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# Expression of three gonadotropin subunits and gonadotropin receptor mRNA during male-to-female sex change in the cinnamon clownfish, *Amphiprion melanopus*

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ABSTRACT

To quantify the sex-change progression from male to female in the cinnamon clownfish, *Amphiprion melanopus*, we divided gonadal development into three stages (I, mature male; II, male at 90 days after removal of the female; and III, mature female), and the expression of GTH subunits and GTH receptors during each of these stages was investigated. The mRNA of the three GTH subunits and their receptors increased with progression from male to female. To understand the effect of gonadotropin-releasing hormone (GnRH) on this progression, we examined expression of genes encoding the GTH subunit mRNA in the pituitary and the GTH-receptor mRNA in the gonads in addition to investigating changes in plasma $E_2$ levels after GnRH analogue (GnRHa) injection. GnRHa treatment increased mRNA expression levels of these genes, as well as plasma $E_2$ levels, indicating that GnRH plays an important regulatory role in the brain-pituitary-gonad axis of immature cinnamon clownfish.



## 1. Introduction

Gonadotropins (GTHs), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which are all members of the glycoprotein hormone family, are synthesized in the pituitary gland. GTHs consist of a common α subunit and a hormone-specific β subunit. GTHs effect their biological actions by binding with specific receptors (FSHR and LHR) on target cell surfaces; they then stimulate the synthesis and secretion of steroid hormones from the gonads. It is well known that FSH regulates both vitellogenesis and spermatogenesis and that LH promotes follicular maturation, ovulation, and the synthesis of steroid hormones (Nagahama et al., 1995; Ando and Urano, 2005). Many studies have reported that GTHs play important roles in gonadal development and differentiation and that they stimulate secretion of the gonadal steroid hormones that regulate reproduction in vertebrates, including fish (Van Winkoop et al., 1994; Amano et al., 1997; Colombo and Chicca, 2003). The FSHR gene is expressed in the granulosa cells of the ovary and in the Sertoli cells of the testis, whereas the LHR gene is expressed primarily in the theca and granulosa cells of preovulatory ovarian follicles and in the Leydig cells of the testis (Rocha et al., 2007).

In fish, GTH subunit sequences have been determined for a number of teleosts, such as killifish (Lin et al., 1992), striped bass (Hassin et al., 1995), red seabream (Gen et al., 2000), sea bass (Mateos et al., 2003), greasy grouper (Li et al., 2005) and black porgy (An et al., 2009), and many studies have documented their expression and circulating levels at different developmental stages of the reproductive cycle. In addition, it has been shown that FSH regulates the early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH stimulates the final maturation stages, such as ovulation and spermiation (Yaron et al., 2003; Kobayashi et al., 2006). Indeed, FSHβ and LHβ mRNA levels in nonsalmonids have been observed to increase during the spawning season (Kim et al., 2005).

The regulation of reproduction in fish is a complex process involving the interaction of a number of factors, including gonadotropin-releasing hormone (GnRH), GTHs, and gonadal steroid hormone as well as other neurohormones (Ando and Urano, 2005). GnRH is released from the hypothalamus and regulates the synthesis and release of GTH in the pituitary gland. Thus, gonadal maturation is primarily regulated by the brain-pituitary-gonadal axis, and GnRH, GTH subunits, and GTH receptors play central roles in the regulation of gonadal maturation and reproduction in fish and other species.

In cinnamon clownfish (*Amphiprion melanopus*) inhabits consisting of a mated adult pair and an immature individual, sex is controlled by social rank in the group. The female is largest and is dominant in the group. If the dominant female dies or is absent, the male undergoes a sex change to female (Godwin and Thomas, 1993; Godwin, 1994). Hence, cinnamon clownfish are a good model to study the mechanisms of sex change in protandrous hermaphrodite fish. So far, only studies investigating changes in steroid hormones (Godwin and Thomas, 1993) and histological analyses (Godwin, 1994) have been reported with regard to sex-change processes in cinnamon clownfish; the cloning and expression of GTH subunits and their receptors involved in sex-change progression have not yet been examined.

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With these considerations in mind, we isolated cDNAs for three gonadotropin subunits of cinnamon clownfish and investigated their mRNA expression during the sex-change process with the goal of elucidating the roles of GTHs and GTH receptors in the pituitary-gonad axis in these fish during differentiation and gonadal development. Another aim was to investigate the effects of GnRH on the brain-pituitary-gonad axis by measuring mRNA expression of GTH subunits and GTH receptors following a single injection of GnRHa in immature cinnamon clownfish and to describe the relationships among these factors.

## 2. Materials and methods

### 2.1. Experimental fish

The cinnamon clownfish [immature ($n=12$, $7.1\pm0.8$ g), mature male ($n=12$, $10.5\pm1.2$ g), and mature female ($n=12$, $22.2\pm2.1$ g)] were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea), and we made the groups that consisted of the mated pair (dominate female and male) and the each groups were reared in twelve 50-L circulation filter tanks prior to experiments. The temperature was maintained at $28\pm0.5$ °C, and the photoperiod was a 12:12-h light–dark cycle. Fish were fed a commercial marine aquarium fish feed (jeilfeed company, Kyoungnam, Korea) twice a day.

In the present study, the experimental design for sex reversal was a modified version of methods of Godwin and Thomas (1993). Sex change was induced in male fish by removing their female mate from the each group and adding the immature clownfish. After 90 days from removing the female, we observed the male fish to undergo the sex-change process from male to female. The fish were anesthetized in tricaine methane sulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) and decapitated prior to tissue collection. Pituitary and gonads from the fish of the sex-change process (mature male; male at 90 days after removal of the female; and mature female) were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until the total RNA was extracted for analysis.

### 2.2. GnRHa treatment

To investigate the roles of GTHs and GTH receptors in the reproductive physiology of cinnamon clownfish, immature male fish ($7.1\pm0.8$ g) were treated with GnRH and the expression of GTHs and GTH receptors was measured. The fish were reared in four 50-L circulation filter tanks in the laboratory. They were then anesthetized with tricaine methane sulfonate (MS-222, Sigma, USA) prior to injection. GnRHa (des $Gly^{10}$-[$D$-$Ala^{6}$] luteinizing hormone releasing hormone ethylamide, Sigma) was dissolved in 0.9% physiological saline. Each fish was given an injection of GnRHa (0.1 and 0.5 μg/g body weight [BW]) at a volume of 10 μL/g BW. After injection, the pituitary and gonads were removed from five fish at each of the following time periods: 0, 6, 12, 24, and 48 h. During the experimental period, water temperature and photoperiod were maintained at $28\pm0.5$ °C and 12:12 h light–dark, respectively.

### 2.3. Total RNA extraction and reverse transcription (RT)

Total RNA from the pituitary and gonad of the developmental stage during sex change and the GnRHa-treated clownfish were extracted using the Trizol method according to the manufacturer's instructions (Gibco/BRL, Grand Island, NY, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. The amount of extracted RNA was 20 μL (pituitary and gonad) and the concentrations were 3.2541 μg/μL (pituitary) and 6.4856 μg/μL (gonad). After then we diluted with DEPC water to 3 μg/μL for experiment. Total RNA (3 μg) was reverse transcribed in a total volume of 20 μL using an oligo-$d(T)_{15}$ anchor primer and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. The resulting cDNA was diluted and stored at −20 °C for use in a polymerase chain reaction (PCR) and quantitative PCR (QPCR).

### 2.4. cDNA cloning of GTH-subunit cDNA

The primers used for GTHα, FSHβ, and LHβ amplification were designed using highly conserved regions of other teleost fish (Table 1). Total RNA was extracted from the pituitary using a TRIzol kit (Gibco/BRL), and PCR amplification was performed using the TaKaRa Taq (Takara, Japan) according to the manufacturer's instructions. PCR was performed under the following conditions: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 40 s, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide (Biosesang, Korea). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, USA). The transformed colony was cultivated in DH5α (RBC Life Sciences, Korea), and the plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and *Eco*RI (Fermentas, USA). The GTH-subunit cDNA sequences were analyzed using an ABI DNA Sequencer (Applied Biosystems, USA).

### 2.5. Rapid amplification of cDNA 3′ and 5′ ends

For the RACE reaction, total RNA was extracted from the pituitary using a TRIzol kit (Gibco/BRL). Using 3 μg total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo-$d(T)_{18}$ anchor primer and a CapFishing™ adaptor (Seegene, Korea).

Gene-specific primers were selected from the PCR products obtained by RT-PCR. For the 3′ RACE, 50 μL PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 μM 3′ RACE GTHα-primer, 1 μL of 10 μM 3′ RACE FSHβ-primer, 1 μL of 10 μM 3′ RACE LHβ-primer, and 25 μL of

**Table 1**
Primer sequences for RT-PCR, RACE PCR and QPCR.

| Gene name | Primer sequence |
|---|---|
| GTHα | Forward: 5′-CTG CAC TGT CTC TTC TTT TG-3′ |
| GTHα | Reverse: 5′-TAT GGT TTC TCA CCT TTA T-3′ |
| FSHβ | Forward: 5′-GGT TGT CAT GGC AGC GGT GC-3′ |
| FSHβ | Reverse: 5′-GAA GGG CGG ACA TTT GGG-3′ |
| LHβ | Forward: 5′-CAT GGT GTT GAC TTT GTT TC-3′ |
| LHβ | Reverse: 5′-GTC ATT CAT GCA GAA GTC AGG-3′ |
| 5RACE-GTHα | 5′-CCA GCT ACC TCT GTC GCA TAG CTG TGC-3′ |
| 3RACE-GTHα | 5′-CCA GGG ATC GTC CAG TTT ACC AGT GC-3′ |
| 5RACE-FSHβ | 5′-CTG ATG TGC TTC ACC TCG TAG GAC CAG-3′ |
| 3RACE-FSHβ | 5′-CCA ACA TCA GCA TCC AGG TGG AGA GCT G-3′ |
| 5RACE-LHβ | 5′-CTA CAC CTG GAG GAC AGT CAG GAA GCT C-3′ |
| 3RACE-LHβ | 5′-CTG CCA GCT CAT CAA CCA GAC GT GTC-3′ |
| Q-GTHα | Forward: 5′-AAG TCC ATG AAG ACG ATG ACA ATT CC-3′ |
| Q-GTHα | Reverse: 5′-GTG GCA CTG TGT ATG GTT TCT CAC-3′ |
| Q-FSHβ | Forward: 5′-AGC GGC GAC TGG TCC TAC G-3′ |
| Q-FSHβ | Reverse: 5′-CGT CTC CAT CAA ACC TCC CAC AG-3′ |
| Q-LHβ | Forward: 5′-GGT GTC TCT GGA GAA GGA GGG ATG-3′ |
| Q-LHβ | Reverse: 5′-TGA ACA GCG TCT TGA TGA CTG GAT C-3′ |
| Q-FSHR | Forward: 5′-CCT CTC ATT ACC GTG TCC GAC TC-3′ |
| Q-FSHR | Reverse: 5′-GGG TGA AGA AGG CAT ACA GGA AGG-3′ |
| Q-LHR | Forward: 5′-GGA AAC AGA AAT AGA GCC CAC TAC AG-3′ |
| Q-LHR | Reverse: 5′-CAC TTG ACG AAG GGG TTG TTA AGA C-3′ |
| Q-β-actin | Forward: 5′-GCG ACC TCA CAG ACT ACC TCA TG-3′ |
| Q-β-actin | Reverse: 5′-AAG TCC AGG GCA ACA TAG CAG AG-3′ |

SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, extension at 72 °C for 1 min, and 1 cycle of final extension at 72 °C for 5 min.

For the 5′ RACE, 50 μL PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 μM 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 μM 5′ RACE GTHα-primer, 1 μL of 10 μM 5′ RACE FSHβ-primer, 1 μL of 10 μM 5′ RACE LHβ-primer, and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 1 min, followed by a final extension cycle at 72 °C for 5 min. After PCR, ligation and transformation were conducted using the methods described above.

### 2.6. Phylogenetic analysis

Phylogenetic analyses have been performed on the amino acid sequences deduced from GTH-subunit cDNAs from various fish. The amino acid sequences were aligned using the BioEdit Software (Hall, 1999). The phylogenetic tree was constructed using the neighbor-joining method and the Mega 3.1 software package (Center for Evolutionary Functional Genomics, USA).

### 2.7. Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative expression of GTH-subunit and GTH-receptor mRNA using the total RNA extracted from the pituitary and gonads of cinnamon clownfish. The primers for QPCR were used known sequences of cinnamon clownfish (GenBank accession nos. GTHα: EU908056, FSHβ: FJ868867, LHβ: FJ868868, FSHR: GU722648, and LHR: GU722649). The primers used for the QPCR were shown at Table 1. PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The QPCR conditions were 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 20 s. Each experimental group was run in triplicate and inter- and intra-assay calculation of coefficients of variation (CV) for replicate samples was calculated. As an internal control, experiments were duplicated with β-actin, and all data were normalized to the β-actin calculated threshold-cycle (Ct) level. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR, QPCR data from three replicate samples were analyzed with the software of the cycler system to estimate the transcript copy numbers for each sample.

### 2.8. Histological analysis

The gonad tissues from each developmental group (mature male, male at 90 days after removal of the female, and mature female) for the analysis of gonads during sex change were fixed in Bouin's solution. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin. Sections (5 μm thick) were selected and stained with haematoxylin–eosin for observation under a light microscope (Leica DM 100, Germany) and images captured with a digital camera (Leica DFC 290, Germany).

### 2.9. Plasma parameter analysis

Plasma $E_2$ levels were analyzed by radioimmunoassay (RIA) using an $E_2$ RIA kit (Adaltis, Bologna, Italy).

### 2.10. Statistical analysis

A one-way analysis of variance followed by a *post hoc* Newman Keuls' test was used to compare differences at a significance level of $P<0.05$. The data were analyzed with the Statistical Package for the Social Sciences version 10.0 (SPSS, Chicago, IL, USA). Results are presented as mean ± standard deviation (SD). The inter- and intra-assay CV were calculated as the SD divided by the mean.

## 3. Results

### 3.1. Identification of cDNA of GTH subunits

The GTHα cDNA contained 354 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 117 amino acids. The FSHβ cDNA consisted of 360 nucleotides, including an ORF that was predicted to encode a protein of 119 amino acids. The LHβ cDNA contained 420 nucleotides, including an ORF that was predicted to encode a protein of 139 amino acids. The three GTH subunits contained cysteine residues and a highly conserved N-linked glycosylation site (Fig. 1).

Using the Basic Local Alignment Search Tool (BLAST) algorithm (BLASTp) of the National Center for Biotechnology Information, we found that the three GTH-subunit sequences displayed a high similarity with those of other teleosts; these sequences were then compared to those deduced from the cDNA of other teleost species (Fig. 1). The amino acid similarities were as follows: GTHα, 88% with red seabream, 86% with Bengal eel, 86% with striped bass, and 86% with black porgy; FSHβ, 69% with red seabream, 67% with blotched snakeheadfish, 67% with bastard halibut, and 65% with black porgy; and LHβ, 85% with largemouth grouper, 85% with striped bass, 82% with black porgy, and 81% with yellowfin seabream.

### 3.2. Phylogenetic analysis

Phylogenetic analysis indicated the expected relationship among the GTH subunits. Therefore, we named these genes based on our proposed nomenclature and phylogenetic analysis. The cinnamon clownfish GTH subunits were most closely related to halibut (GTHα), red seabream (FSHβ), and yellowfin seabream (LHβ) (Fig. 2).

### 3.3. Expression of pituitary GTH-subunit mRNA during sex change

All GTH-subunit mRNA levels began to increase in the male clownfish at 90 days after removal of the female and were the highest at the mature female stage (Fig. 3). The inter-assay CV was less than 8% and the inter-assay CV was less than 9% for repeated measurements of expression levels from QPCR.

### 3.4. Expression of gonadal GTH receptors mRNA during sex change

As shown in Fig. 4, the FSHR mRNA was significantly higher at the male and female stages, and LHR mRNA was significantly highest at the mature female stage. The inter-assay CV was less than 6% and the inter-assay CV was less than 8% for repeated measurements of expression levels from QPCR.

### 3.5. Histological analysis

The testis of mature male mainly consisted of testicular tissue, with oogonia and primary oocytes (Fig. 5A). And then the oocytes had developed and increased in size (Fig. 5B), the diameter of oocytes was increased and the testicular tissue was regressed, and finally the fish became females (Fig. 5C).

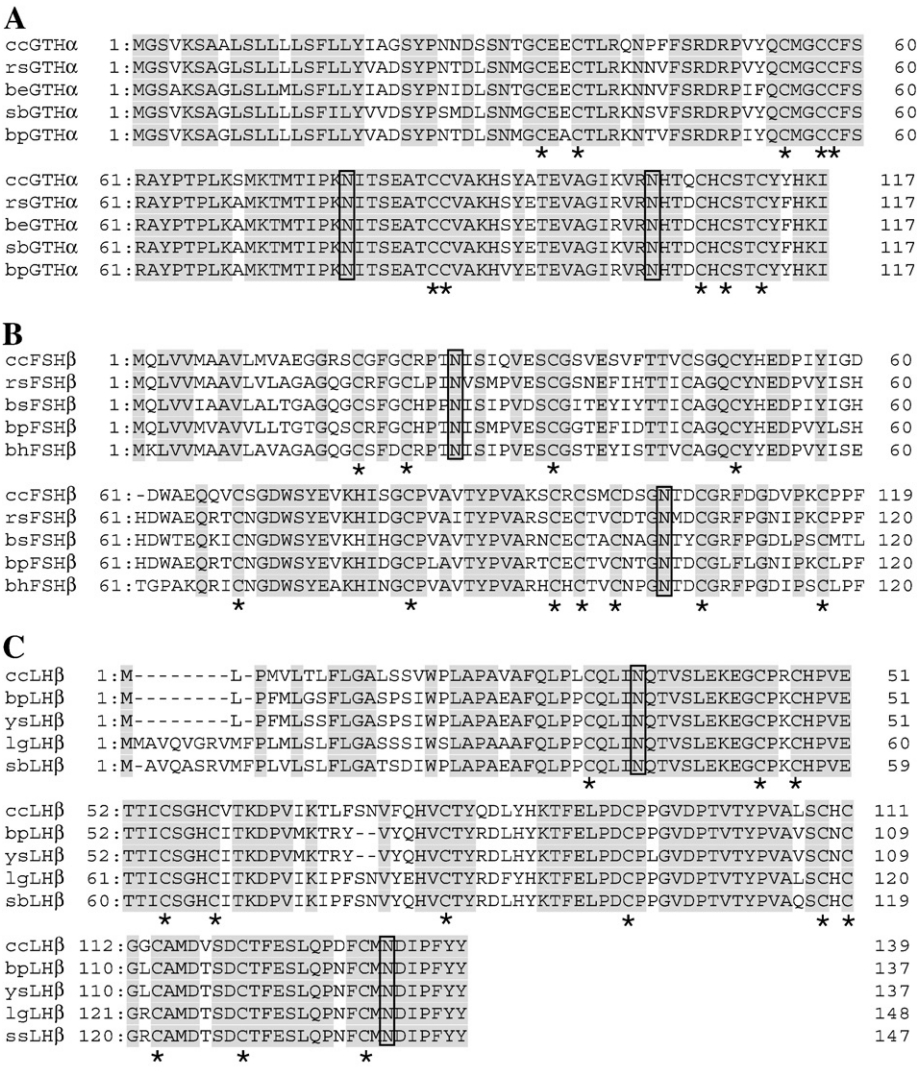


**Fig. 1.** Comparison of the amino acid sequences of GTHα (A), FSHβ (B), and LHβ (C). The sequences are from the GenBank/EMBL/DDBJ sequence databases. GenBank accession numbers of the sequences are: (A) GTHα—cinnamon clownfish (ccGTHα, EU908056), red seabream (rsGTHα, AB028211), Bangel eel (beGTHα, AF502394), striped bass (sbGTHα, L35071), and black porgy (bpGTHα, EF605275); (B) FSHβ—cinnamon clownfish (ccFSHβ, FJ868867), red seabream (rsFSHβ, AB028212), blotched snakeheadfish (bsFSHβ, AY447038), black porgy (bpFSHβ, AY921613), and bastard halibut (bhFSHβ, AB042422); and (C) LHβ—cinnamon clownfish (ccLHβ, FJ868868), black porgy (bpLHβ, EF605276), yellowfin seabream (ysLHβ, L11722), longtooth grouper (lgLHβ, EF583920), and striped bass (sbLHβ, L35096). These sequences are optimally aligned to match identical residues, indicated by the shaded box. The two potential N-linked glycosylation sites are boxed, and cysteine residues of the N- and C-terminal cysteine-rich regions are indicated by asterisk.

### *3.6. Effects of GnRH analogue (GnRHa) on GTH-subunit mRNA expression in the pituitary*

The mRNA of each of the GTH subunits increased significantly after GnRHa injection. In the 0.1 μg/μL GnRHa treatment group, GTHα and FSHβ mRNA were highest at 12 h after injection and then decreased (Fig. 6A and B). LHβ mRNA was the highest at 24 h after injection and then decreased (Fig. 6C). In the 0.5 μg/μL GnRHa treatment group, GTHα mRNA was highest at 12 h after injection and then decreased (Fig. 6A), and FSHβ and LHβ mRNA were highest at 24 h and then decreased (Fig. 6B and C). The inter-assay CV was less than 7% and the inter-assay CV was less than 9% for repeated measurements of expression levels from QPCR.

### *3.7. Effects of GnRHa on GTH-receptor mRNA expression in the gonads*

The mRNA of both GTH receptors increased significantly after GnRHa injection. In the 0.1 μg/μL GnRHa treatment group, FSHR and LHR mRNA were the highest after 12 h, and in the 0.5 μg/μL GnRHa treatment group, the mRNA of these receptors was the highest at 24 h after GnRHa injection (Fig. 7). The inter-assay CV was less than 8% and the inter-assay CV was less than 9% for repeated measurements of expression levels from QPCR.

### *3.8. Effects of GnRHa on $E_2$ levels in plasma*

The plasma $E_2$ level was $35.3 \pm 1.7$ pg/mL at the start of the experiment. In the 0.1 μg/μL GnRHa treatment group, $E_2$ level increased to a maximum of $55.3 \pm 4.3$ pg/mL after 48 h. In the 0.5 μg/μL GnRHa treatment group, $E_2$ level increased to a maximum of $132.1 \pm 7.1$ pg/mL after 24 h (Fig. 8). The inter-assay CV was less than 7% and the inter-assay CV was less than 8% for repeated measurements of expression levels from QPCR.

## 4. Discussion

In the present study, we isolated GTH subunits (GTHα, FSHβ and LHβ) from the pituitary of the cinnamon clownfish and then investigated the changes in mRNA expression of these genes during the processes of sex change from male to female. We also

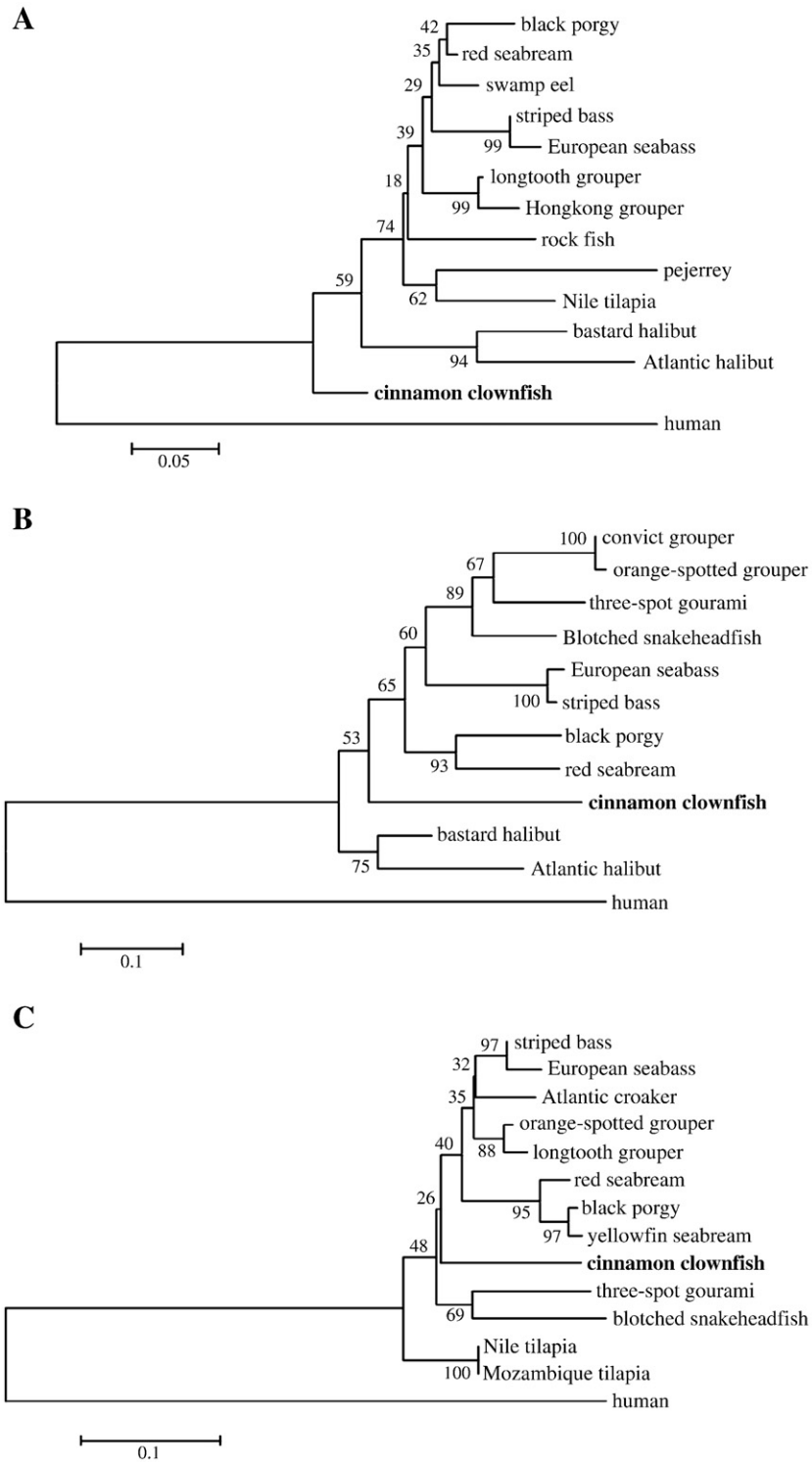


**Fig. 2.** Phylogenetic tree based on an amino acid alignment for GTH subunits in teleost fish. Bootstrap values (%) are indicated for 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: (A) GTHα—cinnamon clownfish (EU908056), black porgy (EF605275), red seabream (AB028211), European sea bass (AF269157), striped bass (L35071), swamp eel (AF502395), longtooth grouper (EF583918), Hong Kong grouper (AY207430), rockfish (AY609078), pejerrey (DQ382280), Nile tilapia (AY294017), bastard halibut (AF268692), and Atlantic halibut (AJ417770), human (NM_000735); (B) FSHβ—cinnamon clownfish (FJ868867), black porgy (AY921613), red seabream (AB028212), striped bass (L35070), European sea bass (AF543314), blotched snakehead (AY447038), three-spot gourami (AF157630), convict grouper (AB111457), orange-spotted grouper (AY186242), bastard halibut (AB042422), Atlantic halibut (AJ417768), and human (NM_000510); and (C) LHβ—cinnamon clownfish (FJ868868), black porgy (EF605276), yellowfin seabream (L11722), red seabream (AB028213), Atlantic croaker (EF433429), striped bass (L35096), European sea bass (AF543315), orange-spotted grouper (AF507939), longtooth grouper (EF583920), Nile tilapia (AY294016), Mozambique tilapia (AY541609), three-spot gourami (AF157631), blotched snakehead (AY447037), and human (NM_000894).

demonstrated the role of GnRHa by documenting the changes of mRNA expression after GnRHa injection in immature cinnamon clownfish.

The expression of GTH-subunit mRNA was low in the male. However, expression levels increased as the sex changed from male to female after the removal of the female, and the mRNA level was

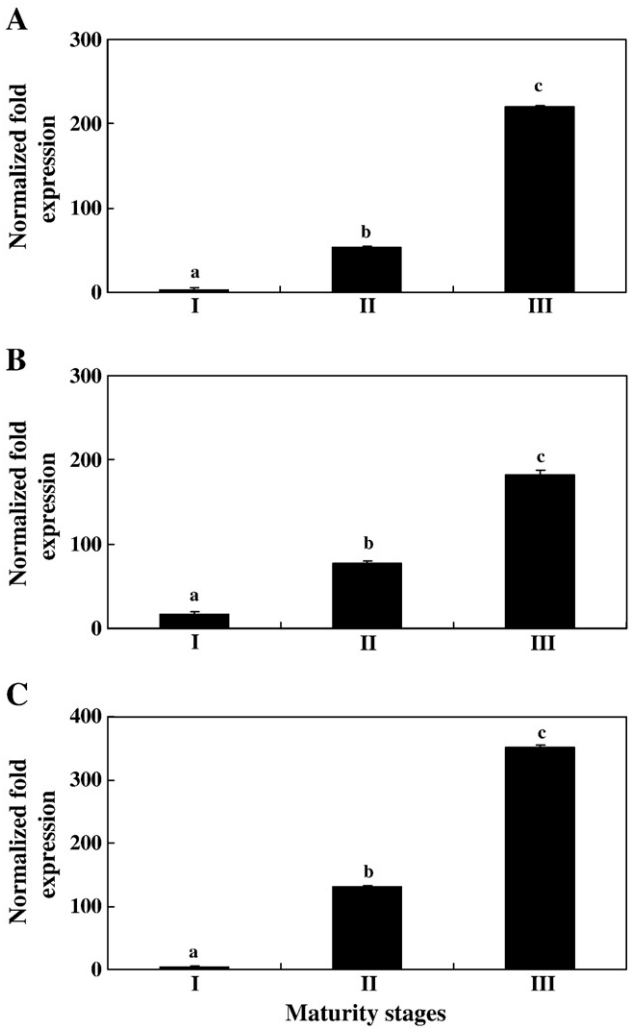


**Fig. 3.** Expression of GTHα (A), FSHβ (B), and LHβ (C) mRNA in the pituitary of cinnamon clownfish during sex change (I, mature male; II, male at 90 days after removal of the female; and III, mature female) using quantitative real-time PCR. We reverse transcribed 3 μg of total RNA prepared from the pituitary and amplified the sample using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=5$).

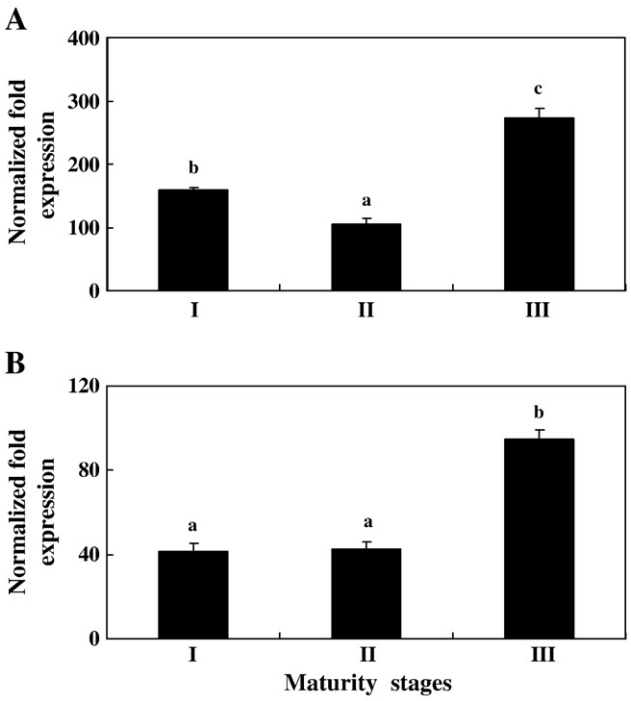


**Fig. 4.** Expression of FSHR (A) and LHR (B) mRNA in the gonads of cinnamon clownfish during sex change (I, mature male; II, male at 90 days after removal of the female; and III, mature female) using quantitative real-time PCR. We reverse transcribed 3 μg of total RNA prepared from gonads and amplified the sample using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=5$).

highest in the mature female (Fig. 3). The increasing expression of GTH mRNA during the male-to-female sex-change process indicates that GTHs are related to the sex change and also suggests their involvement in the development and maturation of oocytes. GTHα also increased during the sex-change process from male to female after removal of the cinnamon clownfish female, suggesting that it, too, may be involved in the sex change; however, GTHα is a common subunit among TSH, FSH, and LH in the pituitary, making it difficult to draw specific conclusions about its role (Salmon et al., 1993). FSH was expressed at an early stage in advance of expression of LH. This may be because FSH is generally involved in the formation of yolk and gamete, and LH is involved in final ovulation and spermiation (Nagahama, 1994; Swanson et al., 2003). We therefore suggest that FSH is expressed in the early stage, while LH increases in the final maturation stage, closer to the female state during the sex-change process. These results agree with those of previous studies reporting that FSH was significantly increased at the early maturation stage (Gen et al., 2000; Hassin et al., 2000; An et al., 2009). Furthermore, An et al. (2008a) reported that the size and the number of oocytes were increased during the sex change to female in the black porgy and that the increases of $E_2$ and LH in plasma also play important roles in this process (Lee et al., 2000, 2001; An et al., 2008b).

As in the case of GTHs, the expression of FSHR and LHR mRNA in the gonads was detected at significant levels during the sex-change process to female (Fig. 4). These results are similar to those of a previous study that found an increase in mRNA of GTH receptors during the sex-change process to female in the black porgy (An et al., 2009). We also found that GTH receptors were involved in development and maturity of the gonads through their interaction with GTHs, as did Rocha et al. (2007, 2009). Notably, Rocha et al. (2009) reported that the level of FSHR was increased from the early vitellogenesis stage and was highest at late vitellogenesis and ovulation, whereas LHR mRNA expression was increased from late vitellogenesis and was highest at ovulation during the maturation process in sea bass. In addition, FSHR mRNA was detected at an early stage of vitellogenesis, and its level increased during vitellogenesis of the channel catfish (*Ictalurus punctatus*) and zebrafish (*Danio rerio*) (Kumar and Trant, 2004; Kwok et al., 2005). Considering these previous and our present results, we suggest that FSH is increased at early vitellogenesis and promotes oocyte development by combining with FSHR, while LH is associated with gonad maturation and sex change when combined with LHR in late vitellogenesis. In addition, we examined gonadal histology during sex change in cinnamon clownfish. As a result, we found that the oocytes were developed and testicular tissue regressed in the ovary as the cinnamon clownfish were changed from male to female (Fig. 5). This result was similar with the previous study (Godwin, 1994; An et al., 2008a). We therefore suggest that gonad development and maturation are controlled by the pituitary-gonad axis through increases in the levels of GTH-subunit and GTH-receptor mRNA in cinnamon clownfish.

It is well known that GnRH plays important roles in the regulation of gonad development and maturation by modifying the synthesis and secretion of GTHs and steroid hormones (Ando and Urano, 2005). In

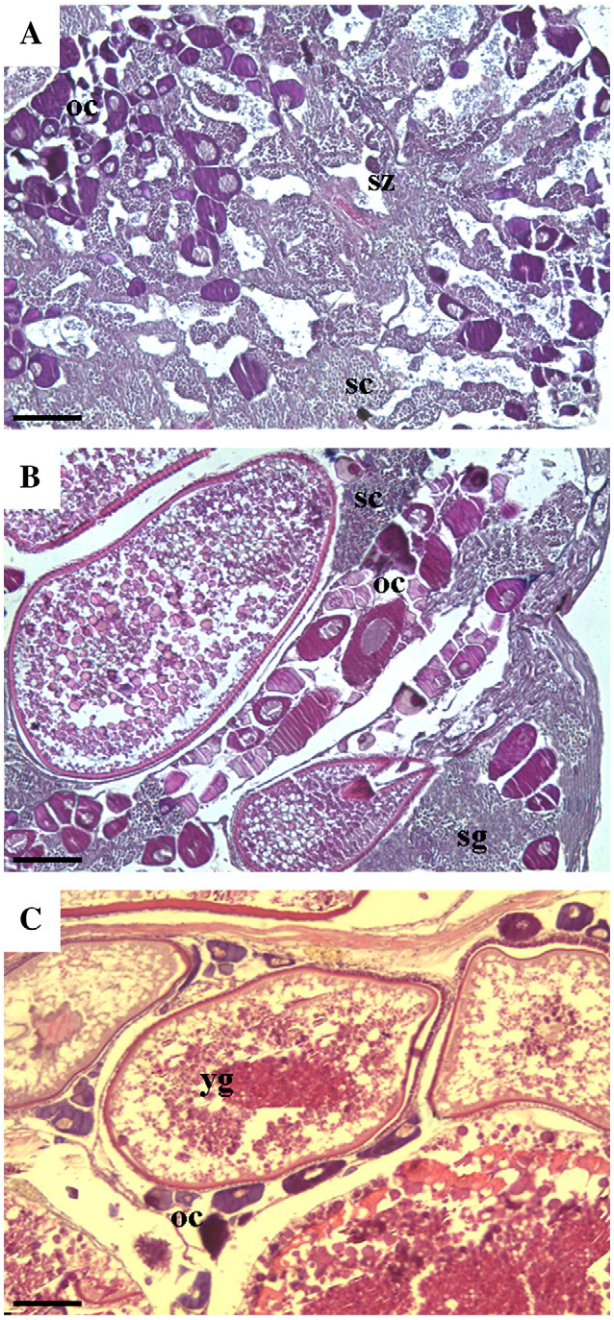


**Fig. 5.** Photomicrograph of gonad maturity stages during sex change of cinnamon clownfish. A: mature male (stage I in Fig. 3), B: male at 90 days after removal of the female (stage II in Fig. 3), C: mature female (stage III in Fig. 3). st: spermatides, sc: spermatocytes, sg: spermatogonia, oc: oocytes, yg: yolk granules. Scale bar = 100 μm.

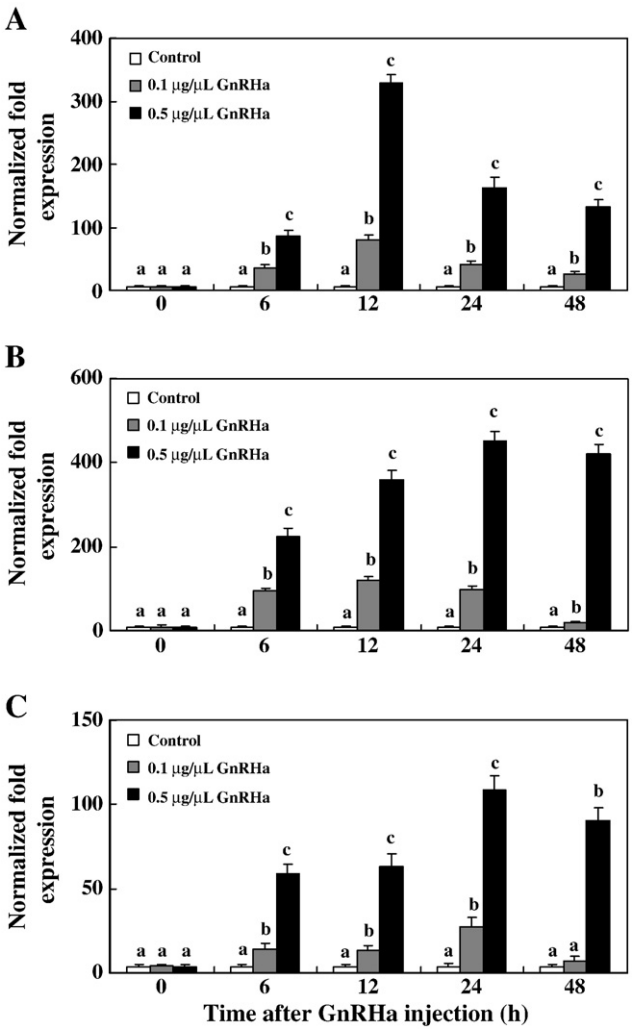


**Fig. 6.** Expression of GTHα (A), FSHβ (B), and LHβ (C) mRNA in the pituitary of cinnamon clownfish using quantitative real-time PCR after GnRHa injection. We reverse transcribed 3 μg of total RNA prepared from pituitary and amplified the sample using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=5$).

order to understand the function and role of GnRH, we investigated GTHs and GTH-receptor mRNA expression levels after injecting GnRHa into immature cinnamon clownfish. Six hours after GnRHa injection, mRNA of all GTH subunits was increased and was maintained at a high level until the end of the experiment. The increase of GTH mRNA expression after GnRHa treatment has been previously reported in striped sea bass (Hassin et al., 1998), sockeye salmon (Ando and Urano, 2005), coho salmon (Dickey and Swanson, 2000), and black porgy (An et al., 2008b). Moreover, three types of GTH were increased by sGnRH and cGnRH-II in goldfish (Huggard-Nelson et al., 2002; Klausen et al., 2002). We found that GTH mRNA expression level was especially high in the high-dose GnRHa treatment group and that this result corresponds to previous studies in goldfish (Sohn et al., 2001; Huggard-Nelson et al., 2002). In addition, plasma $E_2$ levels, as well as the expression of GTH mRNA, were increased by GnRHa injection in red seabream (Kumakura et al., 2004) and black porgy (An et al., 2008b). These results indicate that GTHs are increased in the pituitary by GnRHa and that gonad development is evidently controlled through the combination of GTH and GTH-receptor activity in the gonads. We therefore suggest that GnRHa activates a brain-pituitary-gonad axis pathway in cinnamon clownfish. Furthermore, increasing $E_2$ in plasma, which results in oocyte development and maturation, also plays an important role in the sex change to female (Lee et al., 2000, 2001; An et al., 2008b, 2009).

In summary, we isolated the cDNA of GTH subunits (GTHα, FSHβ, and LHβ) and partial GTH receptors (FSHR and LHR) from mature female cinnamon clownfish and observed an increase in the expression of these genes during the sex-change process from male to female. We also documented that GnRHa is a factor in gonadal development by injecting GnRHa, which increased the levels of mRNA for GTHs and GTH receptors through its activation of the brain-pituitary-gonad axis. This study provides basic data regarding the interaction between GTHs–GTH receptors and GnRH in teleost fish. Further studies are required to demonstrate the action of the brain-pituitary-gonad axis in reproduction and maturity in protandrous fish.

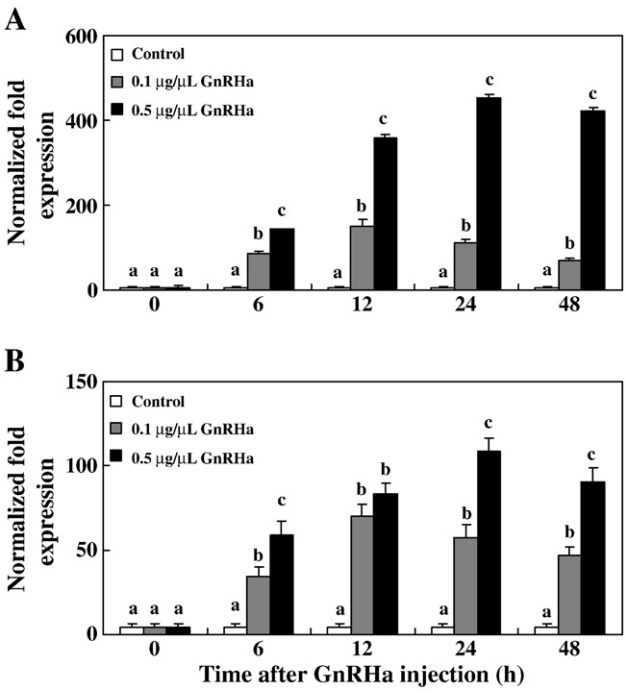


**Fig. 7.** Expression of FSHR (A) and LHR (B) mRNA in the gonads of cinnamon clownfish using quantitative real-time PCR after GnRHa injection. We reverse transcribed 3 μg of total RNA prepared from gonads and amplified the sample using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=5$).

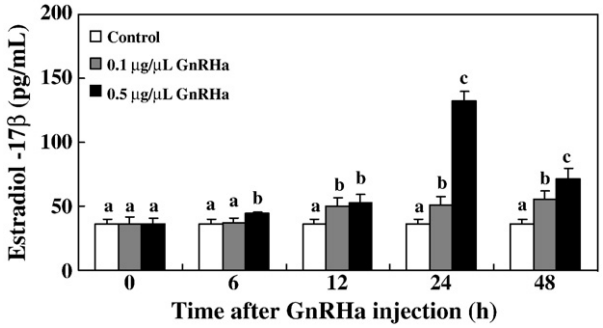


**Fig. 8.** Plasma estradiol-17β ($E_2$) levels after GnRHa injection in cinnamon clownfish. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means ± SD ($n=5$).

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