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Effects of recombinant gonadotropin hormones on the expression of vitellogenin, gonadotropin subunits and gonadotropin receptors in cinnamon clownfish, *Amphiprion melanopus*

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ABSTRACT

Gonadotropins (GTHs) are the key regulators of reproduction in vertebrates. The present study investigated autoregulatory effects of gonadotropins, using recombinant FSH (rFSH) and LH (rLH) in cinnamon clownfish (*Amphiprion melanopus*). Experiments were carried out to investigate the actions of cinnamon clownfish rFSH and rLH on expression of GTH subunits, GTH receptors, and vitellogenin (*Vtg*) mRNA *in vivo* and *in vitro*. Plasma estradiol-17β (E_2) level was also measured in immature fish following treatments with rFSH and rLH. The results demonstrate increasing levels of GTH subunits, GTH-receptors, *Vtg* mRNA levels, as well as plasma E_2 levels following injection with rFSH and rLH. The findings support the hypothesis that LH and FSH stimulate reproduction, in part, by autoregulatory mechanisms leading to upregulation of GTH receptors and GTH hormone production in cinnamon clownfish. The results provide a framework for better understanding of the mechanisms of GTH-mediated control of reproduction in cinnamon clownfish and other vertebrates.

1. Introduction

The regulation of reproduction is a complex process involving the interaction of a number of factors including gonadotropin-releasing hormones (GnRHs), gonadotropin hormones (GTHs), gonadal steroid hormones, and other neurohormones in fish and other vertebrates (Habibi and Matsoukas, 1999; Lee et al., 2001; Habibi and Andreu-Vieyra, 2007; Chang et al., 2009; Zohar et al., 2010). The pituitary GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are members of the glycoprotein hormone family consisting of a common α subunit and a specific β subunit (Pierce and Parsons, 1981). The α/β subunits are highly complex in structure and are identified by the presence of different N-linked oligosaccharide chains and the formation of cross-linked disulfide bonds between cysteine residues. The non-covalent association of the two subunits determines the formation and release of the bioactive dimeric hormone. These hormones carry highly conserved N-linked glycosylation sites, and the attached oligosaccharides are important in hormone bioactivity in teleosts (Kamei et al., 2003). FSH regulates both E_2 for vitellogenesis and spermatogenesis, and LH promotes follicular maturation, ovulation, and the synthesis of steroid hormones in teleosts (Nagahama et al., 1995; Ando and Urano, 2005; Kobayashi et al., 2006). Specific FSH-receptor (FSHR) and LH-receptor (LHR) on target cells mediate FSH and LH-induced stimulation of gametogenesis and gonadal steroidogenesis (Nagahama et al., 1995). There is evidence that 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). It is established that LH and FSH are key regulators of gonadal development and differentiation and control the synthesis of gonadal hormones that regulate reproduction in vertebrates, including fish (Amano et al., 1997; Colombo and Chicca, 2003; An et al., 2008a, 2009). Furthermore, gonadal steroid hormones such as estrogen and testosterone play an important role in sex-related gonadal development and sexual characteristics (Dickey and Swanson, 2000).

A key step in control of reproduction in fish and other oviparous vertebrates is the production of vitellogenin (*Vtg*), a precursor yolk protein, under the regulation of estradiol-17β (E_2). The GTH-induced ovarian E_2 production in turn stimulate synthesize and release of *Vtg* from hepatocytes into the bloodstream, where it is taken up and incorporated into the growing oocytes via receptor-mediated endocytosis (Sawaguchi et al., 2006). The E_2-mediated expression of *Vtg* and ER-mediated pathways have been investigated in fish (Pakdel et al., 2000; Nelson and Habibi, 2010). The *Vtg* gene is also present in male fish and can be stimulated by E_2. However, under normal condition *Vtg* level in male is very low, and can be used as an effective biomarker for environmental contaminants

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with estrogen-like activity (Filby et al., 2006; Jeffries et al., 2008, 2010; Kim et al., 2010).

Cinnamon clownfish (*Amphiprion melanopus*) is a protandrous hermaphroditic fish, typically found as a mated adult pair and an immature individual. Social ranking in the group normally controls fish gender. In general, the female is the largest in size and is dominant in the group. If a dominant female dies or is absent, the male partner undergoes a sex change to become a female (Godwin and Thomas, 1993).

Currently, we know little about hormonal control of reproduction and gonadal sex change in cinnamon clownfish, which is used in this study as a suitable experimental model. Also, we produced recombinant GTHs (rFSH and rLH) to investigate GTH-mediated control of reproduction in cinnamon clownfish. However, it is unclear whether the maturational GTH is important for regulation of gonadal steroidogenesis.

The results provide the information on autoregulatory mechanisms of GTH-mediated control of reproduction and vitellogenic/spermatogenic synthesis in immature cinnamon clownfish.

2. Materials and methods

2.1. Experimental fish

The study was conducted with immature clownfish ($n = 80$, mass 7.1 ± 0.8 g). Fish were purchased from CCORA (Center of Ornamental Reef and Aquarium, Jeju, Korea), and reared in the six 50-L circulation filter tanks in the laboratory. All fish were anesthetized in tricaine methane sulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) and decapitated prior to tissue collection. Gonads and pituitaries were removed from the fish, immediately frozen in liquid nitrogen, and stored at −80 °C until total RNA was extracted for analysis.

2.2. Production of rFSH and rLH

Open reading frames (ORF) of the cinnamon clownfish FSHβ and LHβ were amplified by polymerase chain reaction (PCR) using previously cloned cinnamon clownfish cDNAs for the FSH and LH subunits (An et al., 2010). The GenBank accession numbers for GTHα, FSHβ, LHβ are EU908056, FJ868867, and FJ868868, respectively. The strategy to construct the tethered single-chain FSHα/β and LHα/β are shown in Fig. 1. At first, the ORF regions of mature β subunits encoding the FSHβ (26 amino acids of signal sequence and 118 amino acids of the mature protein without stop codon) or the LHβ (25 amino acids of signal sequence and 138 amino acids of the mature protein without stop codon), and mature GTHα were generated by overlapping PCR method. Recombinant protein included the maltose binding protein (MBP) in the N-terminals and a synthetic *N*-linked glycosylation sequence (NCS) (Fig. 1). In each cDNA construct, a synthetic DNA encoding Ser-Gly-Ser-Asn-Ala-Thr-Gly-Ser-Gly-Ser-Asn-Ala-Thr-Ser-Gly-Ser (NCS) was inserted between the β and α chain by overlapping PCR strategy. In addition, an EcoRI site (FSHβ and LHβ) was placed at the 5′-end of the DNA constructs and a HindIII (FSHβ), and SalI (LHβ) site was placed at the 3′-end of the DNA constructs immediately following the terminator codon of the common GTHα subunit. PCR was performed in 50 μL final volume containing the subunit cDNA templates (An et al., 2010), 50 μL 10× reaction buffer, 2 mM $MgCl_2$, 200 mM dNTP, 2 mM each primer, and 2.5 U Pfu-Taq DNA polymerase (Fermentas, Vilnius, Lithuania). After an initial 5 min denaturing step at 94 °C, 30 cycles of amplification were performed using a cycle profile of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. After the last cycle, elongation was extended to 5 min at 72 °C. Next, the PCR products were cut with EcoRI and HindIII (FSHβ-GTHα), EcoRI and SalI (LHβ-GTHα), purified by gel-extraction and inserted into EcoRI-HindIII sites and EcoRI-SalI of a transfer vector (pYNG: Katakura Industry, Sayama, Saitama, Japan). Finally, the plasmid DNA was sequenced in both strands by chaintermination method using a Big Dye Terminator Ready Reaction Mix (Applied Biosystems, Boston, MA, USA) and an Applied Biosystems' Prism 377 DNA Sequencer.

The recombinant proteins pre-test for activation assay was overexpressed in *Escherichia coli* BL21 (DE3) pLysS cells. Briefly, 10 mL of *E. coli* BL21 (DE3) pLysS cells starter culture was inoculated into 1000 mL Luria broth with 1 mL ampicillin (50 mg/mL). The culture was incubated at 37 °C with shaking at 180 rpm until the cell count reached 0.7 at an optical density of 600 nm. Then, the culture was shifted to 20 °C for 15 min and induced with 0.5 mM isopropyl-β-thiogalactopyranoside for 6 h at FSHβ-GTHα 28 °C and LHβ-GTHα 37 °C. After 6 h of induction, the cells were cooled on ice for 30 min and harvested by centrifugation at 6000*g* for 15 min at 4 °C. The cells were re-suspended in 30 mL 1× PBS buffer and frozen at −70 °C. After thawing, the bacterial cells were placed in an ice-water bath and sonicated 10 times using short 10 s pulses. After centrifugation at 10,000*g* for 20 min and 4 °C, the recombinant proteins were purified in the form of fusion protein as MBP using a pMAL protein fusion and purification system (vector: p2x, amylose resin [E8021S], Cosmo Genetech, Seoul, South Korea). Briefly, amylose resin was poured into a poly-chromatography column and washed three times with PBS, and the fusion protein was eluted with an elution buffer (2–10 mM maltose). The purified proteins were run on 10–15% SDS-PAGE gel with a protein marker (Fermentas, Burlington, Ontario, Canada). Gels were stained using 0.05% Coomassie Blue R-250, followed by a standard de-staining procedure.

2.3. Electrophoresis and Western blot analysis

SDS-PAGE was performed using 12% polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane (Pall Corp., Ann

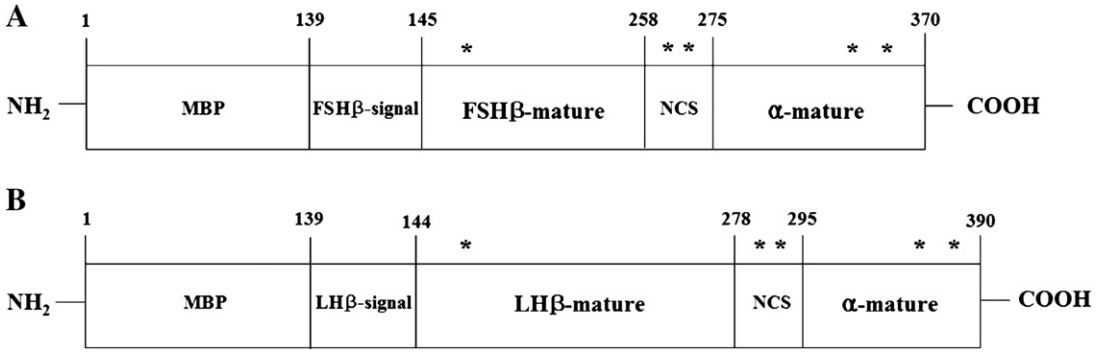

Fig. 1. Schematic diagrams of recombinant single-chain cinnamon clownfish follicle stimulating hormone (rFSH) (A) and recombinant luteinizing hormone (rLH) (B). The numbers above each box refer to the amino acid position in each fragment or gonadotropin subunit. Asterisks above each box indicate the putative N-linked glycosylation sites (Asn-X-Ser/Thr).

Arbor, MI, USA). After blocking, the membrane was sequentially incubated with a 1:2000 dilution of polyclonal mouse anti-MBP (Abcam, Cambridge, MA, rFSHβ-GTHα and rLHβ-GTHα) overnight at 4 °C and a peroxidase-conjugated polyclonal antibody to mouse IgG (1:2000; goat anti-mouse IgG-HRP; Santa Cruz Biotechnology, Santa, Cruz, CA, USA) for 2 h at room temperature. After washing, the membrane was incubated with an enhanced chemiluminescent detection reagent (Amersham Biosciences, Fairlawn, CT, USA) according to the manufacturer's directions. Bands were visualized by exposure to X-ray film (Amersham Biosciences).

2.4. Treatment procedure

To investigate the effects of rLH and rFSH, *in vivo*, fish were reared in 220-L circulating filter tanks in the laboratory and were anesthetized with tricaine methane sulfonate prior to injection. rLH and rFSH were dissolved in 0.9% physiological saline, and each fish was given an injection of the either rLH or rFSH (0.1 and 1 μg/g, body mass [BW]) at a volume of 10 μL/g BW and sham group of fish was injected with a equal volume of 0.9% NaCl (10 μL/g BW). After the injection, gonad samples were removed from five fish at 0, 6, 12, 24, and 48 h. During the experimental period, the water temperature and photoperiod were maintained at 26 ± 1 °C and 12L/12D, respectively.

2.5. Gonadal culture, in vitro

For *in vitro* experiments, the gonads were dissected in to small pieces in cold culture phenol red free Medium 199 (Invitrogen, Carlsbad, CA, USA) from 10 immature cinnamon clownfish. Approximately 5 mg of testes slices (20 μm thick) were added per well for 1 h prior to hormone treatment in 24-well culture plates (SPL Life Sciences, Seoul, South Korea). Cultured gonadal tissues were treated with 0.1 and 1 μg/mL of either rLH or rFSH in M199 for 0, 6, 12, 24, and 48 h at 18 °C, 100% humidity, and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20 °C, 10,000*g*, 15 s), the supernatant and gonadal tissues were removed and stored separately in micro-centrifuge tubes at −80 °C.

2.6. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of GTH subunits (GTHα, FSHβ, and LHβ), GTH receptors (FSHR, LHR), and *Vtg*, using total RNA extracted from the pituitaries (GTH subunits), gonad (GTH receptors), and liver (*Vtg*) of cinnamon clownfish, respectively. Primers for QPCR are shown in Table 1. QPCR amplification was conducted as described previously (Kim et al., 2010) using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following conditions: 0.5 μL of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, Sybr Green and Taq polymerase in buffer (10 mM Tris–HCl [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein) to a total volume of 25 μL. QPCR was conducted as follows: one cycle of denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 20 s and annealing at 55 °C for 20 s. After the PCR program, QPCR data from three replicate samples were analyzed with system analysis software (Bio-Rad) to estimate transcript copy numbers for each sample. Each experimental group was run in triplicate to ensure consistency. Experiments were duplicated with β-actin (accession no. JF273495) as an internal control, and all data are expressed as the change with respect to the corresponding β-actin calculated threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated $\Delta\Delta Ct = 2\text{^}-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$. Also, to ensure that the primers amplified a specific product, we performed a melt curve, and a single melting point was observed for the products of each primer pairs.

Table 1
Primers used for QPCR amplification.

GenBank ID	Gene name	Primer sequences
EU908056	GTHα-F	5′-AAG TCC ATG AAG ACG ATG ACA ATT CC-3′
	GTHα-R	5′-GTG GCA CTG TGT ATG GTT TCT CAC-3′
FJ868867	FSHβ-F	5′-AGC GGC GAC TGG TCC TAC G-3′
	FSHβ-R	5′-CGT CTC CAT CAA ACC TCC CAC AG-3′
FJ868868	LHβ-F	5′-GGT GTC TCT GGA GAA GGA GGG ATG-3′
	LHβ-R	5′-TGA ACA GCG TCT TGA TGA CTG GAT C-3′
GU722648	FSHR-F	5′-CCT CTC ATT ACC GTG TCC GAC TC-3′
	FSHR-R	5′-GGG TGA AGA AGG CAT ACA GGA AGG-3′
GU722649	LHR-F	5′-GGA AAC AGA AAT AGA GCC CAC TAC AG-3′
	LHR-R	5′-CAC TTG ACG AAG GGG TTG TTA AGA C-3′
HM185181	*Vtg*-F	5′-GAG ATT CTG AAA CAC CTG-3′
	Vtg-R	5′-ACA GCG TCT TGA TGA CTG GAT C-3′
JF273495	β-actin-F	5′-GGA CCT GTA TGC CAA CAC TG-3′
	β-actin-R	5′-GCTGAA ATA ATT CCA CAA ACT T-3′

2.7. Plasma parameters analysis

Plasma E_2 levels were analyzed by radioimmunoassay (RIA) using an E_2 RIA kit (DIASORIN, Antony, France). Duplicate 200 μL aliquots of plasma samples were extracted in 3 mL of ethyl-acetate/cyclohexane (v/v) mixed for 5 min. After 2 h, the tubes were centrifuged for 15 min and frozen in liquid nitrogen. The solvent layer was decanted, transferred into glass tubes, and evaporated under nitrogen. Standards and samples were reconstituted in 150 μL of 0.1 M PBS (pH 7.4) that contained 1 g/mL BSA and 1 mM EDTA. Samples were incubated in primary antibody (150 μL) for 24 h at 4 °C, and ^{125}I estradiol (100 μL, 30,000 cpm) was added to each tube for 24 h at 4 °C. The secondary antibody (500 μL of precipitating solution) was added, mixed, and incubated at 4 °C for 1 h. Subsequently, 2 mL of 0.025 M Tris buffer (pH 7.4) was added, the tubes were centrifuged at 1000*g* for 30 min, the supernatant was discarded, and the radioactivity was measured.

2.8. Statistical analysis

All data were analyzed using the SPSS statistical package version 10.0 (SPSS, Inc., Chicago, IL, USA). A one-way analysis of variance followed by Tukey's *post hoc* test was used to compare differences. A P-value <0.05 was considered significant. Values are expressed as mean ± SD.

3. Results

3.1. Production of rGTHs

To study the biological activities and physiological significance of the two cinnamon clownfish GTHs, we produced rLH and rFSH using the pMAL protein fusion and purification system with *E. coli*. The result showed that the concentration of the purified MBP-tagged FSHβ-GTHα and LHβ-GTHα protein was 0.2 mg/mL and 0.3 mg/mL by Bradford assay, respectively. Specific bands corresponding to molecular sizes of 68 kDa for rFSHβ-GTHα and 69 kDa for rLHβ-GTHα were obtained, using MBP tagging of *E. coli* BL21 (DE3) pLysS cells resolved by SDS-PAGE (Fig. 2A). We also used anti-MBP antibodies (FSHβ-GTHα and LHβ-GTHα) to detect the same bands in a Western blot analysis (Fig. 2B). In the present study, rLH and FSH were used, *in vivo* and *in vitro* experiments. For *in vitro* experiments, we used rLH and rFSH at 0.1 and 1 μg/mL. These doses correspond to 1.5 and 15 nM, respectively. For *in vivo* experiments, fish were injected with rLH and rFSH at 0.1 and 1 μg/g BW which corresponds to 1.5 and 15 nM/g of BW.

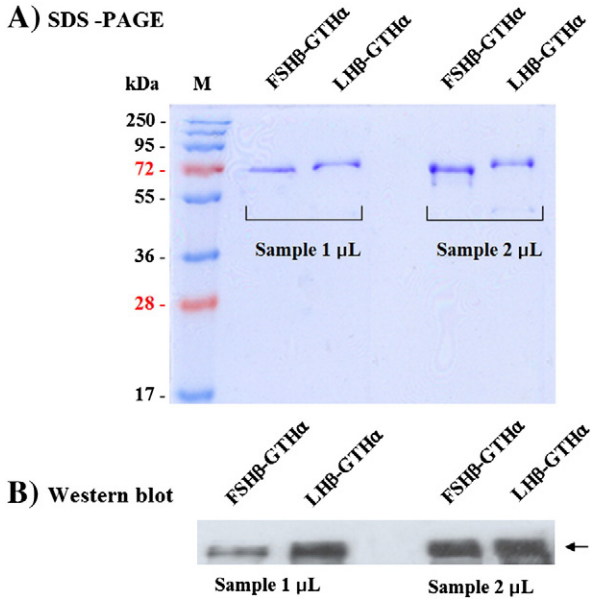

Fig. 2. Production of recombinant follicle stimulating hormone (rFSH) and recombinant luteinizing hormone (LH). (A) SDS-PAGE analysis of purified rFSH and rLH. (B) Western blot analysis of purified rFSH and rLH using a MBP-Tag probe. M, protein markers.

3.2. Time and dose-related effects of rLH and rFSH on pituitary GTH subunits mRNA levels, in vivo

Pituitary expression levels of GTH subunits (GTHα, FSHβ, and LHβ) were measured following treatment with rLH and rFSH (Fig. 3). The results provide information on time and dose related effects of FSHβ and LHβ and their autoregulatory properties on GTH subunit mRNA levels *in vivo*. Treatments with both rLH and rFSH significantly increased GTHα, mRNA level, although rFSH exerted a greater effect, especially at higher dose tested (Fig. 3A). Treatments with both rLH and rFSH significantly increased FSHβ, and LHβ mRNA levels. The results demonstrate that rFSH exerted greater stimulation of FSHβ than LHβ mRNA level, and rLH exerted greater stimulation of LHβ than FSHβ mRNA levels (Fig. 3B,C).

3.3. Time and dose-related effects of rLH and rFSH on gonadal LH and FSH receptor mRNA levels, in vivo

To further elucidate the autoregulatory effects of LH and FSH, we measured LH and FSH receptor (LHR and FSHR) mRNA levels in the gonadal tissues following treatments of rLH and rFSH, *in vivo* in cinnamon clownfish. Injection with rFSH, significantly increased FSHR after 6 h and the rFSH-induced response at the 15 nM/g BW remained significant even after 48 h of treatment. At the lower dose of rFSH (1.5 nM/g BW) tested, significant increase in FSHR was observed after 12 and 24 h of injection (Fig. 5). Injection with rLH resulted in small but significant increase in FSHR mRNA level at the higher dose tested after 12 and 24 h of administration (Fig. 5A). For LHR, only injection with rLH significantly increased LHR mRNA level, with the exception of very small increase in LHR following 12 h of higher dose of rFSH treatment. In terms of magnitude of response, rLH-induced increase in LHR mRNA level was detected earlier, following 6 h of the higher dose of rLH injection, compared to rFSH-induced increase in FSHR observed after 24 h of injection (Fig. 5).

3.4. Time and dose-related effects of rLH and rFSH on gonadal GTH subunits and receptors mRNA levels, in vitro

In this experiment, direct actions of rLH and rFSH on GTH subunits (GTHα, FSHβ, and LHβ) and receptors (FSHR and LHR) mRNA were

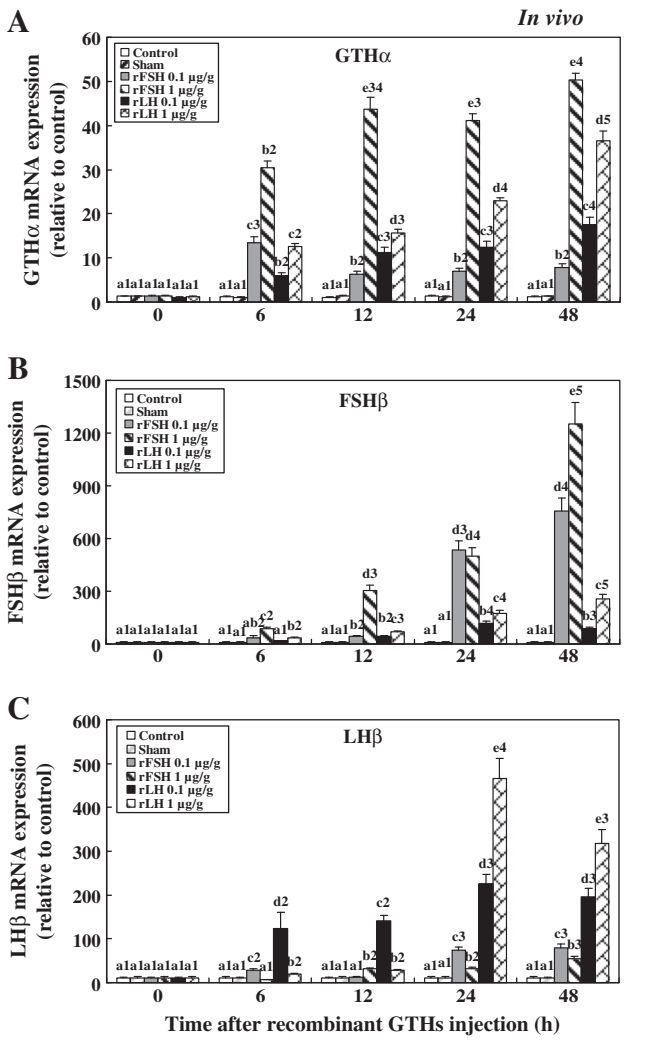

Fig. 3. Time-related effect of recombinant follicle stimulating hormone (rFSH) and recombinant luteinizing hormone (rLH) (0.1 and 1 μg/g) on gonadotropin (GTHα) (A), FSHβ (B), and LHβ (C) mRNA levels in cinnamon clownfish pituitary. Total RNA was extracted at 0, 6, 12, 24, and 48 h after treatment, and 3 μg of RNA was used for PCR. The expression level of each sample was normalized with respect to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P<0.05$). All values are means ± SD ($n=5$).

tested in cultured gonads (Figs. 4 and 6). Overall, treatments with both rLH and rFSH significantly increased all three GTH subunits mRNA levels. Treatments with both rLH and rFSH significantly increased GTHα mRNA level in the cultured gonadal tissue. However, rFSH exerted a greater effect on GTHα after 12 and 48 h of treatment (Fig. 4A). Similar to pituitary, treatment with rFSH exerted greater stimulation of FSHβ than LHβ mRNA level, and rLH treatment exerted greater stimulation of LHβ than FSHβ mRNA levels, although not consistently at all time points (Fig. 4B,C). In all cases, no significant change was observed following treatments with rLH and rFSH after 6 h in cultured cinnamon clownfish gonads (Fig. 4).

Both rLH and rFSH significantly increased FSHR mRNA levels after 12 h of incubation, *in vitro*. However, the magnitude of rFSH-mediated response on FSHR mRNA level was greater for rFSH than rLH at all time points (Fig. 6A). The response for LHR was somewhat different. Treatment with rLH significantly increased LHR after 12 h with maximum effect observed at higher level of rLH after 24 h of incubation. Treatment with rFSH also caused a significant increase in LHR after 24 h, although to a much lesser extent compared to rLH

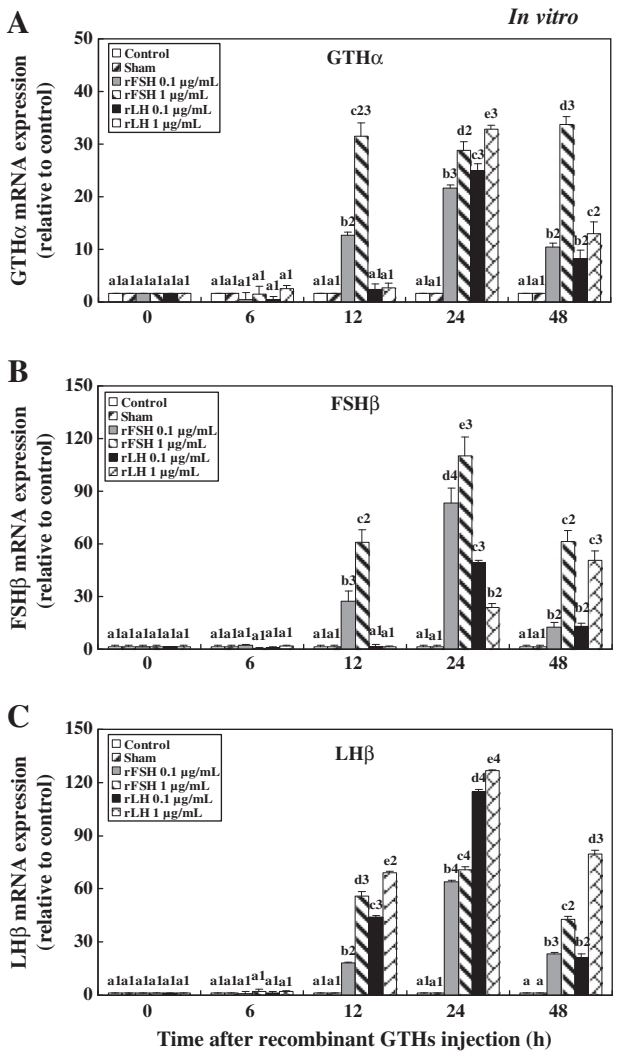

Fig. 4. Time-related effect of recombinant follicle stimulating hormone (rFSH) and recombinant luteinizing hormone (rLH) (0.1 and 1 μg/mL) on gonadotropin (GTHα) (A), FSHβ (B), and LHβ (C) mRNA levels in cinnamon clownfish cultured gonads. Total RNA was extracted at 0, 6, 12, 24, and 48 h after treatment, and 3 μg RNA was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P<0.05$). All values are means ± SD ($n=5$).

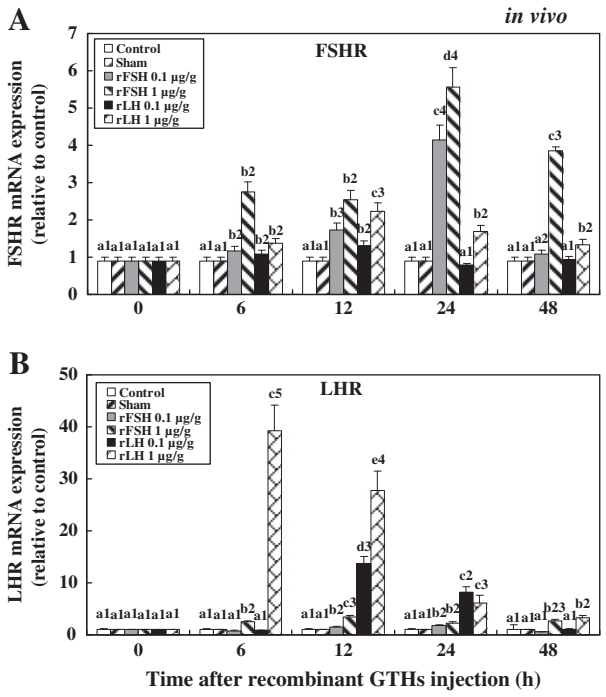

Fig. 5. Time-related effect of recombinant follicle stimulating hormone (rFSH) and recombinant luteinizing hormone (rLH) (0.1 and 1 μg/g) on follicle stimulating hormone receptor (FSHR) (A) and luteinizing hormone receptor (LHR) (B) mRNA levels in the cinnamon clownfish gonad. Total RNA was extracted 0, 6, 12, 24, and 48 h after treatment, and 3 μg RNA was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P<0.05$). All values are means ± SD ($n=5$).

(Fig. 6B). A small but significant increase in LHR was also observed following incubation with rFSH after 48 h.

3.5. Time and dose-related effects of rLH and rFSH on liver Vtg mRNA level, in vivo

Production of *Vtg* is estrogen dependent and provides a suitable parameter to estimate efficacy of rLH and rFSH, *in vivo*. Injection with both rLH and rFSH significantly increased *Vtg* mRHA level after 6 h of treatment (Fig. 7). rFSH-induced increase in *Vtg* mRNA level appeared earlier than rLH induced response. After 24 h of injection, however, rLH stimulated *Vtg* expression to a greater extent than rFSH. After 48 h, only rLH significantly increased *Vtg* mRNA level (Fig. 7).

3.6. Time and dose-related effects of rLH and rFSH circulating E_2 concentration, in vivo

As a follow up to measuring *Vtg* production, we also measured circulating level of E_2 in cinnamon clownfish following injection with rFSH and rLH. Injection with both rLH and rFSH significantly increased circulating E_2 concentration after 6 h. As in *Vtg* level, the effect of rFSH peaked earlier and the effect of rLH was greater at later time course by 24 h. After 48 h, the rLH and rFSH induced response were lower relative to earlier time points, but remained statistically significant (Fig. 8).

4. Discussion

Teleosts are among the most diverse species and display a wide range of plasticity in reproduction and gonadal development. There are both hermaphroditic and gonochoristic species that reproduce once a year, once in a lifetime or several times per year. While reproduction of gonochoristic teleosts has been studied extensively over many decades, much less information is available on reproductive biology of hermaphroditic species. Among hermaphroditic teleosts more information is available on reproductive endocrinology of protogynous hermaphrodite teleosts than protandrous species that begins life as a male and then changes into a female at a later stage. Cinnamon clownfish (*A. melanopus*) is a protandrous hermaphroditic teleosts, and study of its reproductive biology as a model species would be important to understand evolution and diversity of reproductive endocrinology among teleosts. To better investigate control of reproduction of cinnamon clownfish, we have provided molecular characterization of gonadotropins and GTH receptors (An et al., 2010), and GnRH forms (Kim et al., 2012), respectively. Here we describe a method to produce biologically active recombinant gonadotropins, rLH and rFSH, which was used to provide novel information on autoregulation of LH and FSH-induced responses. Given the size of these fish, it would be very difficult to extract and purify pituitary LH and FSH, and production of recombinant LH and FSH are the best

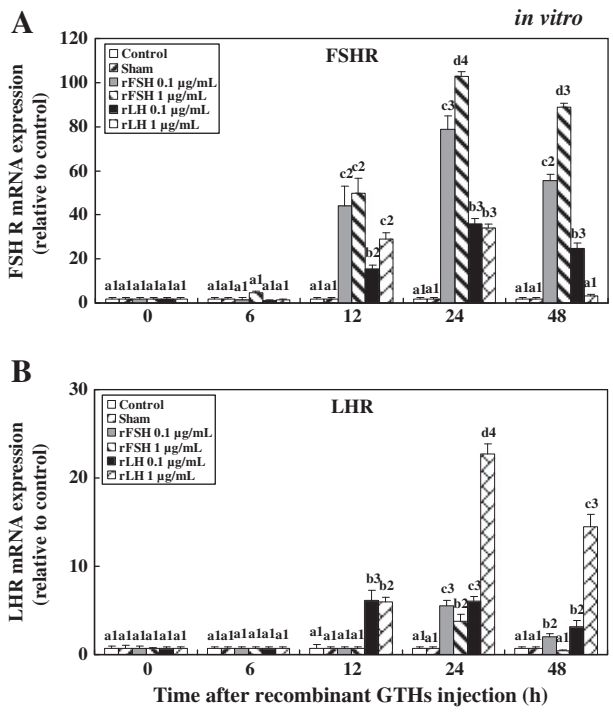

Fig. 6. Time-related effect of recombinant follicle stimulating hormone (rFSH) and recombinant luteinizing hormone (rLH) (0.1 and 1 μg/mL) on follicle stimulating hormone receptor (FSHR) (A) and luteinizing hormone receptor (LHR) (B) mRNA levels in cinnamon clownfish cultured gonad. Total RNA was extracted at 0, 6, 12, 24, and 48 h after treatment, and 3 μg RNA was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P<0.05$). All values are means ± SD ($n=5$).

approach. The present study is also important as it validate previously described approach to produce single chain recombinant LH and FSH (Kobayashi et al., 2010).

Our previous studies provided molecular characterization and cDNA sequence for FSHβ and LHβ of cinnamon clownfish, encoding predicted proteins of 119 and 139 amino acids, respectively (An et al., 2010). The three GTH subunits contain cysteine residues and a highly conserved N-linked glycosylation site, which are reportedly

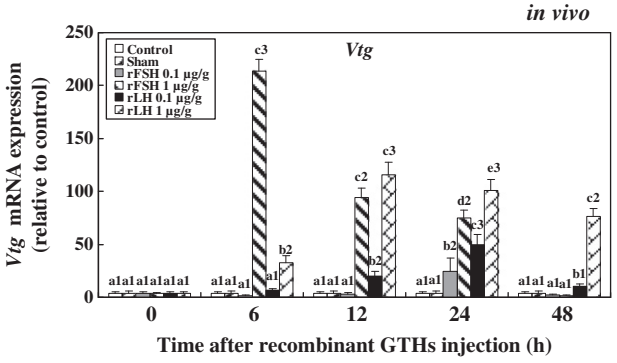

Fig. 7. Time-related effects of recombinant follicle stimulating hormone (rFSH) and recombinant luteinizing hormone (rLH) (0.1 and 1 μg/g) on *Vtg* mRNA levels in the cinnamon clownfish liver *in vivo*. Total RNA was extracted at 0, 6, 12, 24 and 48 h after treatment, and 3 μg RNA was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P<0.05$). All values are means ± SD ($n=5$).

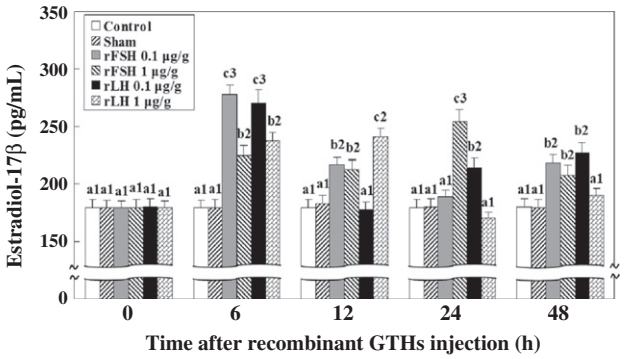

Fig. 8. Plasma estradiol-17β (E_2) levels during treatment with recombinant follicle stimulating hormone (rFSH) and recombinant luteinizing hormone (rLH). The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P<0.05$). All values are means ± SD ($n=5$).

sites for receptor binding in mammals and fish (Gen et al., 2000). Phylogenetic analyses suggest that the clownfish GTH subunits are closely related to those of other teleost species such as Atlantic salmon *Salmo salar* (Ando and Urano, 2005), rainbow trout *Oncorhynchus mykiss* (Sambroni et al., 2007), black porgy *Acanthopagrus schlegeli* (An et al., 2009), and red seabream *Pagrus major* (Gen et al., 2000).

In the present study, we produced single chain recombinant FSH and LH (rFSH and rLH), consisting FSHβ-GTHα and LHβ-GTHα, fusion proteins, respectively. Each fusion protein contains N-glycosylated protein, which is important for extending its half-life (Klein et al., 2003). The present results demonstrate that the single-chain rFSH and rLH as heterodimeric fusion proteins are biologically active and exert specific LH and FSH-like activity at least up to 48 h post treatment. This is consistent with pervious report on production of recombinant Manchurian trout FSH and LH (Ko et al., 2007). Overall, there is evidence that pituitary glycoprotein hormones carry highly conserved N-linked glycosylation sites, and the attached oligosaccharides are important for hormone bioactivity in teleosts (Klein et al., 2003).

Production of rFSH and rLH enabled us to investigate autoregulatory control of LH and FSH by investigating expression of pituitary and gonadal GTHα, FSHβ, and LHβ, gonadal LHR and FSHR, liver *Vtg* mRNA levels as well as circulating E_2 level in cinnamon clownfish. The results indicate that single chain rLH and rFSH are biologically active and exert specific autoregulatory actions by upregulation of GTH subunits in the pituitary and gonads. The present results demonstrate that rFSH has greater potency in up regulating FSHβ transcript level as well as FSHR in cinnamon clownfish. Likewise, rLH was found to have greater potency in up regulating LHβ transcript level as well as LHR. These observations provide novel information on homologous upregulatory mechanisms underlying GTH-mediated control of reproduction in cinnamon clownfish. We hypothesized that the results provide indirect evidence for LH and FSH receptor specificity. The observed autoregulatory mechanism may be particularly important during ovulation to facilitate maximum gonadotropic response to LH and FSH by upregulating LHR and FSHR in gonadal tissue as well as increasing production of GTHs in cinnamon clownfish. The present results are also consistent with previous observations on specificity of LH and FSH in Coho salmon, *Oncorhynchus kisutch* (Miwa et al., 1994), channel catfish, *Ictalurus punctatus* (Zmora et al., 2003), and zebrafish, *Danio rerio* (So et al., 2005), which suggested that FSH and LH exert their effects on ovarian functions through membrane receptors (GTHR) on the granulosa and theca cells.

The observed action of rFSH in immature clownfish is consistent with the view that FSH is generally involved in the formation of yolk and gametes, and LH is involved in final oocyte maturation and spermiation (Nagahama, 1994; Swanson et al., 2003). The present results are in agreement with previous report that FSH increases

significantly at the early maturation stage in red seabream, *Pagrus major*, striped bass, *Morone saxatilis*, and Black porgy, *Acanthopagrus schlegeli* (Gen et al., 2000; Hassin et al., 2000; An et al., 2009). Specially, Ko et al. (2007) reported that a single injection with rFSH and rLH significantly increased mean GSI and follicle diameters as compared with those of immature Manchurian trout (Ko et al., 2007).

As expected, plasma E_2 level increased following injection with rLH and rFSH which is consistent with previous studies on goldfish, *Carassius auratus* (Kobayashi et al., 2006), and African catfish (Vischer et al., 2003). Also, Ko et al. (2007) suggested that rmtFSH elevated plasma E_2 levels and in turn E_2 stimulated VTG synthesis and to some extent its uptake into immature ovarian follicles of immature Manchurian trout. The observed increases in GTH-induced E_2 level as well as LHR and FSHR suggest that gonad development is controlled through the combination of GTH and GTH-receptor activity in the gonads. Furthermore, increasing plasma E_2 levels results in oocyte development and maturation in immature teleosts, and plays an important role in sex change from male to female (Lee et al., 2000, 2001; An et al., 2008b, 2009). Thus, rGTH activates the brain–pituitary–gonad axis pathway in cinnamon clownfish.

A likely consequence of increase in rLH, and rFSH-induced circulating E_2 concentration was increased level of *Vtg* mRNA. The present results provide a better understanding on the mechanisms of LH and FSH stimulated production of E_2 and vitellogenesis which are critical steps in hormonal control of reproduction. The multistep autoregulatory process may in part explain the observed temporal increase in FSHR mRNA content during early vitellogenesis in other species, including channel catfish (Kumar and Trant, 2004), zebrafish (Kwok et al., 2005), and Manchurian trout (Ko et al., 2007). These previous studies suggest that FSH/FSHR increases during early vitellogenesis and promotes oocyte development, whereas LH/LHR is associated with gonadal maturation and sex change during late vitellogenesis (Rocha et al., 2007).

In summary, the present study provides information on the production of biologically active single chain recombinant cinnamon clownfish LH and FSH. The rLH and rFSH were subsequently used in experiments that provide valuable insight into autoregulatory mechanisms of gonadotropin control of reproduction in cinnamon clownfish. The finding will enhance our understanding of hormonal control of reproduction in cinnamon clownfish which is used here as a suitable model to investigate reproductive endocrinology in a protandrous hermaphroditic teleost.

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