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Effects of gonadotropin inhibitory hormone or gonadotropin-releasing hormone on reproduction-related genes in the protandrous cinnamon clownfish, *Amphiprion melanopus*

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ABSTRACT

Hypothalamic peptide neurohormones such as gonadotropin-releasing hormones (GnRHs) and gonadotropin-inhibitory hormone (GnIH) play pivotal roles in the control of reproduction and gonadal maturation in teleost fish. To study the effects of GnIH on fish reproduction, we investigated the influence of seabream GnRH (sbGnRH) and GnIH (both alone and in combination) on levels of reproductive genes (GnIH, GnIH-receptor [GnIH-R], melatonin receptor [MT_3], sbGnRH, and gonadotropic hormones [GTHs]) during different stages of gonadal maturation in male, female, and immature cinnamon clownfish, *Amphiprion melanopus*. The results showed that the expression levels of GnIH, GnIH-R, and MT_3 genes increased after the GnIH injection, but decreased after the sbGnRH injection. In addition, these gene expression levels gradually lowered after GnIH3 and sbGnRH combination treatment, as compared to the MT_3 mRNA levels of GnIH treatment alone. However, the expression levels of the HPG (hypothala mus–pituitary–gonad) axis genes (sbGnRH and GTHs) decreased after the GnIH injection, but increased after the sbGnRH injection. In all cinnamon clownfish groups, HPG axis gene mRNA levels gradually decreased after mixed GnIH3 and sbGnRH treatment, compared to GnIH treatment alone. The present study provides novel information on the effects of GnIH and strongly supports the hypothesis that GnIH plays an important role in the negative regulation of the HPG axis in the protandrous cinnamon clownfish.

1. Introduction

The discovery of gonadotropin-inhibitory hormone (GnIH) in the brain of Japanese quail, *Coturnix japonica* (Tsutsui et al., 2000), reinforced the concept of the multifactorial control of reproduction in vertebrates. In most species tested, GnIH appears to impair the gonadotropic axis and works in opposition to gonadotropin-releasing hormone (GnRH). However, in some species, especially gonochoristic fishes such as goldfish, *Carassius auratus* and salmonid, GnIH has both stimulatory and inhibitory effects on reproduction. Thus, our current understanding is that GnIH may be an important regulatory hormone in the multifactorial control of gonadotropin production (Tsutsui et al., 2012; Tsutsui and Ubuka, 2014). GnIH belongs to the family of Arg-Phe (RF)-amide peptides and a class of amidated peptides with an arginine and phenylalanine motif at the C-terminal end ([Ser-Ile-Lys-Pro-Ser-Al a-Tyr-Leu-Pro-Leu-Arg-Phe-NH2, SIKPSAYLPLRFamide]) (Tsutsui et al., 2000). This peptide plays a role in the control of reproduction and maturation in vertebrate species (Tsutsui and Ubuka, 2014). In vertebrates, neurons containing RF-amide peptides project to the hypothalamic region that is near the pituitary gland, suggesting that RF-amide peptides have hypophysiotropic actions and GnIH was shown to inhibit the release of luteinizing hormone (LH) (Tsutsui et al., 2010).

Studies on birds demonstrated that GnIH acts on the anterior pituitary gland through a specific GnIH-receptor (GnIH-R or GPR147), and inhibits the synthesis and release of LH, thereby controlling the reproductive cycle by influencing the brain–pituitary–gonadal axis (Bentley et al., 2006; Tsutsui et al., 2006; Ubuka et al., 2008).

Abbreviations: BM, body mass; GnIH, gonadotropin-inhibitory hormone; GnRHs, gonadotropin-releasing hormones; GSI, gonadosomatic index; GTH, gonadotropic hormone; FSH, follicle-stimulating hormone; HPG axis, hypothalamus–pituitary–gonad axis; LH, luteinizing hormone; MT, melatonin receptor; RT, Reverse transcription; QPCR, Quantitative PCR.

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Fishes are among the most variable species with respect to the control of sex differentiation, gonadal development, and reproductive function, ranging from gonochoristic species that develop to either male or female, to hermaphroditic organisms. Studies in fish revealed that GnIH plays a more complex role in the control of gonadotropin production, exerting both inhibitory and stimulatory actions (Amano et al., 2006; Moussavi et al., 2012, 2013). In sockeye salmon, all three goldfish LPXRFamide peptides were found to stimulate the release of gonadotropin hormones and growth hormone in a dose-dependent manner, with no effect on somatolactin or prolactin (Amano et al., 2006). In goldfish, GnIH exerts stimulatory and inhibitory actions, both directly at the pituitary level and indirectly by affecting other neurohormones, depending on sex, season, and mode of administration on LH, follicle-stimulating hormone (FSH) (Moussavi et al., 2012, 2013), and growth hormone (Moussavi et al., 2014). Intraperitoneal injections of GnIH in female goldfish were found to significantly decrease salmon GnRH (sGnRH) mRNA expression in the hypothalamus and FSHβ mRNA levels in the pituitary (Qi et al., 2013).

The multifactorial control of sexual development in fish involves GnRH, GnIH, FSH, and LH, gonadal steroids and peptides, and a number of other neurohormones (Lee et al., 2001; Moussavi et al., 2012). A key regulator of this system is GnRH, which stimulates the synthesis and release of pituitary LH and FSH (Tsutsui and Ubuka, 2014). In most species investigated, FSH regulates both estrogen for vitellogenesis and spermatogenesis, and LH promotes follicular maturation, ovulation, and the synthesis of steroid hormones in teleosts (Ando and Urano, 2005; Kobayashi et al., 2006; Okubo and Nagahama, 2008).

In teleost fish, GnRH-expressing neurons are distributed among three distinct GnRH populations (sGnRH, chicken GnRH-II [cGnRH-II], and seabream GnRH [sbGnRH]) within the brain (Andersson et al., 2001). sbGnRH is produced as the third form in neuronal groups localized in the ventral forebrain along the terminal nerve and controls gonadotropic hormone (GTH) secretion, and has also been implicated in the regulation of spawning behavior (Senthilkumaran et al., 1999). Okuzawa et al. (1997) reported that the expression of sbGnRH mRNA was approximately 17-fold and 9-fold higher than that of sGnRH and cGnRH-II mRNA, respectively, in the spawning period of the red seabream, *Pagrus major*.

Photoperiod is one of the key environmental factors that regulate reproduction. Photoperiod-mediated changes in reproductive hormone production are mainly mediated by melatonin, which is released from the pineal gland and retina. Melatonin levels increase at night and decrease during the day, acting as a neuroendocrine signal that is closely associated with the regulation of circadian rhythms (Falcón et al., 2007). Specific melatonin receptors (MTs) mediate the actions of melatonin, which in some species leads to the impairment of sexual maturation by inhibiting FSH and LH release (McGuire et al., 2011; Sébert et al., 2008). Teleost fish have three subtypes of MTs: MT_1, MT_2, and MT_3 (Ikegami et al., 2009; Shin et al., 2011). A previous study found that the melatonin-mediated responses elicited by the activation of MT_1 and MT_2 are dependent on circadian time, duration, and mode of exposure to endogenous or exogenous melatonin, as well as functional receptor sensitivity (Dubocovich and Markowska, 2005). In contrast, melatonin acts via MT_3 to affect the neurogenic function of GnIH gene expression in the testis and interacts with other hypothalamic peptides in the reproductive control system via its response to light levels and photoperiod, and stimulates GnIH synthesis and secretion (McGuire et al., 2011; Ubuka et al., 2005).

Cinnamon clownfish, *Amphiprion melanopus* typically exist as a mated adult pair or an adult pair with an immature individual. In this species, social ranking in the group influences the sex of the fish (Godwin and Thomas, 1993). In general, the female is larger and more dominant than the male. If a dominant female dies or is absent, the male partner undergoes a sex change to become female, and the immature fish develops into a male (Godwin and Thomas, 1993).

The purpose of this study was to evaluate the effect of GnIH on cinnamon clownfish during the period of sex change and the relationship between hypothalamus–pituitary–gonad axis (HPG axis) regulation and sexual maturation. We tested the hypothesis that in this protandrous hermaphroditic fish, treatment with GnIH and other reproductive hormones will affect mature females more than male and immature fish. In the present study, we investigated the effects of GnIH, sbGnRH, and combination treatment (GnIH [0.1 μg/g] + sbGnRH [0.1 μg/g]) in immature, male, and female cinnamon clownfish. We measured the mRNA expression of GnIH, GnIH-R, GnRH, GTHs, and MT_3, as well as plasma GnRH, GTHs, and melatonin. To our knowledge, this study is the first of its kind to focus on the sex change of teleost fish, and in particular, the cinnamon clownfish.

2. Materials and methods

2.1. Experimental conditions and fish

The study was conducted with families of artificially created cinnamon clownfish (immature fish, $n = 100$, length, 4.6 ± 0.5 cm, weight, 6.3 ± 0.4 g, gonadosomatic index [GSI; gonad weight/body weight × 100] = 0.03 ± 0.03; male, $n = 100$, length, 6.5 ± 0.4 cm, weight, 11.5 ± 0.3 g, GSI = 0.11 ± 0.03; female, $n = 100$, length, 8.4 ± 0.5 cm, weight, 14.6 ± 0.5 g, GSI = 0.15 ± 0.03).

The experimental design used for artificially created immature, male, and female cinnamon clownfish in the present study was modified from the method described by Kim et al. (2014). Briefly, fish were reared in 100-L circulating filter tanks in the laboratory and were anesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO, USA) prior to injection. Kiss (metastin 45–54 amide; M2816; Sigma) was dissolved in physiological saline (0.85% NaCl in distilled water), and male and female fish were injected intraperitoneally with either Kiss (0.1 μg/g, body mass [BM]) at a volume of 10 μL/g BM (experimental group), or an equal volume of 0.9% NaCl (10 μL/g BM) (sham group) once a week for 9 weeks.

Fish were purchased from CCORA (Corea Center of Ornamental Reef & Aquarium, Jeju, Korea). The water temperature was 27 ± 1 °C, and the photoperiod was a 12 h light:12 h dark cycle (lights on 07:00–19:00 h). The fish were fed a commercial diet twice a day (09:00 h and 17:00 h). The fish were reared under these conditions for 9 weeks.

2.2. Hormone treatment and sampling

The next step was to investigate the effects of reproductive hormones (GnIH and sbGnRH) on the clownfish. The artificially created cinnamon clownfish family (immature fish, male, and female) was anesthetized with 2-phenoxyethanol prior to injection. GnIH3 (goldfish LPXRF-3 [SGTGLSATLPQRF-NH_2]; courtesy of H.R. Habibi [Moussavi et al., 2012]) and sbGnRH (des Gly^{10}-[D-Leu^6] LHRH, H4284; Bachem, Torrance, CA, USA) were dissolved in physiological saline (0.85% NaCl in distilled water), and each fish was injected intraperitoneally with GnIH3 (0.1 μg/g, BM), sbGnRH (0.1 μg/g BM), and GnIH3 (0.1 μg/g BM) + sbGnRH (0.1 μg/g BM) at a volume of 10 μL/g BM. The sham group was injected with an equal volume of physiological saline (0.85% NaCl in distilled water, 10 μL/g BM). The control samples (non-treatment groups) were removed prior to injection. Each tank (i.e., experimental group) contained an artificially created cinnamon clownfish family (immature fish, male, and female). After injection, samples (brain, pituitary, retina, gonad, liver, kidney, gill,

and muscle) of the artificially created cinnamon clownfish family (immature fish, male, and female) were removed from the fish at 0, 6, 12, and 24 h, immediately frozen in liquid nitrogen, and stored at −80 °C until the total RNA was extracted for analysis. Plasma samples were separated from blood by centrifugation (4 °C, 10,000 × *g*, 5 min) and stored at −80 °C until analysis. During the experimental period, the water temperature and photoperiod were maintained at 27 ± 1 °C and 12 h light:12 h dark, respectively.

2.3. Tissue distribution of GnIH and GnIH-R mRNAs

To examine the tissue distribution of the mRNA of select GnIH and GnIH-R, total RNA was extracted from the brain, pituitary, retina, gonad, liver, kidney, gill, and muscle. Total RNA was extracted from the tissues using Tri-Reagent® (MRC, Cincinnati, OH, USA). Reverse transcription (RT) with RNA samples was performed to synthesize cDNA using M-MLV reverse transcriptase (Promega, OH, USA), according to the instructions of the manufacturer. The following RT-PCR primers were designed with reference to the known sequences of the cinnamon clownfish (GenBank accession numbers: GnIH, **KT455505**; GnIH-R, **KT455506**; β-actin, **JF273495**): GnIH forward (5′-CTC CCT CTT CGC TTC GGG CG-3′) and reverse (5′-GCC GAA TCG CTG AGG GAG GT-3′) primers; GnIH-R forward (5′-CTG GTG GAC AAC CTC ATC AC-3′) and reverse (5′-TTG GAG AAG GCC AAC CAG TG-3′) primers; β-actin forward (5′-AGC ACG GTA TTG TGA CCA AC-3′) and reverse (5′-ACG GAA CCT CTC ATT GCC AG-3′) primers. PCR amplification was performed with specific primer sets with the Ex × Taq (RR001A, TaKaRa, Tokyo, Japan). PCR was carried out as follows: initial denaturation at 95 °C for 2 min; then 40 cycles consisting of denaturation at 95 °C for 20 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1 min; followed by 7 min at 72 °C for the final extension. Amplification of β-actin mRNA was used to verify the quality of the RT products, using a primer set specific for cinnamon clownfish β-actin cDNA. The amplified PCR products (10 μL) were electrophoresed on 1% agarose gels with 5 μL of lane marker (Labo Pass™, 1 kb ladder, catalog no. CMM7001; Seoul, Korea), detected by staining with ethidium bromide, and visualized by illumination with the UV light of a Molecular Imager ChemiDoc XRS+ System (Bio-Rad). Each reaction was run in triplicate.

2.4. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of GnIH, GnIH-R, MT_3, sbGnRH, and GTHs subunits (GTHα, FSHβ, and LHβ) mRNA using the total RNA extracted from the tissue samples. Primers for the QPCR are shown in Table 1. For the QPCR, we followed the MIQE (Minimum Information for Publication of qRT-PCR experiments) guidelines (Bustin et al., 2009). PCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad), according to the instructions of the manufacturer. QPCR was performed as follows: 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. Each experimental group was run in triplicate to confirm consistency. As an internal control, experiments were duplicated with β-actin. The efficiencies of the reactions were determined by performing QPCR. Amplification of a single product from PCR was confirmed by melt curve analysis, and representative samples were electrophoresed to verify that only a single product was present; a single melting curve was observed for the products of each primer pair. The amplification efficiencies were found to be as follows: β-actin = 99.7%, GnIH = 96.2%, GnIH-R = 95.9%, MT_3 = 90.3%, sbGnRH = 98.3%, GTHα = 91.2%, FSHβ = 92.6%, and LHβ = 92.2%. Transcript levels of genes were normalized to the levels of β-actin; the data are expressed as relative mRNA levels. All data were expressed as change with respect to the corresponding β-actin-calculated cycle threshold (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated as $\Delta\Delta Ct = 2\text{^}-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$. Based on QPCR assays, the intra- and inter-assay coefficients of variation (CV) for GnIH, GnIH-R, MT_3, sbGnRH, GTHα, FSHβ, and LHβ mRNAs were less than 10% and 15%, respectively.

Table 1
Primers used for amplification of QPCR.

Genes	Primer	DNA sequences
GnIH (**KT455505**)	Forward	5′-CCC TCT TCG CTT CGG GCG GGA TG-3′
	Reverse	5′-GAA TCG CTG AGG GAG GTT GAT A-3′
GnIH-R (**KT455506**)	Forward	5′-AAC CAC AGC GGC TCA GTG TGT CC-3′
	Reverse	5′-ACC AGA CAG AGG AAG ACA AA-3′
MT_3 (**HM107821**)	Forward	5′-CTG CTG GTG GTG ATG ATG-3′
	Reverse	5′-GGT CTC TCT TCC CTC CTG-3′
sbGnRH (**HQ883476**)	Forward	5′-CTG CTG GTG GTG ATG ATG-3′
	Reverse	5′-GGT CTC TCT TCC CTC CTG-3′
GTHα (**EU908056**)	Forward	5′-AAT GTT CCC GCC AGA GAA-3′
	Reverse	5′-AGA GGT TGG AGA AGG CAG-3′
FSHβ (**FJ868867**)	Forward	5′-CTC ATC CTG TCC GCA CTT-3′
	Reverse	5′-GAG AAG CAG CAG CCT GTA-3′
LHβ (**FJ868868**)	Forward	5′-ACC ATC ATC GTG GAG AGA G-3′
	Reverse	5′-GAT AGT TCA GGT CCG TTG TTT C-3′
β-actin (**JF273495**)	Forward	5′-GGA CCT GTA TGC CAA CAC TG-3′
	Reverse	5′-TGA TCT CCT TCT GCA TCC TG-3′

2.5. Plasma parameters analysis

Plasma melatonin, GnRH, FSH, and LH levels were analyzed using the immunoassay technique with the following ELISA kits: melatonin (catalog no. MBS013211; Mybiosource, USA), GnRH (catalog no. CSB-E08810f; Cusabio Biotech, China), FSH (catalog no. MBS035576; Mybiosource, USA), and LH (catalog no. MBS283097; Mybiosource, USA).

An anti-antibody that was specific to the antibody of the hormones (melatonin, GnRH, FSH, and LH) was pre-coated onto a microplate. Next, 50 μL of plasma, 50 μL of horseradish peroxidase-conjugate, and 5 μL of antibody were added to each well. The solution was mixed thoroughly and incubated for 2 h at 37 °C. Fifty microliters of Substrate A and Substrate B was then added to each well, and the mixture was incubated for 15 min at 37 °C in the dark. After incubation, 50 μL of stop solution was added to each well. Finally, the optical density of each well was determined within 10 min using a microplate reader set to 450 nm.

To assess assay parallelism, we compared the standard curve with a duplicate, and the standard curve was constructed with the following ranges: melatonin (3.12–100 pg/mL), GnRH (0.5–20 pg/mL), and GTHs (FSH and LH; 0.625–20 and 3.56–100 mIU/mL, respectively). The results of the ELISA kits indicated that there was no significant cross-reactivity or interference between these analytes and their analogues, and the intra-assay and inter-assay precisions were less than 10% and 15%, respectively.

2.6. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). Two-way analysis of variance followed by Tukey's post hoc test was used to evaluate for significant differences in the data ($P < 0.05$). Values are expressed as mean ± standard error (SE) of three independent measurements.

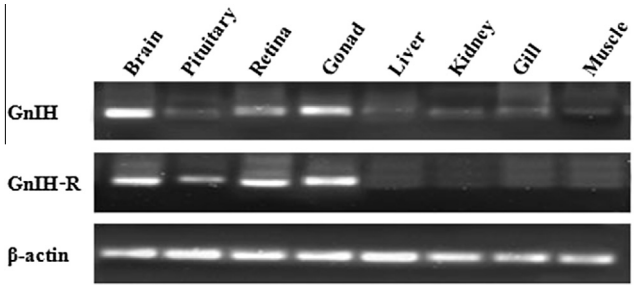

Fig. 1. Tissue distribution of cinnamon clownfish gonadotropin inhibitory hormone (GnIH) and GnIH-receptor (GnIH-R). Reverse transcriptase-PCR analysis of GnIH and GnIH-R transcripts in different tissues, as shown in a 1.0% agarose electrophoresis gel with ethidium bromide. The sizes of the RT-PCR products were 84 bp for GnIH, 701 bp for GnIH-R, and 560 bp for β-actin. The product of the β-actin gene as an internal control was amplified using the same tissue samples.

3. Results

3.1. Tissue distribution of GnIH and GnIH-R mRNAs

The tissue-specific expression patterns of cinnamon clownfish GnIH and GnIH-R mRNA are shown in Fig. 1. GnIH mRNA was primarily detected in whole tissue (brain, pituitary, retina, gonad, liver, kidney, gill, and muscle), and the highest levels of GnIH mRNA were detected in the brain, retina, and gonad of the cinnamon clownfish. GnIH-R mRNA was primarily detected in the brain, pituitary, retina, and gonad.

3.2. Time-course effects of hormone on GnIH and GnIH-R

In all cinnamon clownfish experimental groups, GnIH mRNA levels gradually increased to approximately 1.3-fold (Fig. 2A;

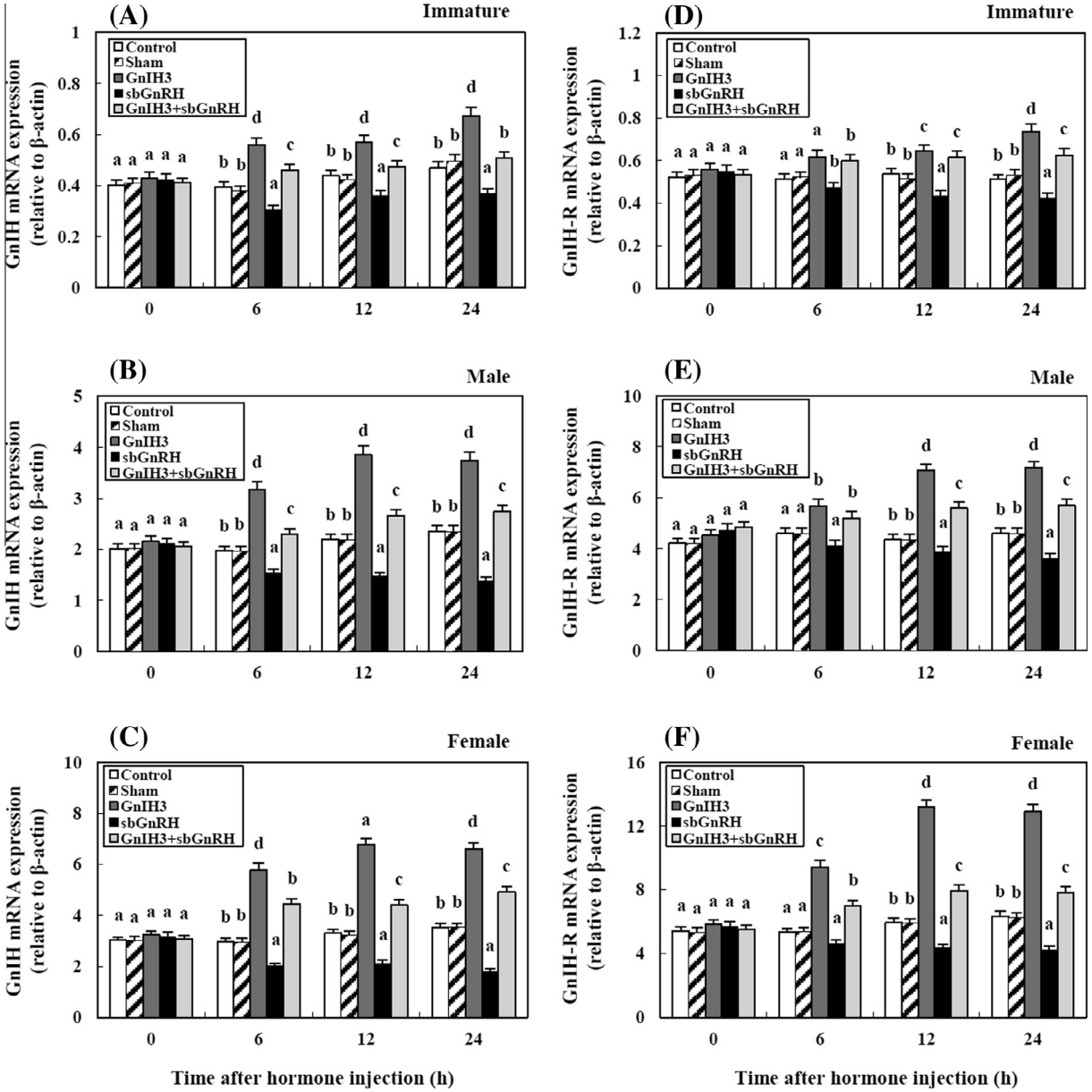

Fig. 2. Expression of gonadotropin inhibitory hormone (GnIH; A, B, C) and GnIH-receptor (GnIH-R; D, E, F) mRNA in cinnamon clownfish (immature fish, A and D; male, B and E; female, C and F) after GnIH3 (0.1 μg/g BM), seabream GnRH (sbGnRH) (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH). mRNA levels are relative to the β-actin mRNA levels in the brain of cinnamon clownfish. Values with letters indicate significant differences between treatments ($P < 0.05$). All values are means ± SE ($n = 5$).

immature fish), 2.1-fold (Fig. 2B; male fish), and 2.8-fold (Fig. 2C; female fish) higher at 24 h after GnIH3 treatment than the levels of untreated control fish (Fig. 2). In particular, the GnIH mRNA expression of female cinnamon clownfish was higher than that of immature fish (approximately 10.3-fold) and male fish (approximately 2.1-fold). However, in the sbGnRH treatment experiment, GnIH mRNA levels significantly decreased at 24 h after GnRH treatment in all cinnamon clownfish (Fig. 2). Furthermore, in all clownfish groups, GnIH mRNA levels gradually decreased after GnIH3 and sbGnRH combination treatment, as compared to GnIH mRNA levels after GnIH treatment alone (Fig. 2).

Similar to the expression of GnIH mRNA in cinnamon clownfish, the levels of GnIH-R mRNA expression in the cinnamon clownfish significantly increased with the administration of 0.1 μg/g GnIH (Fig. 2D, E, and F), and decreased with GnRH treatment. The *P* values of all of the experimental groups were less than 0.05.

3.3. Time-related changes in MT_3 and melatonin

In all cinnamon clownfish groups, MT_3 mRNA levels in the brain gradually increased to levels that were approximately 1.2-fold (Fig. 3A; immature fish), 1.4-fold (Fig. 3B; male fish), and 2.3-fold (Fig. 3C; female fish) higher at 24 h after GnIH3 treatment than the levels of the untreated control group (Fig. 3). In particular, the MT_3 mRNA expression of female cinnamon clownfish was greater than that of immature fish (approximately 4.7-fold) and males (approximately 1.8-fold). However, in the sbGnRH treatment experiment, MT_3 mRNA levels significantly decreased at 24 h after GnRH treatment in all fish (Fig. 4). In addition, in all groups, MT_3 mRNA levels gradually decreased after GnIH3 and sbGnRH combination treatment, as compared to the MT_3 mRNA levels of GnIH treatment alone (Fig. 3).

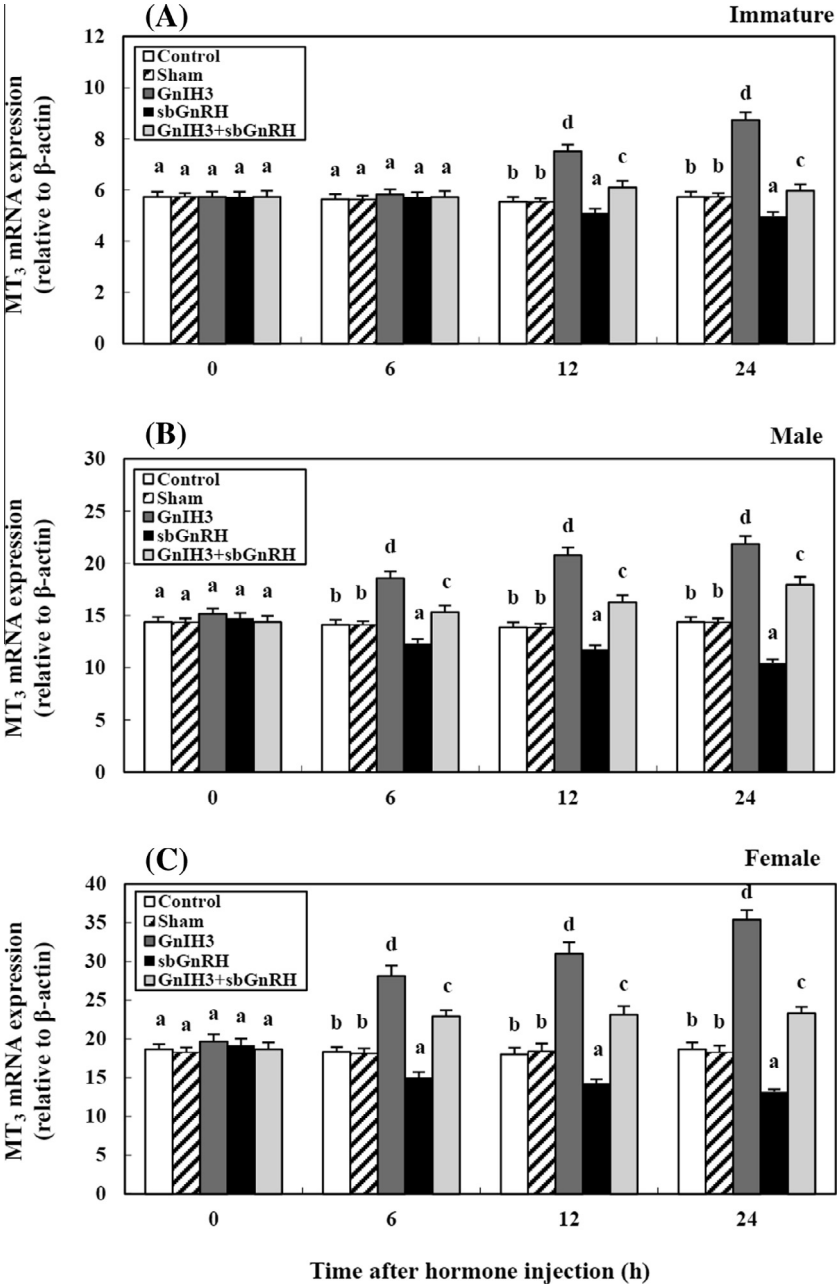

Fig. 3. Expression of melatonin receptor (MT_3) mRNA in the brain (A, B, C) of cinnamon clownfish (immature fish, A; male, B; female, C) after gonadotropin inhibitory hormone (GnIH3) (0.1 μg/g BM), seabream GnRH (sbGnRH) (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH). mRNA levels are relative to the β-actin mRNA levels in the brain of cinnamon clownfish. Values with letters indicate significant differences between treatments ($P < 0.05$). All values are means ± SE ($n = 5$).

Following GnIH injection, plasma melatonin levels increased to reach levels that were approximately 1.5-fold (Fig. 4A; immature fish), 1.3-fold (Fig. 4B; male fish), and 1.2-fold (Fig. 4C; female fish) higher after 24 h compared to the control group (Fig. 4). The plasma melatonin level was 401.2 ± 19.8 pg/mL (Fig. 4A; immature fish), 682.3 ± 17.5 pg/mL (Fig. 4B; male fish), and 896.7 ± 22.4 pg/mL (Fig. 4C; female fish) at the start of the experiment. The levels of plasma melatonin then increased to 463.2 ± 19.8 pg/mL (Fig. 4A; immature fish), 786.2 ± 29.8 pg/mL (Fig. 4B; male fish), and 1094.7 ± 30.1 pg/mL (Fig. 4C; female fish) after 24 h of GnIH3 treatment. However, plasma melatonin levels decreased following GnRH treatment. Furthermore, in all cinnamon clownfish groups, plasma melatonin levels gradually decreased after administration of combined GnIH3 and sbGnRH treatments, compared to plasma melatonin levels following GnIH treatment alone. The *P* values of all of the experimental groups were less than 0.05.

3.4. Time-related changes in GnRH

In all cinnamon clownfish groups, GnRH mRNA levels in the brain slowly increased to levels that were approximately 1.5-fold (Fig. 5A; immature fish), 4.4-fold (Fig. 5B; male fish), and 5.1-fold (Fig. 5C; female fish) higher at 24 h after sbGnRH treatment than the levels of the untreated control group (Fig. 5). In particular,

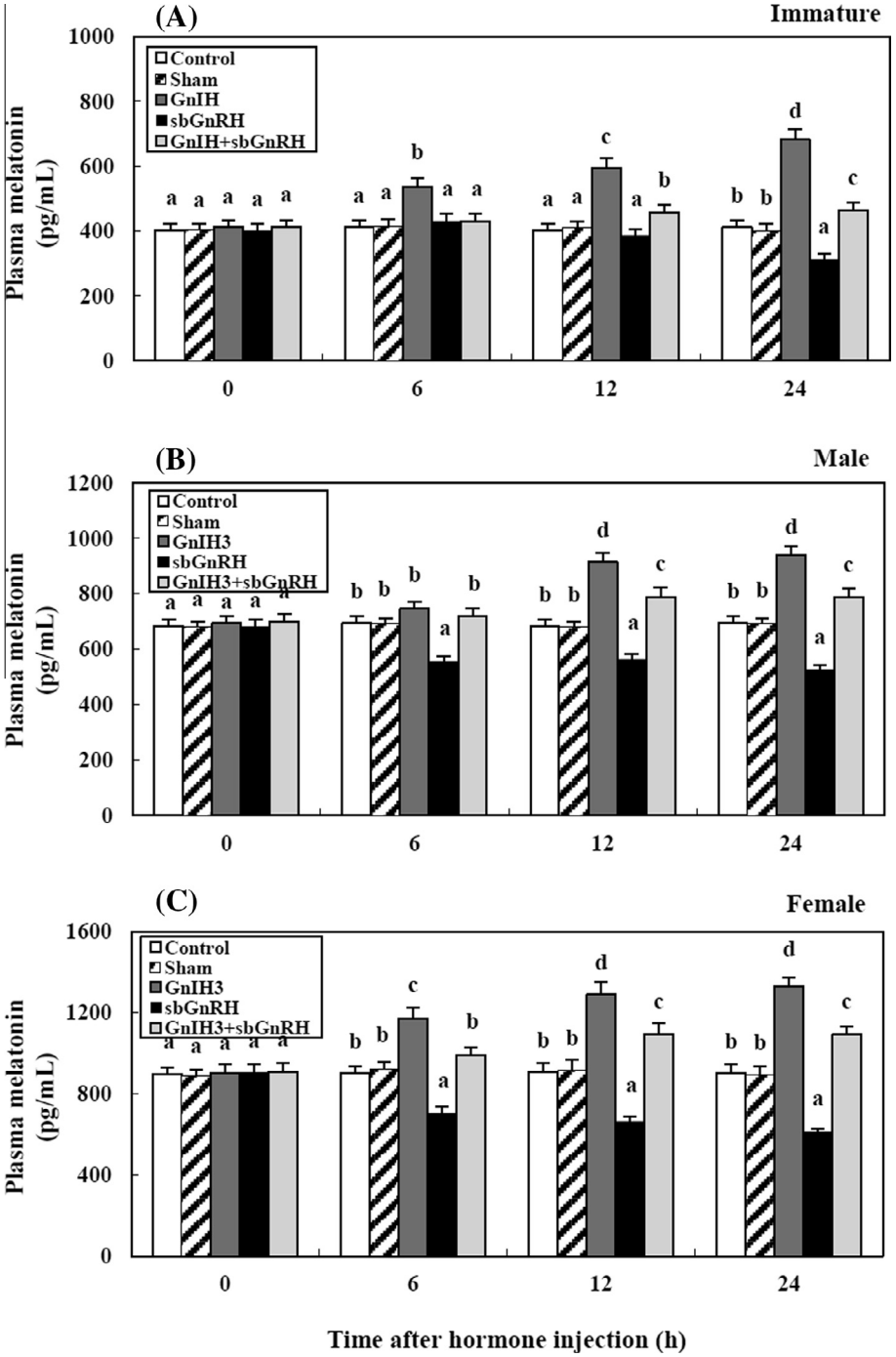

Fig. 4. The levels of plasma melatonin of cinnamon clownfish (immature fish, A; male, B; female, C) after gonadotropin inhibitory hormone (GnIH3) (0.1 μg/g BM), seabream GnRH (sbGnRH) (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH) treatment as analyzed with a plate reader. Values with letters indicate significant differences between treatments ($P < 0.05$). All values are means ± SE ($n = 5$).

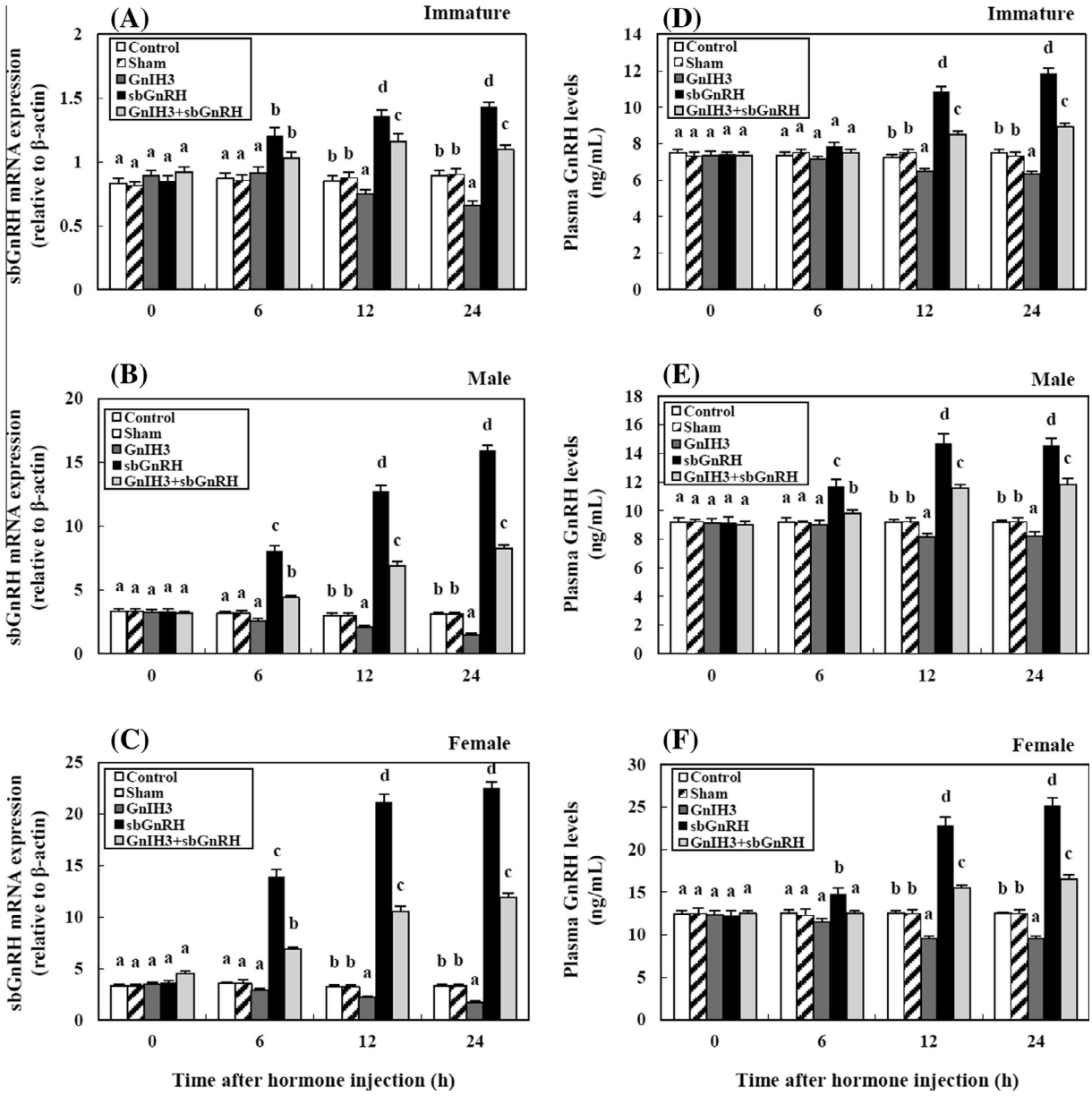

Fig. 5. Expression (A, B, C) and activity (D, E, F) of gonadotropin releasing hormone (GnRH) in cinnamon clownfish (immature fish, A and D; male, B and E; female, C and F) after gonadotropin inhibitory hormone (GnIH3) (0.1 μg/g BM), seabream GnRH (sbGnRH) (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH). sbGnRH mRNA levels are relative to the β-actin mRNA levels in the brain of cinnamon clownfish. The activity of plasma GnRH of cinnamon clownfish after hormone injection was also analyzed with a plate reader. Values with letters indicate significant differences between treatments ($P < 0.05$). All values are means ± SE ($n = 5$).

the GnIH mRNA expression of female cinnamon clownfish was higher than that of immature fish (approximately 11.2-fold) and male fish (approximately 1.3-fold). However, in the GnIH3 treatment study, sbGnRH mRNA levels significantly decreased at 24 h after GnRH treatment in all cinnamon clownfish (Fig. 5). In all clownfish groups, sbGnRH mRNA levels gradually decreased after mixed GnIH3 and sbGnRH treatment, compared to GnIH treatment alone (Fig. 5).

Similarly, the levels of plasma GnRH levels of the fish were significantly increased with sbGnRH treatment (0.1 μg/g) (Fig. 5D, E, and F), and decreased with GnIH treatment. The *P* values of all of the experimental groups were less than 0.05.

3.5. Time-related changes in GTHs

The expression pattern of GTHα, FSHβ, and LHβ mRNA in the pituitary of cinnamon clownfish correlated with changes in the sbGnRH mRNA levels in the brain of fish following GnIH3, sbGnRH, and GnIH3 + sbGnRH treatments (Figs. 6 and 7). In particular, GTHα, FSHβ, and LHβ mRNA levels in female cinnamon clownfish were higher than that of immature and male fish.

Treatment with 0.1 μg/g of sbGnRHs significantly increased the GTHα, FSHβ, and LHβ mRNA levels in all cinnamon clownfish groups. However, the levels of these transcripts were significantly decreased following treatment with 0.1 μg/g of the GnIH3. In all groups, GTHα, FSHβ, and LHβ mRNA levels gradually decreased after GnIH3 and sbGnRH combination treatment, compared to GnIH treatment alone (Figs. 6 and 7).

Similar to the mRNA expression of FSHβ and LHβ in the pituitary of cinnamon clownfish, the levels of plasma FSH and LH in these fish were significantly increased with the administration of 0.1 μg/g of sbGnRH and decreased following GnIH treatment (Fig. 8). The *P* values associated with all of the experimental groups were less than 0.05.

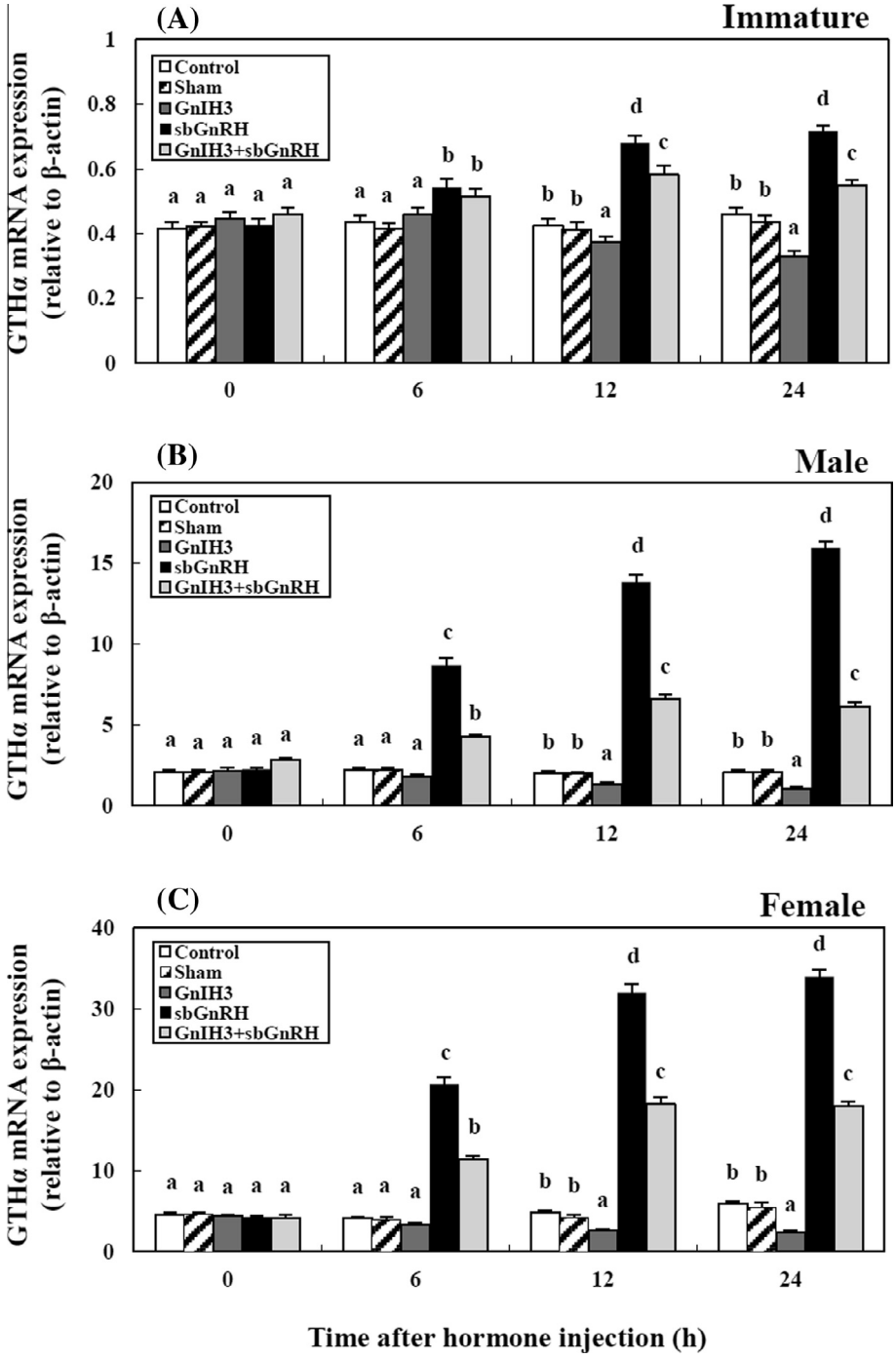

Fig. 6. Expression of gonadotropic hormone α (GTHα) mRNA in the pituitary gland of cinnamon clownfish (immature fish, A; male, B; female, C) after gonadotropin inhibitory hormone (GnIH3) (0.1 μg/g BM), seabream GnRH (sbGnRH) (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH) treatment. mRNA levels are relative to the β-actin mRNA levels in the pituitary of cinnamon clownfish. Values with letters indicate significant differences between treatments ($P < 0.05$). All values are means ± SE ($n = 5$).

4. Discussion

In the present study, we investigated the effects of GnIH in cinnamon clownfish during the period of sex change and sexual maturation in relation to other hormones of the HPG axis.

Clownfish were injected weekly with Kiss (0.1 μg/g) for 9 weeks to induce gonadal differentiation. The females and males were evaluated during the sex change stage.

The results demonstrate that GnIH expression levels in the brain, retina, and gonad were higher than in other tissues. The expression levels of GnIH-R were similar to those of GnIH that were also found in the pituitary. The present results are consistent with those reported by Sawada et al. (2002) and Zhang et al. (2010), with respect to the greater expression of GnIH and GnIH-R in the brain, retina, and gonads compared to other tissues in the zebrafish, *Danio rerio,* and the goldfish, respectively.

We observed a reduction in GnIH mRNA level in all experimental groups (immature, male, and female) following treatment with sbGnRH. Treatment with GnIH, however, resulted in increased GnIH and GnIH-R mRNA levels in all experimental groups

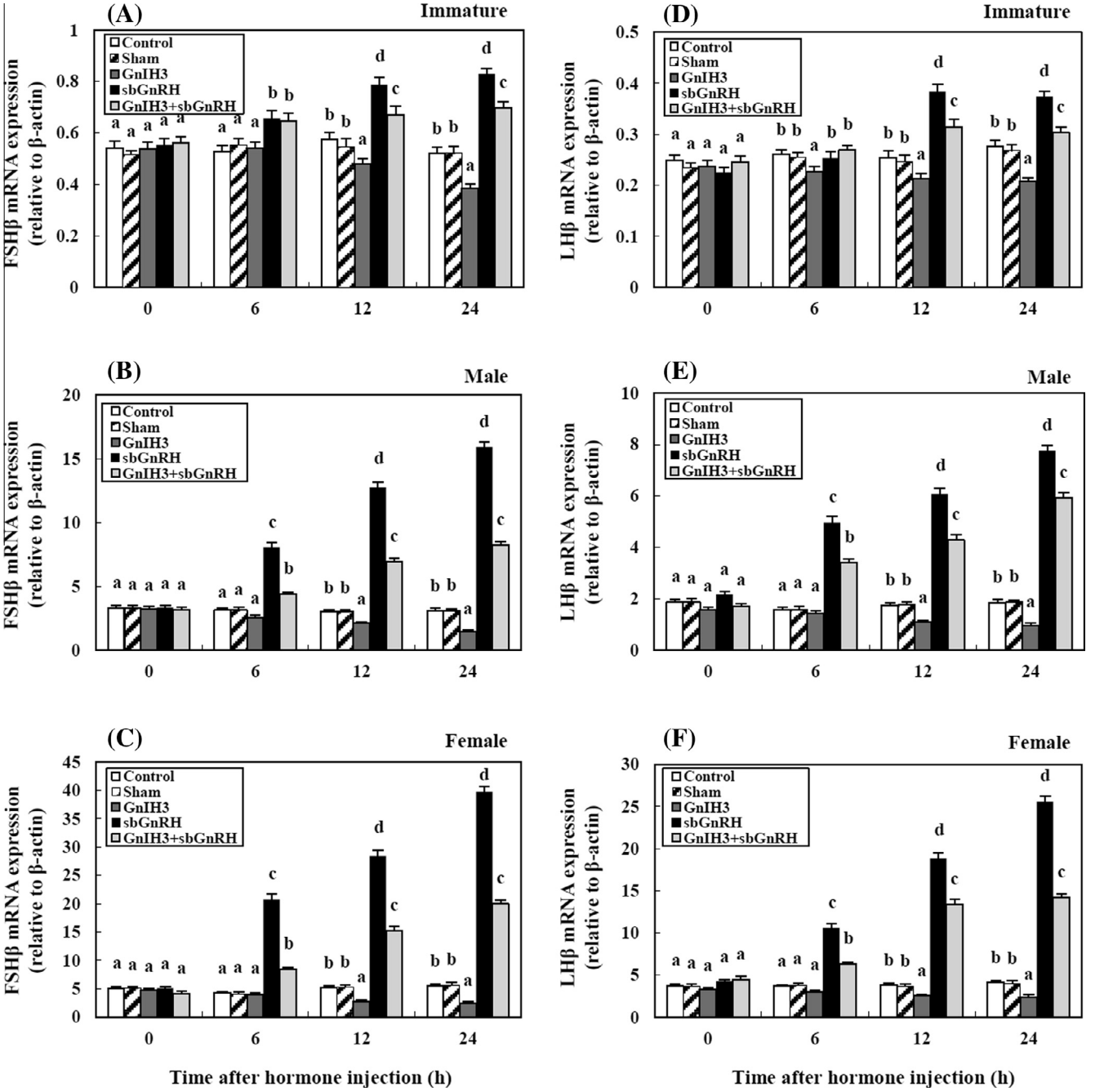

Fig. 7. Expression of follicle stimulating hormone (FSHβ; A, B, C) and luteinizing hormone (LHβ; D, E, F) in cinnamon clownfish (immature fish, A and D; male, B and E; female, C and F) after gonadotropin inhibitory hormone (GnIH3) (0.1 μg/g BM), seabream GnRH (sbGnRH) (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH) treatment. mRNA levels are relative to the β-actin mRNA levels in the pituitary of cinnamon clownfish. Values with letters indicate significant differences between treatments ($P < 0.05$). All values are means ± SE ($n = 5$).

(immature, male, and female). We observed significant differences in GnIH and GnIH-R mRNA levels in the mature individual (male and female), compared to immature fish. An earlier study by Kim et al. (2012) also reported increased levels of sbGnRH gene expression following injection with three GnRHs, and the authors suggested that sbGnRH may play an important role in the regulation of gonadal development and sex change in cinnamon clownfish. In gilthead seabream, *Sparus aurata* and barfin flounder, *Verasper moseri*, the levels of sbGnRH in the pituitary of sexually mature fish were found to be higher than cGnRH-II and sGnRH levels in early recrudescent fish (Amano et al., 2008; Holland et al., 1998). Shahjahan et al. (2010) also reported increased levels of sbGnRH gene expression in the spawning grass puffer, *Takifugu niphobles*, and suggested that sbGnRH may play a central role in final sexual maturation by stimulating GTH secretion in this species. In goldfish, GnIH mRNA was also increased following treatment with GnIH. The present results are also consistent with those in goldfish that demonstrate changes in the expression of GnRH mRNA levels at different stages of gonadal development and maturity (Moussavi et al., 2012). Furthermore, in chickens, the GnIH-R level was significantly lower in the pituitary of sexually mature birds (26 weeks old), compared to their sexually immature counterparts (16 weeks old) (Maddineni et al., 2008). The same study also described that the expression of GnIH-R mRNA significantly decreased following

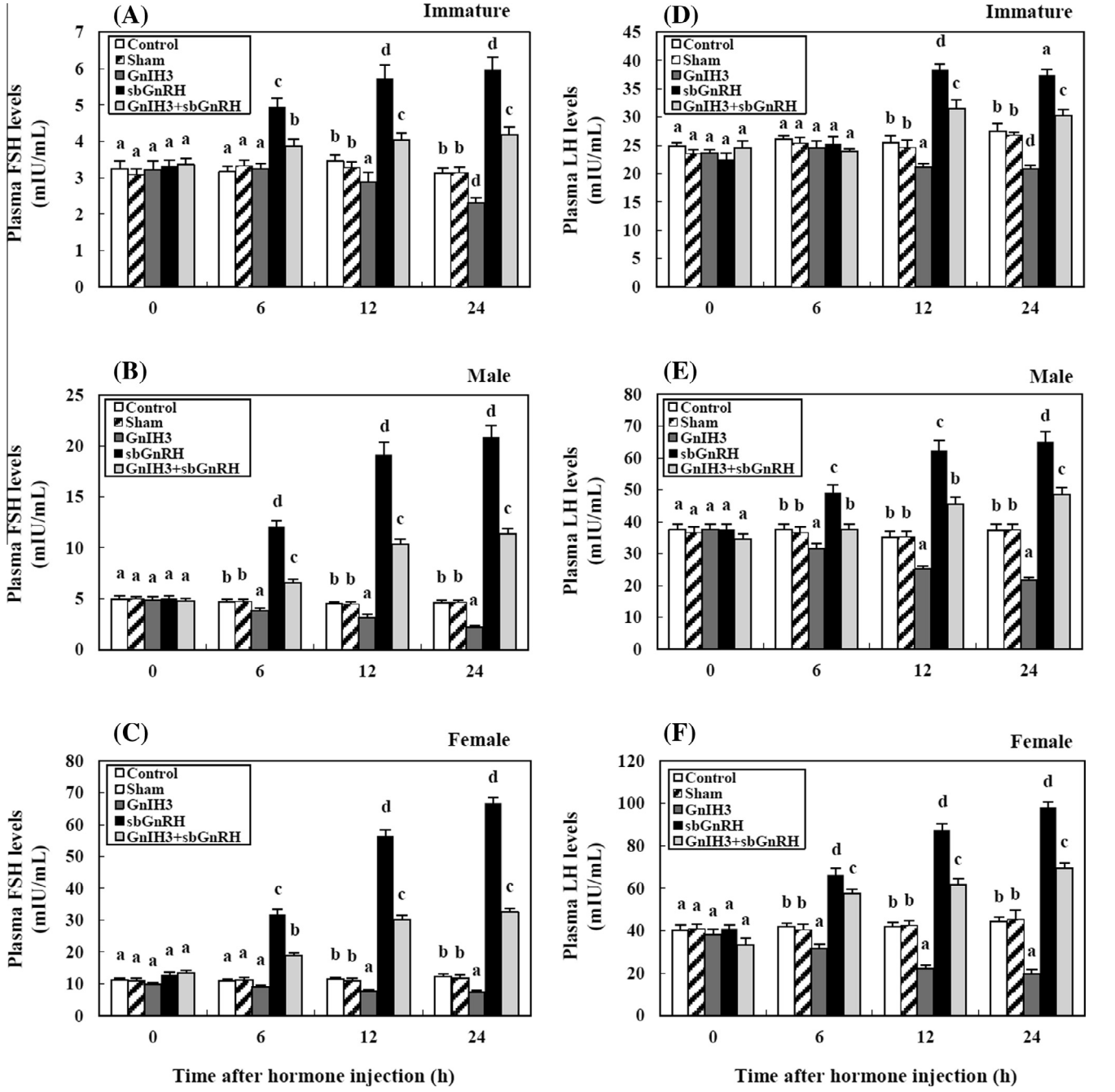

Fig. 8. The activity of plasma follicle stimulating hormone (FSH; A, B, C) and luteinizing hormone (LH; D, E, F) of cinnamon clownfish (immature fish, A and D; male, B and E; female, C and F) after gonadotropin inhibitory hormone (GnIH3) (0.1 μg/g BM), seabream GnRH (sbGnRH) (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH) treatment as analyzed with a plate reader. Values with letters indicate significant differences between treatments ($P < 0.05$). All values are means ± SE ($n = 5$).

treatment with progesterone, progesterone + estradiol, and estradiol, which stimulate gonadal maturation and development (Maddineni et al., 2008).

Collectively, these studies support our findings that GnIH treatment reduced the expression of sbGnIH mRNA in cinnamon clownfish, but increased the expression of GnIH and GnIH-R mRNA. Accordingly, the results demonstrate that GnIH directly inhibits sexual maturation and increases the expression of GnIH, and this effect may vary depending on gonadal development and maturity, particularly in mature male and female fish.

We also investigated MT_3 mRNA level and the plasma concentrations of melatonin, which are important factors in the regulation of GnIH production. In this study, plasma melatonin and MT_3 mRNA levels decreased in all experimental groups (immature, male, and female) following injection with sbGnRH, but increased in all experimental groups after injection with GnIH. In particular, we observed that the plasma melatonin and MT_3 mRNA levels of mature fish (male and female) were affected to a greater extent than those of immature fish receiving similar treatments.

The results of this study are consistent with previous findings that indicated that melatonin directly affects the production of GnIH in a number of vertebrate species (Tsutsui et al., 2010). Sébert et al. (2008) reported that gonadotrophin beta-subunit (FSHβ and LHβ) mRNA expression and sexual steroid (11-ketotestosterone, estradiol) plasma levels decreased in eels treated with melatonin, and they suggested that melatonin

treatment had a negative effect on eel reproductive function. The same study suggested that when melatonin was combined with GnRH, other hormones in the HPG axis were suppressed because MT was presented on GnRH neurons (Sébert et al., 2008). Thus, these findings confirm the relationship between melatonin and reproduction.

The present findings confirm that melatonin production and MT_3 mRNA expression of mature male and female fish are more significantly affected by GnIH than immature fish. In immature fish, however, it is possible that GnIH could inhibit sexual maturation.

The present study also provides information on sbGnRH, GTHα, FSHβ and LHβ mRNA levels, and plasma FSH and LH concentrations following treatment with GnIH. GnIH treatment resulted in a reduction of GnRH, GTHα, FSHβ, and LHβ mRNA levels in all experimental groups (immature, male, and female), whereas sbGnRH treatment had the opposite effect. In particular, these levels in mature male and female fish were higher. In addition, the GnIH-R located on GnRH can control the synthesis and secretion of FSH and LH (Tsutsui et al., 2010). Zhang et al. (2010) also reported that plasma LH levels of zebrafish directly decreased following treatment with goldfish GnIH.

This study provides both novel information on the effects of GnIH and strong support for the hypothesis that GnIH plays an important role in the negative regulation of the HPG axis in cinnamon clownfish, a protandrous hermaphroditic fish. The results are also significant because they enhance the current understanding of the possible role of GnIH in the control of sex change in cinnamon clownfish.

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