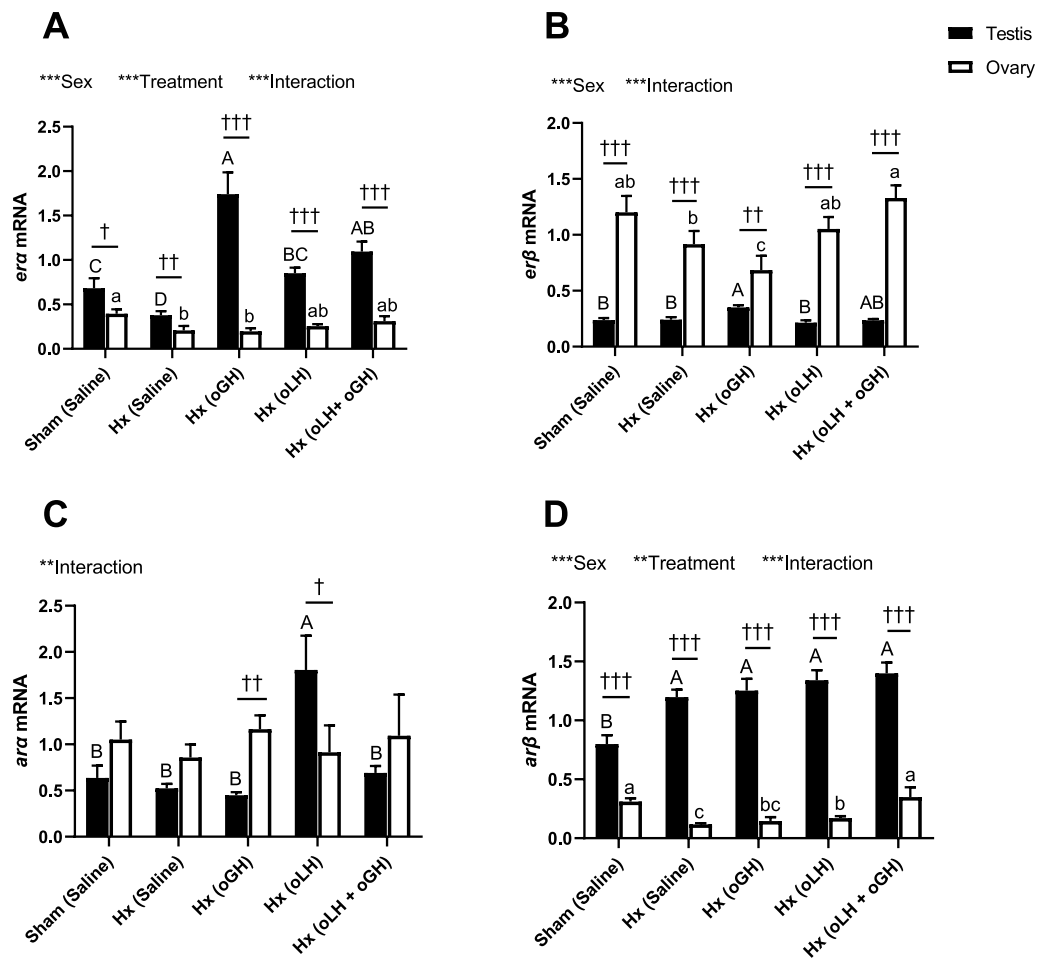


Fig. 5. Effects of hypophysectomy (Hx), and injections of ovine growth hormone (oGH) and ovine luteinizing hormone (oLH) or their combination (oLH + oGH) following Hx on gonadal *era* (A), *erβ* (B), *ara* (C), and *arβ* (D) mRNA levels in male and female Mozambique tilapia. Values represent means $\pm$ S.E.M. ($n = 4-9$). Data were analyzed by two-way ANOVA followed by Fisher's protected LSD test when significant main or interaction effects were detected ($**P < 0.01$, $***P < 0.001$). Means not sharing the same letter are statistically significant at $P < 0.05$. Male and female treatment means not sharing the same uppercase and lowercase letters, respectively, are significantly different. †, ††, ††† indicates significant difference between sexes at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

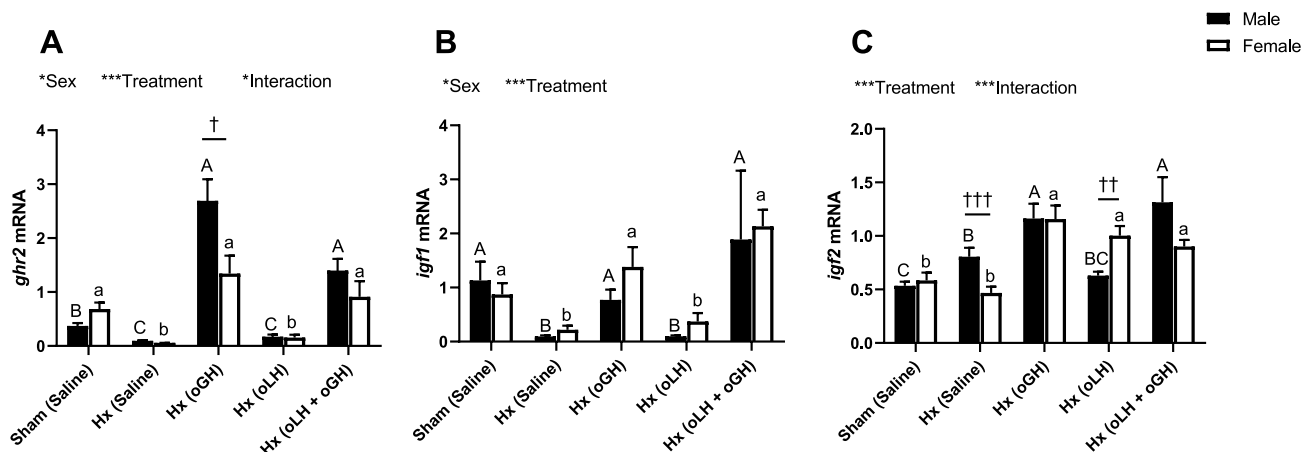


Fig. 6. Effects of hypophysectomy (Hx), and injections of ovine growth hormone (oGH) and ovine luteinizing hormone (oLH) or their combination (oLH + oGH) following Hx on muscle *ghr2* (A), *igf1* (B), and *igf2* (C) mRNA levels in male and female Mozambique tilapia. Values represent means $\pm$ S.E.M. ($n = 4-9$). Data were analyzed by two-way ANOVA followed by Fisher's protected LSD test when significant main or interaction effects were detected ($*P < 0.05$, $***P < 0.001$). Means not sharing the same letter are statistically significant at $P < 0.05$. Male and female treatment means not sharing the same uppercase and lowercase letters, respectively, are significantly different. †, ††, ††† indicates significant difference between sexes at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

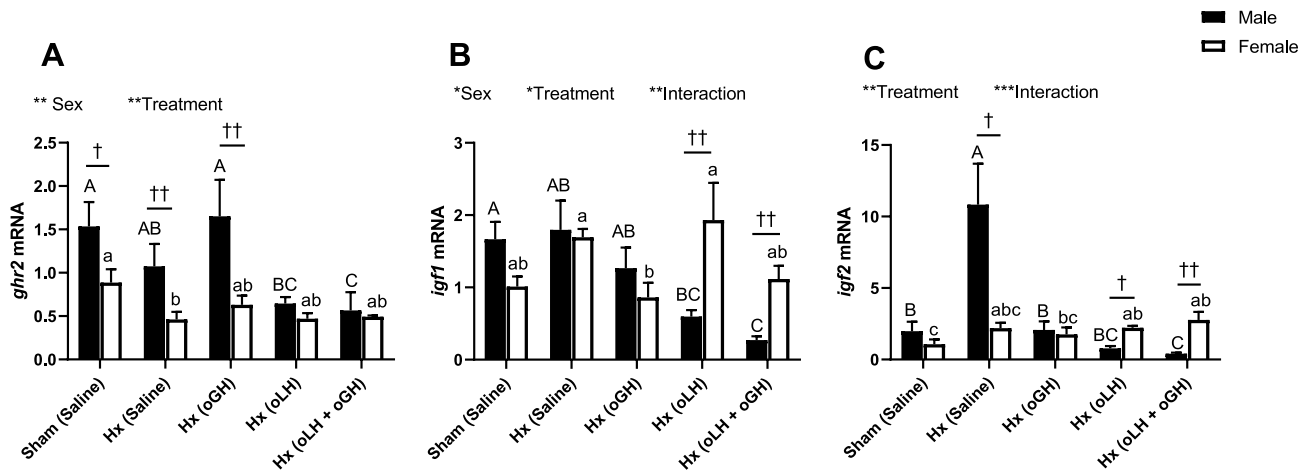


Fig. 7. Effects of hypophysectomy (Hx), and injections of ovine growth hormone (oGH) and ovine luteinizing hormone (oLH) or their combination (oLH + oGH) following Hx on hepatic *ghr2* (A), *igf1* (B), and *igf2* (C) mRNA levels in male and female Mozambique tilapia. Values represent means $\pm$ S.E.M. ($n = 4-9$). Data were analyzed by two-way ANOVA followed by Fisher's protected LSD test when significant main or interaction effects were detected (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Means not sharing the same letter are statistically significant at $P < 0.05$. Male and female treatment means not sharing the same uppercase and lowercase letters, respectively, are significantly different. †, †† indicates significant difference between sexes at $P < 0.05$, and $P < 0.01$, respectively.

the differences in the function of Igf1 in gonadal development between sexes. Igfs are responsive to Gh and direct follicular growth, lipid uptake, and steroid biosynthesis (Campbell et al., 2006; Kagawa et al., 2003; Lokman et al., 2007; Maestro et al., 1997; Paul et al., 2010; Weber and Sullivan, 2001). Igf1 is necessary for the action of the main teleost androgen, 11-ketotestosterone (11-KT), to initiate spermatogenesis. (Miura and Miura, 2001). Moreover, both systemic Igf1 and granulosa cell-derived Igf1 can affect ovarian function (Baroiller et al., 2014; Berishvili et al., 2006; Kagawa et al., 1995; Perrot et al., 2000; Reinecke, 2010). Thus, although both sexes might require Igf1 for normal gonadal development, differences in expression levels could be attributed to the extent to which local Igf1 is required for steroid biosynthesis at each stage of gonadal maturation.

Unlike Igf1, relatively little is known about the physiological roles of Igf2 in fish reproduction. While Igf2 is capable of inducing germinal vesicle breakdown in red sea bream (*Pagrus major*), the effect of Igf1 is more pronounced (Kagawa et al., 1994). Interestingly, among gonadal igfs, the effects of hypophysectomy, oGH, and oLH were most pronounced on *igf3*, especially in males. Moreover, *igf3* responses to hypophysectomy and hormonal treatment in both males and females closely correlated with GSI. Li et al. (2012) reported that gonadal *igf3* expression was higher in male versus female Nile tilapia from 50 to 70 days after hatching, and varied with the age of individuals. Berishvili et al. (2010) also found higher *igf3* in the testis versus the ovary in adult Nile tilapia. The gonad-specific expression of Igf3 and its differing responses to oGH and oLH from Igf1 and Igf2, suggests distinct roles for Igf3 in gonad development (Wang et al., 2008). Our findings, therefore, are consistent with previous studies supporting sex-dependent patterns of *igf3* expression.

Er and Ar play major roles in mediating the responsiveness of target tissues to estrogens and androgens (Park et al., 2007). The neuroendocrine control of reproductive and metabolic homeostasis is affected by changes in receptor expression and/or function (Goksoyr, 2006; Tabb and Blumberg, 2006; Vijayan et al., 2005). Here, we observed sex- and subtype-specific modulation of gonadal steroid hormone receptors in male and female tilapia.

The steroid hormone E_2 functions in both males and females to regulate reproductive processes (Esterhuysen et al., 2010) by binding specific receptors, $Er\alpha$ and $Er\beta$ (Gross and Yee, 2002; Park et al., 2007). *era* levels were higher in the testis than in the ovary, while *er\beta* levels were higher in the ovary than in the testis. Previous studies indicate that

patterns of *er* subtype expression vary according to age, sex, reproductive stage, and species (Chakraborty et al., 2011; Davis et al., 2008; Nagasawa et al., 2014). Transcripts of testicular and ovarian *era* were both diminished following hypophysectomy. While all hormonal treatments strongly upregulated testicular *era* levels, only oLH or its co-administration with oGH, stimulated ovarian *era* levels. In contrast, *er\beta* seems to be less susceptible to the effects of hypophysectomy, oGH, and oLH. Hence, our data indicate both sex-dependent and receptor subtype specific responses by *ers* to hypophysectomy, oGH, and oLH. In a breeding stock of Mozambique tilapia, no sex-specific expression patterns of gonadal *era* and *er\beta* were detected (Esterhuysen et al., 2010). In another study, however, both *ers* were higher in ovaries of sexually mature fish (Davis et al., 2008). In all-male or all-female Nile tilapia fry produced by artificial fertilization, there were no differences in *era* levels between ovary and testis until 70 days after hatch when *era* transcripts were higher in males (Jiri et al., 2008). These differences between studies may indicate a diversity of E_2 signaling requirements of germ cells among tilapia species. The differential responsiveness of *era* and *er\beta* to hypophysectomy, oGH and oLH, as well as the higher level of testicular *era*, indicates an important role for *era* in spermatogenesis. E_2 induced spermatogonial stem cell renewal and spermatogonial proliferation in Japanese eel and threespot wrasse (*Halichoeres trimaculatus*) (Kobayashi et al., 2011; Miura et al., 1999, 2003), thus as mediators of E_2 effects, *Ers* play a role in normal testicular development.

As observed with *ers*, *ars* also exhibited sex- and sub-type dependent patterns of expression, where *ar\beta* was higher in testis than in ovary. In males, 11-KT appears to be the major sex steroid that influences male reproduction (Borg, 1994; Kobayashi et al., 1999; Nagahama et al., 1994; cf Turcu et al., 2018). Moreover, 11-KT can induce all stages of spermatogenesis in *A. japonica* (Miura et al., 1991a, b). In *Astatotilapia burtoni*, *Ar\beta2* is preferentially activated by 11-KT compared with testosterone (Olsson et al., 2005), which is consistent with the elevated levels of *ar\beta* found in the testis of *O. mossambicus*. Although *ar\beta* levels in the ovary were much lower than that of testis, *ar\beta* respond to hypophysectomy; and the co-administration of oLH and oGH strongly induced ovarian *ar\beta* levels in hypophysectomized females. Androgens have been implicated in female sexual development of several teleost species (Lokman et al., 1998). For example, in cod (*Gadus morhua*), androgen treatment promoted primary follicle development (Kortner et al., 2008, 2009), while in coho salmon (*Oncorhynchus kisutch*), 11-KT induced follicle growth and the appearance of cortical alveoli in the cytoplasm

(Forsgren and Young, 2012). Thus, the stimulation of *arβ* by oLH and oGH in female tilapia supports pituitary control of ovarian androgenic responsiveness. The importance of *arβ* signaling during follicle development is further supported by observations in which levels of this isoform, but not *ara*, varied with follicular stages (García-López et al., 2011).

In muscle, *Ghr2* and *Igfs* are stimulated by Gh, which in turn induce a variety of growth-promoting effects (Butler and Le Roith, 2001; Duan et al., 2010; Le Roith, 2003; Mommsen, 2001; Wood et al., 2005). Consistent with these patterns, *ghr2* and *igf1* transcript levels were diminished following hypophysectomy and subsequently stimulated by oGH. The magnitude of muscle *ghr2* induction, however, was greater in males. These results not only reinforce the essential role of Gh in regulating its receptor and *Igf1*, but also imply that the sensitivity of this signaling pathway varies between male and females. A greater sensitivity of the Gh/*Igf* axis in males is consistent with males outgrowing females in this species. Specifically, our previous studies in Mozambique tilapia showed that somatic growth is positively correlated with pituitary *gh* and muscle *ghr2* expression (Moorman et al., 2016) and that pituitary *gh* expression is greater in males than in females (Seale et al., 2020). Thus, the current findings are consistent with sex-specific modulation of *ghr2* in a fashion that supports enhanced somatic growth in males. Both *Igf1* and *−2* have direct effects on muscle growth in fish (Fuentes et al., 2013; Garikipati and Rodgers, 2012a, 2012b; Montserrat et al., 2012;). Specific aspects underlying the control of muscle growth, however, may be differentially controlled by the *Igf* isoforms. *Igf2*, for example, had stronger effects than *Igf1* on myocyte proliferation in sea bream (*Sparus aurata*) (Rius-Francino et al., 2011). Because *igf2* was relatively unresponsive to pituitary control, the underlying differences between *igf1* and *−2* regulation are not clear.

Consistent with a previous experiment in Mozambique tilapia, our results showed higher hepatic *ghr2* expression in males compared with females (Davis et al., 2008). Other than the tendency of hypophysectomy to decrease *ghr2*, and a tendency of oGH to restore *ghr2*, there were no marked effects of hypophysectomy on *ghr2* and *igf1* expression. Previous studies showed that oGH stimulated plasma *Igf1* and hepatic *ghr2* levels, and restored *ghr2* levels in anterior and middle intestine in hypophysectomized fish (Breves et al., 2014; Petro-Sakuma et al., 2020). These previous experiments, however, employed only a single intraperitoneal injection of oGH, while in the present study, three intraperitoneal injections of oGH were administered. There was no effect of sham operation on somatotrophic axis genes, indicating that handling stress may not be directly linked to the lack of a response by *igf1* to oGH treatment. While there was an increase in hepatic *ghr2*, this lack of response of hepatic *igf1* to oGH was also observed by Breves et al. (2014) in hypophysectomized tilapia. In contrast, Pierce et al. (2011) found that oGH injection increased hepatic *igf1* levels. This experiment, however, did not use hypophysectomized fish. Hence, differences in responses could be attributed to the differences in the experimental designs and/or the frequency of injections.

In males, hypophysectomy resulted in increased *igf2*; oGH and oLH suppressed the hypophysectomy-induced rise in hepatic *igf2*. These effects, however, were not seen in females. Hypophysectomy-induced stimulation of *igf2* and its strong suppression by oGH could be explained by a feedback effect of hepatic *igf2* brought about by the loss of Gh. The removal of Gh may trigger the Gh/*Igf* axis to compensate by elevating *igf2*.

In summary, the current study identified regulators of growth and reproduction in tilapia that exhibit sex-specific regulation by oGH and oLH. The high sensitivity of ovarian transcripts to a lack of Lh and Gh compared with males implies important synergistic functions of these hormones on ovarian development. The distinct responses of *igf3* when compared with other *Igf* isoforms indicate that its translated product plays key roles in gonadal development. The higher expression of hepatic *ghr2* and the strong response of *ghr2* to oGH in muscle in males suggests enhanced Gh signaling in support of somatic and systemic

growth promotion, whereas the higher *ghr2* levels in ovary compared to testis indicate a shift towards gonadal development in females. Taken collectively, our data provide new insight into how hormones underlie sexual dimorphism in tilapia by resolving the interactions between somatotrophic and gonadotrophic endocrine axes.

CRediT authorship contribution statement

Fritzie T. Celino-Brady: Conceptualization, Methodology, Investigation, Formal analysis, Validation, Writing – original draft, Writing – review & editing, Visualization. **Jason P. Breves:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing, Funding acquisition. **Andre P. Seale:** Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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

The authors thank Mr. Ryan Chang for laboratory assistance during the course of the study and Ms. Miyoko Bellinger for histological preparation.

Funding

This work was supported in part by grants from the National Oceanic and Atmospheric Administration (NA18OAR4170347), National Institute of Food and Agriculture (HAW02051-H), National Institute of Diabetes and Digestive and Kidney Diseases (R21DK111775) to A.P.S., National Science Foundation (IOS-1755016) to A.P.S. and (IOS-1755131) to J.P.B., and National Institute on Minority Health and Health Disparities (U54MD007601) to Histology and Imaging Core Facility, John A. Burns School of Medicine, University of Hawaii.

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