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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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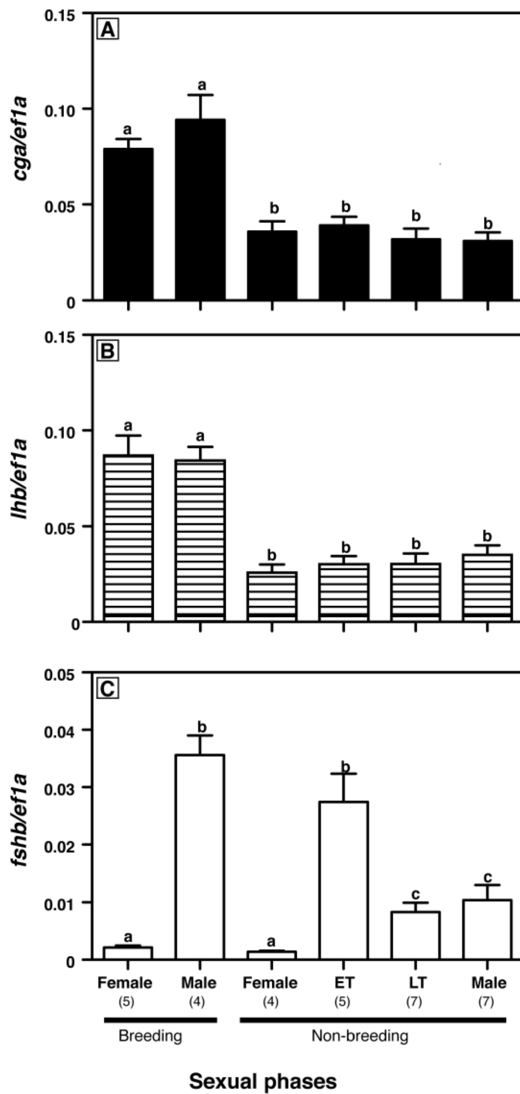


FIG. 1. Changes of GTH subunit transcripts (**A**, *cga*; **B**, *lhb*; **C**, *fshb*) in pituitaries of the different sexual phases in honeycomb grouper, as determined by quantitative real-time RT-PCR. Data are shown as means ± SEM. The parenthetic numbers in the figure indicate total number of fish 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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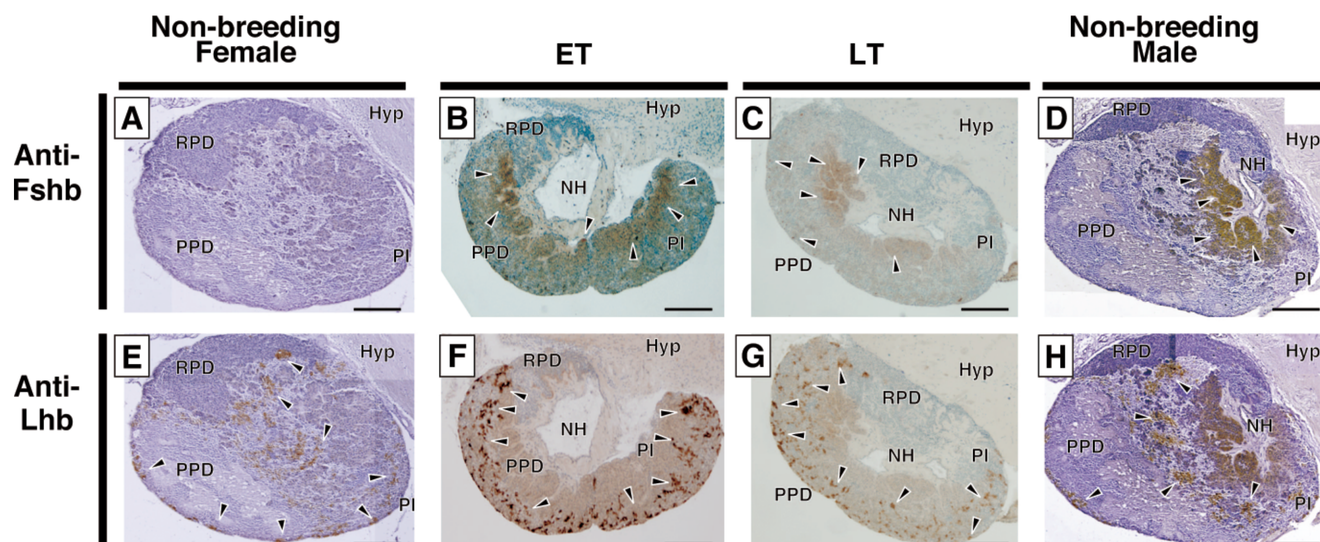


FIG. 2. Representative sagittal sections of pituitary glands from nonbreeding female (**A** and **E**), ET-phase sex-changing (**B** and **F**), LT-phase sex-changing (**C** and **G**), and nonbreeding male (**C** and **D**) honeycomb grouper. Immunostained with anti-Fh Fshb 50–60 (**A**–**D**) and anti-Fh 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

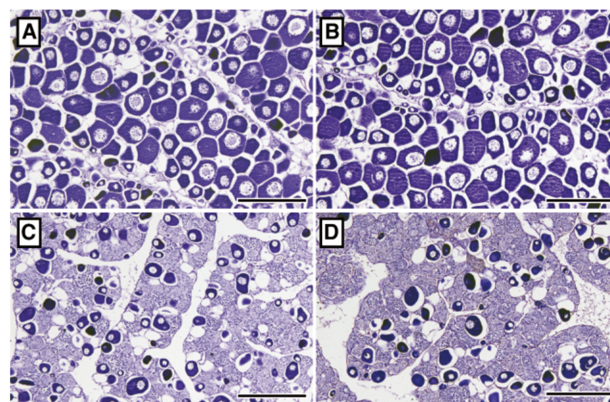


FIG. 3. Gonadal sections of honeycomb grouper, treated with only molten cocoa butter as control (**A**), bovine LH (500 ng/fish) (**B**), low-dose bovine FSH (50 ng/fish) (**C**), and high-dose bovine FSH (500 ng/fish) (**D**) for 3 wk. In **A** and **B**, gonads had many previtellogenic oocytes. In **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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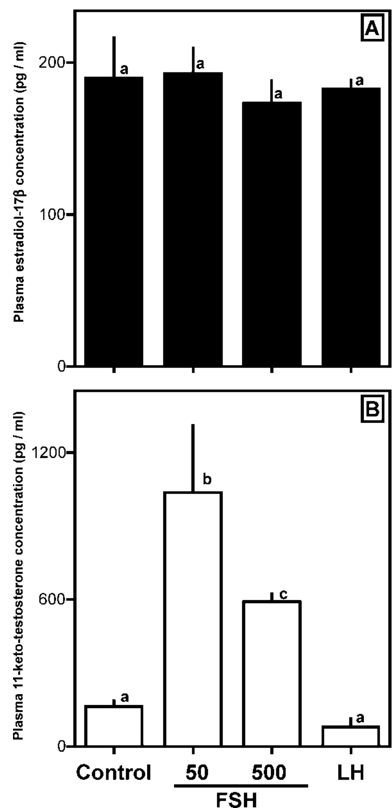


FIG. 4. Effects of treatment with bovine FSH and LH on plasma levels of estradiol-17β (**A**) and 11-keto-testosterone (**B**) in honeycomb grouper. Data are shown as means ± SEM. Data points not sharing a letter (a, b, c) are significantly different by Tukey-Kramer multiple comparison test.

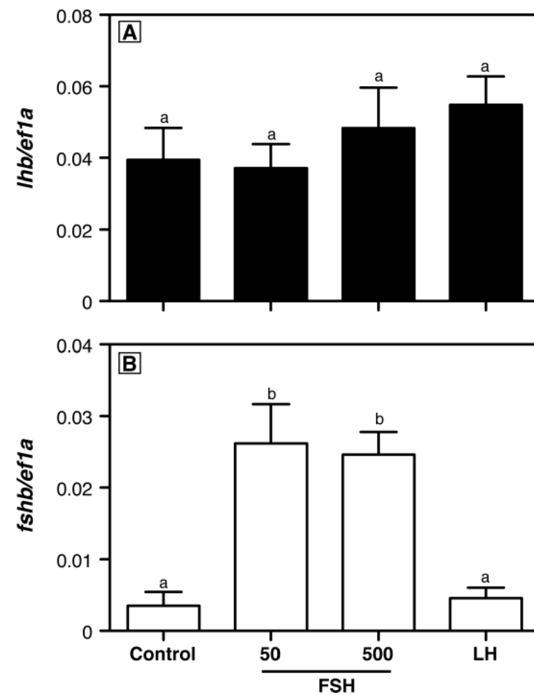


FIG. 5. Effects of treatment with bovine FSH and LH on endogenous *lhb* transcript (**A**) and *fshb* transcript (**B**) in the pituitaries of honeycomb grouper. Data are shown as means ± SEM. Data points not sharing a letter (a, b) are significantly different by Tukey-Kramer multiple comparison test.

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-hydroxylase, 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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