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Sexually Dimorphic Expression of Gonadotropin Subunits in the Pituitary of Protogynous Honeycomb Grouper (*Epinephelus merra*): Evidence That Follicle-Stimulating Hormone (FSH) Induces Gonadal Sex Change[1]

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ABSTRACT

Recent studies have suggested that the hypothalamic-pituitary-gonadal axis is involved in gonadal sex change in sex-changing teleosts. However, its underlying mechanism remains largely unknown. In this study, we focused on the distinct roles of two gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in the protogynous hermaphrodite teleost, honeycomb grouper (*Epinephelus merra*). First, we investigated the expression pattern of mRNAs for GTH subunits (*cga*, *fshb*, and *lhb*) in the pituitaries from fish at the different sexual phases. Real-time RT-PCR analyses showed that *fhsb* mRNA levels in the female pituitary were low. However, *fshb* transcripts increased dramatically in association with testis development. In contrast, levels of *cga* and *lhb* mRNAs did not significantly vary during sex change. In addition, immunohistochemical observations of Fshb- and Lhb-producing cells in the pituitary, through the use of specific antibodies for detections of teleost GTH subunits, were consistent with sexually dimorphic expression of Fshb. In order to identify the role of GTH in gonad of honeycomb grouper, we treated females with bovine FSH (50 or 500 ng/fish) or LH (500 ng/fish) in vivo. After 3 wk, FSH treatments induced female-to-male sex change and up-regulated endogenous androgen levels and *fshb* transcripts, whereas LH treatment had no effect on sex change. These results suggest that FSH may trigger the female-to-male sex change in honeycomb grouper.

androgen, estrogen, follicle-stimulating hormone, gonadotropin, grouper, luteinizing hormone, ovary, pituitary hormones, sex change, teleost

INTRODUCTION

Sex in most animals is determined at an early developmental stage, and is fixed throughout their life span [1]. By contrast, many marine teleosts exhibit sequential hermaphroditism, wherein an individual changes from one sex to the other in adulthood [1–3]. During this process, the gonad changes dramatically from ovary to testis (protogynous), or vice versa (protandrous) [1–3]. Numerous endocrine studies on gonadal steroidogenesis have been performed to reveal the physiological mechanisms of sex change. In several protogynous species, plasma estrogen (estradiol-17β [E2]) levels are high in the female phase, and a rapid decrease of E2 was observed in association with sex change [4–6]. Additionally, androgen treatment of these species during the female phase results in sex change [5, 7, 8]. In contrast, administration of E2 induces male-to-female sex change in the protandrous black porgy [9, 10] and the initial-phase male of protogynous wrasse [11, 12]. Taken together, these results suggest that regulation of the appropriate balance of androgen to estrogen biosynthesis is critical to the process of gonadal sex change. However, the upstream mechanisms for the regulation of gonadal steroidogenesis during sex change are largely unknown.

In teleosts, as in other vertebrates, gonadal steroidogenesis is largely controlled by pituitary-produced gonadotropins (GTHs), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) [13]. These GTHs contain a common glycoprotein hormone α subunit (Cga) that forms a heterodimer with unique β subunits (Fshb and Lhb) (reviewed by Bousfield et al. [14] and Pierce and Parsons [15]). In well-studied salmonids, FSH plays a significant role in puberty and gametogenesis, whereas LH is primarily involved in final maturation of the gametes in both sexes [16–18]. However, variations in the expression profiles and potential roles of GTHs were reported in other teleost species [19–22]. In protogynous wrasse, sexual dimorphic expression patterns of *fshb* and *lhb* transcripts were observed during the spawning season [23]. In protandrous black porgy, plasma Lh levels were higher in male than sex-changing fish [24]. In addition, treatment with exogenous human chorionic GTH or LH induced sex change in the protogynous bluehead wrasse [25] and rice-field eel [26, 27]. These studies indicate that GTHs participate in regulating sex-changing processes. However, information of GTH expression pattern during the sex-changing process is currently absent. Therefore, the detailed biological functions of GTH during sex change are not clear.

In this study, we used the honeycomb grouper (*Epinephelus merra*) as a model. This species is a protogynous hermaphrodite. All individuals mature initially as females. When the females reach a certain age or body size, they change to males [28]. In this species, artificial sex changes have been intensively studied in our laboratory. Either suppression of estrogen biosynthesis with aromatase inhibitors or elevation in androgen levels by treatment with androgen induced sex change in honeycomb grouper [29–31] and other grouper species [32–34]. Since the different sexual-phase individuals of

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TABLE 1. Oligonucleotide primers used in this study.

Target gene	Purpose	Oligonucleotide sequence (5′ to 3′)
cga	RT-PCR	GGMTGTGAGGARTGYAMACTSAA
		GCWACGCAGCATGTRGCTTCAGA
	3′RACE	AAGAACAGTGTTTTCTCAAGGGATCGTC
	5′RACE	ATGTTCTTCGGGATCGTCATTGTCTT
	Real-time PCR	AAACATTGGCTGCGAGGAGT
		CGGGATCGTCATTGTCTTCA
fshb	RT-PCR	TGCAGYTGGTYSTCATGG
		CWYCTCRTAGGACCASTC
	3′RACE	CCACACCGAGTACATCTACACCACCATA
	5′RACE	ATTGCAGACTTTCTGTTCAGCCCAGT
	Real-time PCR	TGCCACTCCGACTGTCATCT
		TCTGTTCAGCCCAGTCATCG
ihb	RT-PCR	ATCTGCAGYGGYCACTGC
		ACAGTCRGAMGTGTCCAT
	3′RACE	GAAGGACCCTGTCATCAAGATACCATTC
	5′RACE	GAGGACAGTCAGGAAGCTCAAATGTCTT
	Real-time PCR	GGAGAAGGAAGGCTGTCCAA
		TGACAGGGTCCTTCGTGATG
ef1a	Real-time PCR	TTGCCCTCTGGAAGTTTGAG
		ACACCAGCAGCAACAATGAG

honeycomb grouper are readily captured from the wild, honeycomb grouper provides an excellent animal model to elucidate the mechanism of sex change.

The aim of this study was to elucidate the involvement of GTH during the process of sex change in honeycomb grouper. As a first step, we examined the pattern of gene expression for GTH subunits in the pituitaries of different sexual phases, including sex-changing fish, using real-time PCR. Additionally, localization of Fshb- and Lhb-producing cells in the pituitaries of males, females, and sex-changing fishes was performed by immunohistochemistry using antisera that recognize Fsh and Lh from several teleost species. Finally, the effects of purified bovine FSH and LH on the nonbreeding female gonad in vivo were examined. The results generated in this study contribute to the understanding of biological functions of GTH on the process of sex change.

MATERIALS AND METHODS

Animals and Histological Observation

Breeding (May 4, 2007) or nonbreeding (February 9, 2007 and November 5, 2009) adult honeycomb groupers were purchased from fishermen of Nakijin, Okinawa, Japan, and then maintained in 500-L tanks with flowthrough seawater at the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Sesoko, Japan. All fish were anesthetized with 2-phenoxyethanol (Wako Chemicals, Osaka, Japan) before sampling. After measurements of total length and body weight, the fish were euthanized by decapitation. Pituitaries of each fish were placed in a tube with RNAlater reagent (Ambion, Austin, TX) and stored at −30°C until RNA extraction.

A piece of gonad from each individual was fixed overnight in Bouin solution, embedded in paraffin, serially sectioned at 7 μm, and stained with hematoxylin and eosin. Based on a previous study [28], we classified the sexual phases of individual fish into six phases, as follows: 1) breeding female—mainly full-grown oocyte and some oogonia were present in the gonad; 2) nonbreeding female—previtellogenic oocytes were present in the gonad; 3) early transitional (ET)—mixture of male germ cells and primary oocytes were present in the gonad; 4) late transitional (LT)—numerous male germ cells with a few primary oocytes were present in the gonad; 5) nonbreeding male—mainly spermatids and some spermatogonia were present in the gonad; and 6) breeding male—mainly spermatozoa were present in the gonad.

All animal handling and experimentation was conducted in accordance with the Guide for Care and Use of Laboratory Animals, and was approved by the Institutional Committee of Laboratory Animal Experimentation, University of the Ryukyus.

Cloning of Grouper GTH Subunits cDNA

Total RNA extracted from pituitary of adult males was used as a template for cloning (RNeasy mini Kit; Qiagen). First, cDNA fragments of *cga*, *fshb*, and *lhb* were isolated by RT-PCR with degenerate primers. Then, full-length cDNAs of GTH subunits were identified by a SMART RACE cDNA amplification kit (Clontech). Primers used in cloning are listed in Table 1. Sequencing was done with BigDye (Version 3.1). In all cases, at least three independent clones were sequenced.

Transcripts of grouper GTH subunits in pituitary were determined using real-time RT-PCR. Described briefly, total RNA was isolated from individual pituitaries stored in RNAlater using the RNAeasy mini kit with the RNase-free DNase kit (Qiagen), according to the manufacturer's instructions. Samples were reverse transcribed from 200 ng of total RNA in a 20-μl volume using random hexamer primers and OmniScript reverse transcriptase (Qiagen). An aliquot of 2 μl of the above diluted cDNA sample was used for a 22-μl PCR reaction with SYBER Premix Ex Taq (Takara Bio Inc., Shiga, Japan) on the ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primer sets for each gene were designed by PrimerExpress Software. Quantitative PCR assay was done with triplicate samples. Copy number in unknown samples was determined by Ct value to the each gene standards. Standard copy number of each gene was estimated based on molecular weight and absorbance of plasmid ligated with target genes. Each transcript level was then normalized on the basis of the expression of the expression values of the constitutive elongation factor 1α (*ef1a*). All the post-PCR samples were analyzed in 3% agarose gels and confirmed the expected single band. Primers used in real-time RT-PCR are listed in Table 1.

Localization of Fshb and Lhb Cells in Pituitaries of Males and Females

For immunohistological staining of pituitary gonadotropes, we used rabbit antiserum developed against mummichog (*Fundulus heteroclitus*) Fshb (anti-Fh Fshb 50–60) and Lhb (anti-Fh Lhb91–106). The antigens for generating these antisera were synthetic peptides (anti-Fh Fshb 50–60, DWTYEVKHFQG; anti-Fh Lhb 91–106, AMATSDCTFESLQPDF) have been previously shown to specifically stain Fsh- and Lh-producing cells from many species of teleost fish [35].

For immunohistochemistry, pituitaries were removed from nonbreeding female (n = 3), ET (n = 2), LT (n = 2), and nonbreeding male (n = 3) fish. After Bouin solution fix, samples were embedded in paraffin, and sagittally sectioned at 7 μm. After removal of paraffin and hydration, the sections of pituitary were washed in 0.1 M PBS (pH 7.2), then soaked in methanol containing 0.3% H_2O_2 for 15 min, and washed with PBS. After blocking nonspecific binding with 10% normal goat serum, the sections were incubated with the primary antibody solution diluted to 1:1000 (Fh Fshb 50–60) or 1:2000 (Fh Lhb 91–106) overnight at 4°C, and washed with PBS. Sections were then incubated with secondary antibody using Histofine SAB-PO (multi) kit (Nichirei Corp., Tokyo, Japan). After washing with PBS, peroxidase activity was visualized

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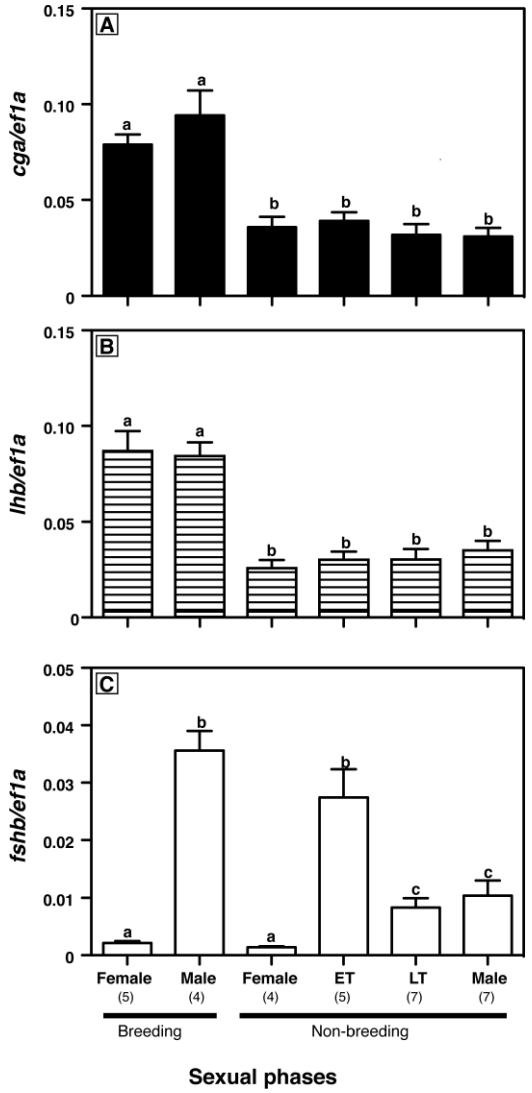


FIG. 1. Changes of GTH subunit transcripts (**A**, *cga*; **B**, *lhb*; **C**, *fshb*) in pituitaries of the different sexual phases in honeycomb grouper, as determined by quantitative real-time RT-PCR. Data are shown as means ± SEM. The parenthetic numbers in the figure indicate total number of fish sampled. Data points not sharing a letter (a, b, c) are significantly different by Tukey-Kramer multiple comparison test. ET, early transitional; LT, late transitional.

with 3,3′-diaminobenzidine (DAB) in Tris-HCl (pH 7.6) buffer containing H_2O_2. Sections were lightly counterstained with hematoxylin.

Hormone Treatment

The sexual phase of experimental fish was determined using a nonlethal gonadal biopsy [36] before treatment with hormones. All gonadal biopsies were examined histologically, as described above, and only nonbreeding female-phase fishes were selected and used for hormone treatment experiment.

Purified bovine FSH and LH (Cosmo Bio Co., Ltd., Tokyo, Japan) were directly dissolved in molten cocoa butter. The purity of the FSH and LH were each >95%. Bovine hormones were used in this study, because sufficient quantities of fish FSH and LH were unavailable at the time of the study.

The fish were divided into four treatment groups: 1) control (n = 5, molten cocoa butter without hormone); 2) FSH-low (n = 8; 50 ng bovine FSH/fish); 3) FSH-high (n = 8; 500 ng bovine FSH/fish); and 4) LH (n = 7; 500 ng bovine LH/fish). After females recovered from the biopsy, fish were anesthetized in 0.05% phenoxyethanol and given intraperitoneal injections of the control or hormone-containing cocoa butter. At 3 wk after the hormone treatment, fish were sampled after being euthanized by decapitation. Gonads were fixed in Bouin and examined histologically, as described above. Blood was collected via a heparinized syringe, and separated plasma was stored frozen at −30°C until analysis of steroid hormone levels. Plasma E2 and 11-keto-testosterone (11KT) concentrations were measured by ELISA, according to procedures described in a previous study [37]. Pituitaries were stored in RNAlater regent, and the *fshb* and *lhb* subunit transcripts were measured using real-time RT-PCR, as described above.

RESULTS

Isolation of GTH Subunits of Honeycomb Grouper

The nucleotide sequences of *cga*, *fshb*, and *lhb* were 675, 535, and 600 base pairs long, respectively. GenBank accession numbers for cDNA sequences are as follows: *gypa*, AB525769; *fshb*, AB525770; and *lhb*, AB525771. The amino acid sequence of these subunits all showed high similarities with those of other grouper species [38, 39].

Furthermore, the amino acid sequences of the grouper subunits showed high similarity to those of the mummichog synthetic peptides that were used to generate antisera for immunohistochemical identification of fish Fsh- and Lh-producing cells. The grouper Fshb fragment (DWTYEV-KHIQG) and Lhb fragment (AMDTSDCTFESLQPNF) were 90.9% and 87.5% identical to those of the mummichog, respectively.

Levels of Transcripts for Pituitary GTH Subunits in Different Sexual Phases

In the present study, the relative expression of the mRNA of GTH subunits in the pituitaries of individuals at various sexual phases was measured by real-time RT-PCR. The levels of *cga* and *lhb* mRNA were high in the breeding season. However, there were no significant differences in these levels among different sexual phases during the nonbreeding season (Fig. 1, A and B). In contrast, the expression pattern of *fshb* transcripts showed a marked sexual dimorphism (Fig. 1C). The *fshb* subunit transcripts were low in the breeding and nonbreeding female phases, and increased 20- to 60-fold during the female-to-male sex change, especially in ET stage (Fig. 1C).

Localization of Fshb and Lhb Immunoreactive Cells in the Pituitaries of All Sexual Phases

To determine if there was sexually dimorphic expression of Fshb protein in the grouper pituitary gland, we examined Fshb and Lhb immunoreactive (ir) cells in pituitaries using antisera against conserved epitopes of the fish Fshb and Lhb subunits

TABLE 2. Effects of bovine FSH and LH on sex change of protogynous honeycomb grouper.

Group	n	Sex-changing fish	Female	TL (cm)	GSI*
Control	5	0	5	16.02 ± 0.43	0.44 ± 0.09
FSH-low (50 ng)	8	6	2	16.21 ± 0.35	0.43 ± 0.07
FSH-high (500 ng)	8	8	0	15.11 ± 0.25	0.45 ± 0.05
LH (500 ng)	7	0	7	15.46 ± 0.33	0.57 ± 0.13

* GSI, Gonadosomatic index (gonad weight/body weight × 100).

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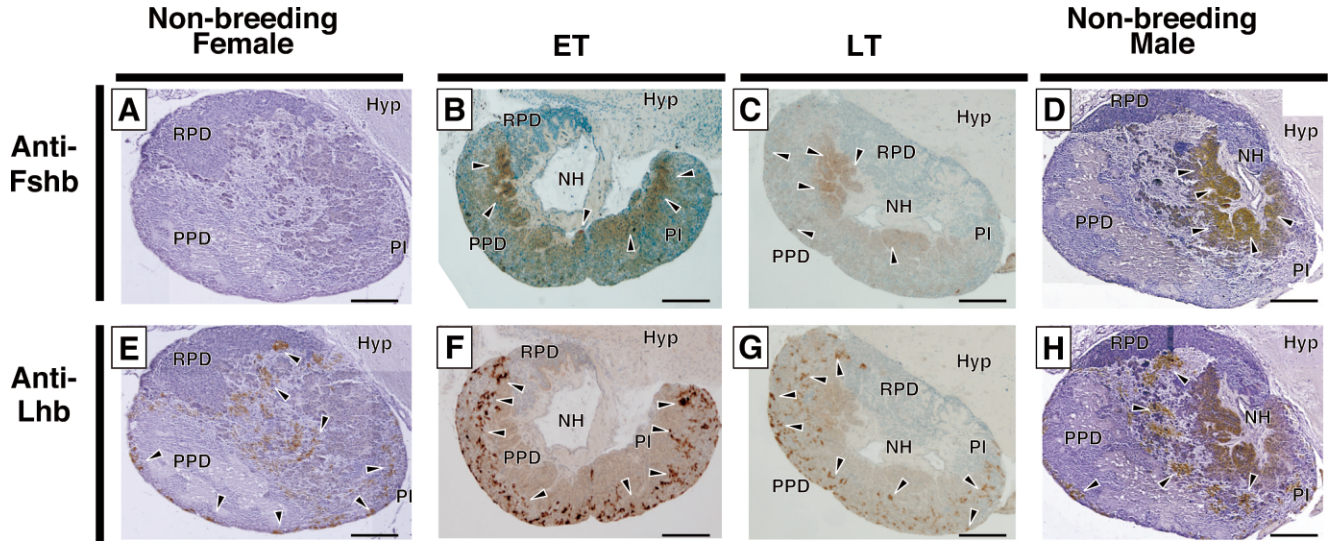


FIG. 2. Representative sagittal sections of pituitary glands from nonbreeding female (**A** and **E**), ET-phase sex-changing (**B** and **F**), LT-phase sex-changing (**C** and **G**), and nonbreeding male (**C** and **D**) honeycomb grouper. Immunostained with anti-Fh Fshb 50–60 (**A**–**D**) and anti-Fh Lhb 91–106 (**E**–**H**). Counterstained with hematoxylin. Arrows indicate positive signals; bar = 200 μm. Hyp, hypothalamus; NH, neurohypophysis; PI, pars intermedia; RPD, rostral pars distalis.

(Fig. 2). The Fshb-ir cells were not detected in the nonbreeding female pituitary (Fig. 2A). In contrast, appreciable Fshb-ir cells were seen around the neurohypophysis of the sex-changing fish and male pituitary (Fig. 2, B–D). In all sexual phases, Lhb-ir cells were observed in the proximal pars distalis (PPD) and pars intermedia, but not in rostral pars distalis (RPD) (Fig. 2, E–H).

Effects of Bovine FSH and LH on Female-to-Male Sex Change

To examine the effects FSH and LH on the female-to-male sex change in grouper, we treated nonbreeding females with purified bovine GTHs and examined the gonad histologically 3 wk posttreatment. All fish in control and LH-treated groups had ovaries containing many previtellogenic oocytes (Table 2 and Fig. 3, A and B). Six out of eight fish treated with the low dose of FSH had bisexual gonads with both ovarian and testicular tissue, indicating that fish were in the process of sex change (Fig. 3C and Table 2). The remaining two fish had ovaries and no testicular tissue. In contrast, gonads of all the fish treated with high-dose FSH contained active spermatogenic tissues mixed with previtellogenic oocytes (Fig. 3D). There were no significant differences in the gonad weight or total length of fish among treatment groups (Table 2).

Effects of Bovine FSH and LH on Sex Steroid Production and Endogenous GTH Subunits Transcript

In order to investigate possible changes in steroid hormone production in response to bovine GTHs, we measured serum levels of E2 and 11KT by ELISA (Fig. 4). Treatment of females with FSH and LH had no effect on E2 production (Fig. 4A). In contrast, treatment with both doses of FSH remarkably enhanced 11KT production (Fig. 4B).

In an attempt to investigate the effects of bovine FSH and LH, we measured the transcripts of subunit of *fshb* and *lhb* in the pituitaries (Fig. 5). No significant effect on *lhb* mRNA levels was observed among the treatment groups (Fig. 5A). However, significant elevations in endogenous *fshb* mRNA were found in the groups treated with both doses of FSH (Fig. 5B).

DISCUSSION

In this study, we provide evidence for a unique function of FSH in the female-to-male sex change and testis development in the honeycomb grouper. First, we cloned the cDNAs and measured levels of transcripts for the subunits of FSH and LH in the pituitary gland from fish at different sexual phases. The amounts of *fshb* mRNA were low in females, and increased significantly in the ET stage of sex change associated with testis development, while transcripts for *cga* and *lhb* did not change. Second, the sexually dimorphic expression of Fshb protein was confirmed by immunohistochemical staining of Fshb- and Lhb-producing cells in pituitaries of all sexual stages using antisera previously shown to recognize Fshb and Lhb from several teleosts. Finally, to provide evidence for a role of

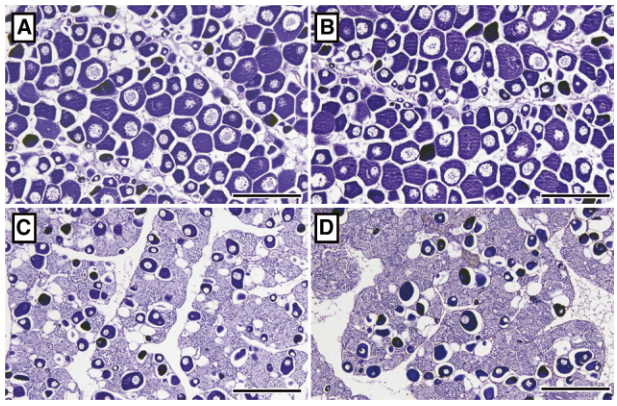


FIG. 3. Gonadal sections of honeycomb grouper, treated with only molten cocoa butter as control (**A**), bovine LH (500 ng/fish) (**B**), low-dose bovine FSH (50 ng/fish) (**C**), and high-dose bovine FSH (500 ng/fish) (**D**) for 3 wk. In **A** and **B**, gonads had many previtellogenic oocytes. In **C** and **D**, primary oocytes and active spermatogonial proliferation were observed in the gonad simultaneously. Thus, we characterized these fishes as sex changing. Bars = 200 μm.

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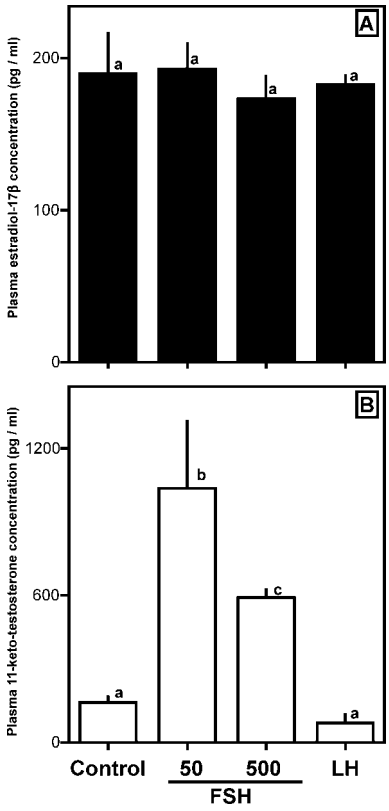


FIG. 4. Effects of treatment with bovine FSH and LH on plasma levels of estradiol-17β (**A**) and 11-keto-testosterone (**B**) in honeycomb grouper. Data are shown as means ± SEM. Data points not sharing a letter (a, b, c) are significantly different by Tukey-Kramer multiple comparison test.

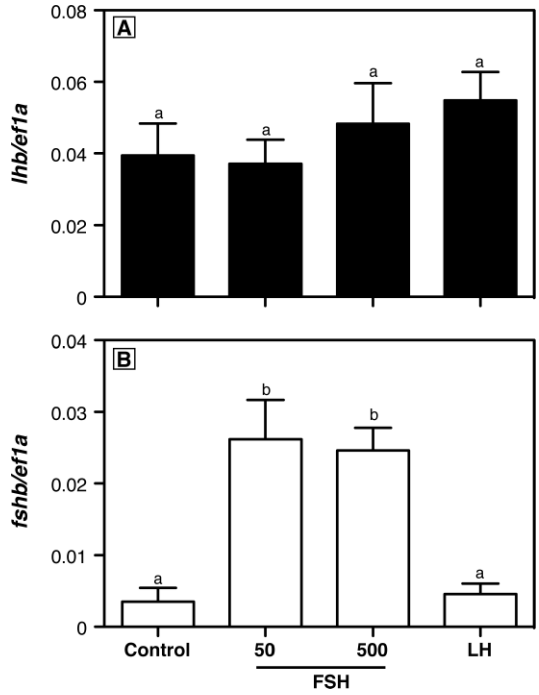


FIG. 5. Effects of treatment with bovine FSH and LH on endogenous *lhb* transcript (**A**) and *fshb* transcript (**B**) in the pituitaries of honeycomb grouper. Data are shown as means ± SEM. Data points not sharing a letter (a, b) are significantly different by Tukey-Kramer multiple comparison test.

GTH in the process of sex change, we investigated changes in gonadal development, steroid production, and pituitary GTH subunit transcripts in nonbreeding females treated with bovine FSH or LH in vivo. Although the present study does not address efficacy of bovine GTHs relative to homologous grouper GTHs in regulating gonadal function of the grouper, bovine FSH treatment clearly induced gonadal sex change. This is the first report to provide evidence for biological functions of GTHs in the process of sex change.

In this study, we showed that *fshb* gene expression in the pituitary of the honeycomb grouper is low in females, and increases during the female-to-male sex change. Similarly, *fshb* transcripts were increased in association with methyl testosterone-induced sex change of orange-spotted grouper, *Epinephelus coioides* [38]. Our results indicate that the pituitary may control gonadal sex change in the grouper, and support the hypothesis that sex change, unlike sex differentiation, involves an alteration of the hypothalamic-pituitary-gonadal (HPG) axis [2, 23]. However, the factors regulating the observed changes in *fshb* gene expression during the onset of sex change are currently unknown. Most of the studies on hypothalamic regulation of fish GTHs have focused on GTH-releasing hormone (GnRH) [40, 41]. In the protandrous wrasse, increases in the size and number of GnRH-secreting cells were observed in the hypothalamus and preoptic area (POA) accompanying sex change [42, 43]. Therefore, future efforts to elucidate involvement of the HPG axis in sex change should focus on the relationship between GnRH cells and secretion of GTH in grouper.

Our study also revealed that FSH might not play a major role in regulating ovarian function in females. In contrast to males, *fshb* mRNA levels were low in females. Levels of *lhb* transcripts were high and not significantly altered during the sex change. The low *fshb* transcript levels were unlikely due to the stage of oogenesis, because *fshb* transcript levels of females were low in the breeding season, while *lhb* mRNA levels were high. These results are similar to those observed in red seabream, in which females had low levels of *fshb* mRNA throughout sexual maturation [44]. Therefore, in some female teleosts, ovarian function and oogenesis are probably controlled primarily by LH.

The sexually dimorphic pattern of pituitary *fshb* and *lhb* gene expression was also reflected in the number of cells producing Fshb and Lhb protein. Immunoreactivities for Fshb and Lhb were localized in separate cells in the pituitary with distinct distributions. These results are similar to those reported for other teleosts [45–48], with the exception of yellowtail [49] and platyfish [50]. In both sexes of the honeycomb grouper, Lhb cells were numerous and localized widely in the PPD. It is of interest that males and sex-changing fishes had numerous Fshb-ir cells, while all nonbreeding females had virtually no Fshb-positive cells in the pituitary. At the present, we do not know whether the sexually dimorphic expression of FSH is a general pattern for grouper species. In another grouper species, *Epinephelus coioides*, where GTHs have recently been studied, both Fshb and Lhb cells were identified immunohistologically in the female pituitary gland, and appeared to be similarly abundant [39]. At this point, more extensive comparative analyses of the role and expression of Fsh and Lh are needed for grouper species to determine how much species variation exists.

To directly address the involvement of GTHs in gonadal sex change, we treated nonbreeding females with purified bovine FSH and LH, since native grouper hormones were not available. A high dose of bovine FSH completely induced

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female-to-male sex change, while LH was ineffective. Treatment with bovine FSH also increased levels of endogenous *fshb* transcripts to levels similar to those in natural male individuals. These results indicate that FSH might be a trigger for gonadal sex change in the honeycomb grouper. However, we do not know if the effects of bovine FSH on the gonad were direct or indirect, via increased endogenous FSH. In tilapia, recombinant or affinity-purified FSH enhanced 11KT secretion [51] and, alternatively, androgen exposure up-regulated the endogenous *fshb* transcripts [52]. Therefore, in the present study, the 11KT that was newly produced in the ovary by bovine FSH treatment could be responsible for the increase in endogenous *fshb* transcripts. Additional studies to analyze the role and effect of bovine FSH on steroid production in the ovary of nonbreeding female honeycomb grouper are required.

Although the amino acid sequences of fish Fshb subunits (including honeycomb grouper) have only about 40% similarity to those of bovine Fshb, fish GTH receptors have been shown to bind bovine FSH. However, the binding specificities of fish GTH receptors appear to be less than what is generally observed in mammals. For example, in sea bass [53] and zebrafish [54], bovine FSH activated both FSH and LH receptors expressed in mammalian cell lines, while bovine LH only activated the LH receptor. If the induction of testicular tissue in the honeycomb grouper is dependent on FSH signaling, this may explain why bovine LH was ineffective in inducing sex change in the present study. Clearly, to elucidate the mechanisms whereby bovine FSH induces sex change in honeycomb grouper, investigations of the ligand specificity of homologous GTH receptors are necessary.

As a first step toward understanding the mechanism of FSH-induced sex change, we measured plasma levels of E2 and androgen (11KT is a major androgen in teleosts) after treatment with bovine GTHs. Previous studies have shown that plasma E2 levels decline and 11KT levels increase during natural female-to-male sex change in grouper [28]. Interestingly, steroid hormone profiles in bovine FSH-treated fish were similar to those of natural sex-changing fish in that 11KT levels increased; however, unlike the natural sex change, E2 levels remained unchanged. In contrast, bovine LH did not significantly alter either E2 or 11KT levels. These results suggest that FSH is a potent stimulator of androgen production, which is similar in a number of teleost species [23, 46, 55–58]. Together with histological observation, these results further strengthen our conclusion that the function of FSH is to induce gonadal sex change and spermatogenesis by stimulating androgen production. Previously, we observed that cell clusters immunostained with antibodies to P450 11β-hydoroxylase, which is the key steroidogenic enzyme for production of 11KT, were localized around blood vessels in the tunica albuginea of the ovary [59]. Based on this observation and data from the present study, we hypothesize that FSH acts on these cell clusters at the beginning of the sex change process to stimulate 11KT production.

In summary, the results of the present study provide the first evidence that FSH may have a unique function in sex-changing fish. FSH, but not LH, displays a highly sexually dimorphic pattern in the pituitary of honeycomb grouper, with males having higher levels of *fshb* transcript and protein than females. Furthermore, development of testicular tissue in the ovary and up-regulation of plasma androgen levels are induced by bovine FSH in vivo. These results suggest that FSH is involved in gonadal sex change in honeycomb grouper. Future studies will address the regulation of FSH and distribution of gonadal GTH receptors during sex change in this species.

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REFERENCES

1. Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 2002; 208:191–364.
2. Frisch A. Sex-change and gonadal steroids in sequentially-hermaphroditic teleost fish. Rev Fish Biol Fish 2004; 14:481–499.
3. Mitcheson YSd, Liu M. Functional hermaphroditism in teleosts. Fish Fish (Oxf) 2008; 9:1–43.
4. Nakamura M, Bhandari RK, Higa M. The role of estrogens plays in sex differentiation and sex changes of fish. Fish Physiol Biochem 2003; 28: 113–117.
5. Cardwell JR, Liley NR. Hormonal control of sex change and color change in the stoplight parrotfish, *Sparisoma viride*. Gen Comp Endocrinol 1991; 81:7–20.
6. Chan STH, Yeung WSB. Sex steroids in intersexual fishes. Fish Physiol Biochem 1989; 7:229–235.
7. Higa M, Ogasawara K, Sakaguchi A, Nagahama Y, Nakamura M. Role of steroid hormones in sex change of protogynous wrasse. Fish Physiol Biochem 2003; 28:149–150.
8. Kroon FJ, Liley NR. The role of steroid hormones in protogynous sex change in the blackeye goby, *Coryphopterus nicholisii* (Teleostei: Gobiidae). Gen Comp Endocrinol 2000; 118:273–283.
9. Chang C-F, Lin B-Y. Estradiol-17β stimulates aromatase activity and reversible sex change in protandrous black porgy, *Acanthopagrus schlegeli*. J Exp Zool 1998; 208:165–173.
10. Wu GC, DU JL, Lee YH, Lee MF, Chang CF. Current status of genetic and endocrine factors in the sex change of protandrous black porgy, *Acanthopagrus schlegeli* (Teleostean). Ann N Y Acad Sci 2005; 1040: 206–214.
11. Kojima Y, Bhandari RK, Kobayashi Y, Nakamura M. Sex change of adult initial-phase male wrasse, *Halichoeres trimaculatus* by estradiol-17 beta treatment. Gen Comp Endocrinol 2008; 156:628–632.
12. MiyakeY, Fukui Y, Kuniyoshi H, Sakai Y, Hashimoto H. Examination of the ability of gonadal sex change in primary males of the diandric wrasses *Halichoeres poecilopterus* and *Halichoeres tenuispinis*: estrogen implantation experiments. Zool Sci 2008; 25:220–224.
13. Swanson P, Dickey JT, Campbell B. Biochemistry and physiology of fish gonadotropins. Fish Physiol Biochem 2003; 28:53–59.
14. Bousfield GR, Perry WM, Ward DN. Gonadotropins: chemistry and biosynthesis. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1994:1749–1792.
15. Pierce JG, Parons TF. Glycoprotein hormones: structure and functions. Annu Rev Biochem 1981; 50:465–495.
16. Suzuki K, Kawauchi H, Nagahama Y. Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. Gen Comp Endocrinol 1988; 71:292–301.
17. Swanson P, Suzuki K, Kawauchi H, Dickhoff WW. Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biol Reprod 1991; 44:29–38.
18. Part F, Sumpter JP, Tyler CR. Validation of radioimmunoassay for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biol Reprod 1996; 54:1375–1382.
19. Elizur A, Meiri I, Rosenfeld H, Zmora N, Knibb WR, Zohar Y. Seabream gonadotropins: sexual dimorphism in gene expression. In: Goetz FW and Thomas P (eds.), Proceeding of the 5th International Symposium on Reproductive Physiology of Fish, Austin, Texas: University of Texas at Austin, Marine Science Institute; 1998:13–15.
20. Kajimura S, Yoshiura Y, Suzuki M, Aida K. cDNA Cloning of two gonadotropin β subunits (GTH-Iβ and -IIβ) and their expression profiles during gametogenesis in the Japanese flounder (*Paralichthys olivaceus*). Gen Comp Endocrinol 2001; 122:117–129.
21. Moteos J, Mananos E, Martinez-Rodrigues G, Carrillo M, Querat B, Zanuy S. Molecular characterization of sea bass gonadotropin subunits (alpha, FSH beta, and LH beta) and their expression during the reproductive cycle. Gen Comp Endocrinol 2003; 133:216–232.
22. Hellqvist A, Schmitz M, Mayer I, Borg B. Seasonal changes in expression of LH-β and FSH-β in male and female three-spined stickleback, *Gasterosterus aculeatus*. Gen Comp Endocrinol 2006; 145:263–269.
23. Ohta K, Mine T, Yamaguchi A, Matsuyama M. Sexually dimorphic

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expression of pituitary glycoprotein hormones in a sex-changing fish (*Pseudolabrus sieboldi*). J Exp Zool A 2008; 309:534–541.
24. Lee YH, Lee FY, Yueh WS, Tacon P, Du JL, Chang CN, Jeng SR, Tanaka H, Chang CF. Profiles of gonadal development, sex steroids, aromatase activity, and gonadotropin II in the controlled sex change of protandrous black porgy, *Acanthopagrus schlegeli*. Gen Comp Endocrinol 2000; 119: 111–120.
25. Koulish S, Kramer CR. Human chorionic gonadotropin (hCG) induces gonad reversal in a protogynous fish, the bluehead wrasse, *Thalassoma bifasciatum* (Teleostei, Labridae). J Exp Zool 1989; 252:156–168.
26. Tang F, Chan ST, Lofts B. Effect of mammalian luteinizing hormone on the natural sex reversal of the rice-field eel, *Monopterus albus* (Zuiew). Gen Comp Endocrinol 1974; 24:242–248.
27. Yeung WS, Chen H, Chan ST. Effects of LH and LHRH-analog on gonadal development and in vitro steroidogenesis in the protogynous *Monopterus albus*. Gen Comp Endocrinol 1993; 89:323–332.
28. Bhandari RK, Komuro H, Nakamura S, Higa M, Nakamura M. Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (*Epinephelus merra*). Zool Sci 2003; 20:1399–1404.
29. Bhandari RK, Higa M, Nakamura S, Nakamura M. Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). Mol Reprod Dev 2004; 67:303–307.
30. Alam MA, Bhandari RK, Kobayashi Y, Soyano K, Nakamura M. Induction of sex change within two full moons during breeding season and spawning in grouper. Aquaculture 2006; 255:532–535.
31. Bhandari RK, Alam MA, Soyano K, Nakamura M. Induction of female-to-male sex change in the honeycomb grouper (*Epinephelus merra*) by 11-ketotestosterone treatments. Zool Sci 2006; 23:65–69.
32. Sarter K, Papadaki M, Zanuy S, Mylonas CC. Permanent sex inversion in 1-year-old juveniles of the protogynous dusky grouper (*Epinephelus marginatus*) using controlled-release 17a-methyltestosterone implants. Aquaculture 2006; 256:443–456.
33. Yeh SL, Dai QC, Chu YT, Kuo CM, Ting YY, Chang CF. Induced sex change, spawning and larviculture of potato grouper, *Epinephelus tukula*. Aquaculture 2003; 228:371–381.
34. Kuo CM, Ting YY, Yeh SL. Induced sex reversal and spawning of blue-spotted grouper, *Epinephelus fario*. Aquaculture 1988; 74:113–126.
35. Shimizu A, Sakai T, Nashida K, Honda H. Universal antisera for immunocytochemical identification of the different gonadotrophs in acanthopterygian fishes. Fish Physiol Biochem 2003; 29:275–287.
36. Alam MA, Nakamura M. Determination of sex and gonadal maturity in the honeycomb grouper, *Epinephelus merra*, through biopsy. Aquaculture 2008; 16:27–32.
37. Rahaman MS, Takemura A, Takano K. Correlation between plasma steroid hormones and vitellogenin profiles and lunar periodicity in the female golden rabbitfish, *Siganus guttatus* (Bloch). Comp Biochem Physiol B 2000; 127:113–122.
38. Zhang W, Zhang Y, Zhang L, Zhao H, Li X, Huang H, Lin H. The mRNA expression of P450 aromatase, gonadotropin β-subunits and FTZ-F1 in the orange-spotted grouper (*Epinephelus coioides*) during 17α-methyltestosterone-induced precocious sex change. Mol Reprod Dev 2007; 74:665–673.
39. Li CJ, Zhou L, Wang Y, Hong YH, Gui JF. Molecular and expression characterization of three gonadotropin subunits common α, FSHβ and LHβ in groupers. Mol Cell Endocrinol 2005; 233:33–46.
40. Yaron Z, Gur G, Melamed P, Rosenfeld H, Levavi-Sivan B, Elizur A. Regulation of gonadotropin subunit genes in tilapia. Comp Biochem Physiol B 2001; 129:489–502.
41. Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B. Regulation of fish gonadotropins. Int Rev Cytol 2003; 225:131–185.
42. Grober MS. Socially controlled sex change: integrating ultimate and proximate levels of analysis. Acta Ethologica 1998; 1:3–17.
43. Foran CM, Bass AH. Preoptic GnRH and AVT: axes for sexual plasticity in teleost fish. Gen Comp Endocrinol 1999; 116:141–152.
44. Gen K, Okuzawa K, Senthilkumaran B, Tanaka H, Moriyama S, Kagawa H. Unique expression of gonadotropin-I and -II subunit genes in male and female red seabream (*Pagrus major*) during sexual maturation. Biol Reprod 2000; 63:308–319.
45. Nozaki M, Naito N, Swanson P, Miyata K, Nakai Y, Oota Y, Suzuki K, Kawauchi H. Salmonid pituitary gonadotrophs. I. Distinct cellular distributions of two gonadotropins, GTH I and GTH II. Gen Comp Endocrinol 1990; 77:348–357.
46. Kagawa H, Kawazoe I, Tanaka H, Okuzawa K. Immunocytochemical identification of two distinct gonadotropic cells (GTH I and GTH II) in the pituitary of bluefin tuna, *Thunnus thynnus*. Gen Comp Endocrinol 1998; 110:11–18.
47. Shimizu A, Tanaka H, Kagawa H. Immunocytochemical applications of specific antisera raised against synthetic fragment peptides of mummichog GtH subunits: examining seasonal variations of gonadotrops (FSH cells and LH cells) in the mummichog and applications to other acanthopterygian fishes. Gen Comp Endocrinol 2003; 132:35–45.
48. Pandolfi M, Nostro FLL, Shimizu A, Pozzi AG, Meijide FJ, Vazquez GR, Maggese MC. Identification of immunoreactive FSH and LH cells in the cichild fish *Cichlasoma dimerus* during the ontogeny and sexual differentiation. Anat Embryol 2006; 211:355–365.
49. García-Hernández MP, García A, Agulleiro B, Gracía A, van Dijk W, Schulz RW. Development of a homologous radioimmunoassay for Mediterranean yellowtail (*Seriola dumerilli*; Risso 1810) LH. Aquaculture 2002; 210:203–218.
50. Magliulo-Cepriano L, Schreibman MP, Blüm V. Distribution of variant forms of immunoreactive gonadotropin-releasing hormone and beta-gonadotropins I and II in the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. Gen Comp Endocrinol 1994; 94:135–150.
51. Aizen J, Kasuto H, Golan M, Zakay H, Levavi-Sivan B. Tilapia follicle-stimulating hormone (FSH): immunochemistry, stimulation by gonadotropin-releasing hormone, and effect of biologically active recombinant FSH on steroid secretion. Biol Reprod 2007; 76:692–700.
52. Melamed P, Gur G, Rosenfeld H, Elizur A, Yaron Z. The mRNA levels of GtH Iβ, GtH IIβ and GH in relation to testicular development and testosterone in pituitary cells of male tilapia. Fish Physiol Biochem 1997; 17:93–98.
53. Rocha A, Gómez A, Zanuy S, Cerdá-Reverter J, Carrillo M. Molecular characterization of two sea bass gonadotropin receptors: cDNA cloning, expression analysis, and function activity. Mol Cell Endocrinol 2007; 272: 63–76.
54. Kwok HF, So WK, Wang Y, Ge W. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors—evidence for their distinct functions in follicle development. Biol Reprod 2005; 72:1370–1381.
55. Planas JV, Swanson P. Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) in vitro. Biol Reprod 1995; 52:697–704.
56. Weltzien F-A, Norberg B, Helvik JV, Andersen O, Swanson P, Andersson E. Identification and localization of eight distinct hormone-producing cell types in the pituitary of male Atlantic halibut (*Hippoglossus hippoglossus* L.). Comp Biochem Physiol A 2003; 134:315–327.
57. Kamei H, Kawazoe I, Kaneko T, Aida K. Purification of follicle-stimulating hormone from immature Japanese eel, *Anguilla japonica*, and its biochemical properties and steroidogenic activities. Gen Comp Endocrinol 2005; 143:257–266.
58. Aizen J, Kasuto H, Levavi-Sivan B. Development of specific enzyme-linked immunosorbent assay for determining LH and FSH levels in tilapia, using recombinant gonadotropins. Gen Comp Endocrinol 2007; 153:323–332.
59. Alam MA, Komuro H, Bhandari RK, Nakamura S, Soyano K, Nakamura M. Immunohistochemical evidence identifying the site of androgen production in the ovary of the protogynous grouper *Epinephelus merra*. Cell Tissue Res 2005; 320:323–329.

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