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Kisspeptin regulates the hypothalamus–pituitary–gonad axis gene expression during sexual maturation in the cinnamon clownfish, *Amphiprion melanopus*

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ABSTRACT

Kisspeptins (Kiss) have been recognized as potent regulators of reproduction in teleosts, and Kiss is suggested to be a key regulator of the hypothalamus–pituitary–gonad axis (HPG). However, its regulatory role on reproduction in fish remains unclear. Therefore, to investigate the role of Kiss on fish reproduction, this study aimed to test differences in the hormones of the HPG axis, Kiss as neuropeptides, and sex steroids on the sexual maturation of paired cinnamon clownfish, *Amphiprion melanopus*, following treatment with Kiss. We investigated the actions of sex maturation hormones, including HPG axis hormones and sex steroid hormones, such as gonadotropin-releasing hormones, gonadotropin hormones (GTHs), GTH receptors, estrogen receptors, and vitellogenin in the pituitary, gonads, and liver following treatment with Kiss. The expression levels of HPG axis genes increased after the Kiss injection. In addition, the levels of plasma 17α-hydroxypregnenolone, estradiol-17β, and 11-ketotestosterone increased. These results support the hypothesis that Kiss play important roles in the regulation of the HPG axis and are most likely involved in gonadal development and sexual maturation in cinnamon clownfish.

1. Introduction

Sexual development and maturation in fish are regulated by various sex hormones in the hypothalamus–pituitary–gonad (HPG) axis, including gonadotropin-releasing hormone (GnRH), gonadotropin (GTH), steroid hormones, and other neurohormones (Lee et al., 2001). A key regulator of this system is GnRH, which stimulates the synthesis and the release of pituitary hormones, most notably follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In turn, FSH and LH stimulate gonadal gametogenesis and steroidogenesis (Andrews et al., 1988). This process involves a complex interplay between neuroendocrine and endocrine inputs from multiple receptors, local paracrine and autocrine regulation, and feedback controls (Taranger et al., 2010).

In vertebrates, GTH release is primarily under the stimulatory control of GnRH. At least 23 molecular forms of GnRH have been identified in various vertebrate and protochordate species (Adams et al., 2002, 2003; Millar et al., 2004). Many studies suggest that GnRH-expressing neurons are distributed among three distinct GnRH populations [salmon GnRH (sGnRH), chicken GnRH-II (cGnRH-II), and sea bream GnRH (sbGnRH)] within the brain, most likely reflecting distinct phylogenetic relationships and functions (Andersson et al., 2001). sGnRH is produced in neuronal groups localized in the ventral forebrain along the terminal nerve, controls GTH secretion, and has been implicated in the regulation of spawning behavior (Senthilkumaran et al., 1999). The cGnRH-II neurons are localized in the midbrain tegmentum, project their axons widely throughout the central nervous system, and modulate sexual and feeding behaviors (Millar, 2003). sbGnRH neuronal cell bodies are localized in the pre-optic area (POA), project their axons throughout various brain loci, have neuromodulatory activities (Oka, 2009), and exert effects on pheromone production (Steven et al., 2003).

Recently, kisspeptin (Kiss), a neuropeptide that regulates sexual differentiation and spawn time in vertebrates, and its receptor, GPR54 (G protein coupled receptor 54), have been shown to play major roles in the central regulation of the HPG axis (Roa et al., 2011; Chang et al., 2012).

Kiss, a member of the Arg-Phe (RF)-amide peptide family, is located in the POA of the hypothalamus and regulates sexual maturation and regulation factors (Roa et al., 2011). Mammalian Kiss1 is suggested to be a key regulator of the HPG axis, and in teleosts, its ortholog is named Kiss1 (Kanda et al., 2008). In addition, the Kiss-GPR54 signal system is one of the circuits regulating GnRH secretion in the hypothalamus (Colledge, 2009). According to a recent study performed in goldfish, both signal peptides could stimulate or inhibit the HPG axis dependent on the stage of gonadal development (Zmora et al., 2012). Many findings have led a number of authors to suggest an important role played by the Kiss system in the activation of the HPG axis in fish (Parhar et al., 2004; Filby et al., 2008; Kitahashi et al., 2009). The hypophysiotropic actions of Kiss1 was also confirmed in vitro on primary pituitary cell culture from goldfish (Yang et al., 2010), and results also

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showed that Kiss1 could stimulate prolactin and growth hormone secretion and gene expression in goldfish pituitaries (Yang et al., 2010). The synthesis and secretion of FSH and LH in the pituitary are stimulated by Kiss protein synthesis and secretion in the hypothalamus; in particular, Kiss plays an important role in regulating reproduction and stimulates LH secretion before reproduction and ovulation (Chang et al., 2012). In addition, LH stimulates the conversion of cholesterol to 17α-hydroxypregnenolone, the precursor of gonadal steroid hormones, which is secreted and affects the theca folliculi and granulosa cells during the final stage of maturation. 17α-Hydroxypregnenolone also plays an important role in the synthesis of sex hormones [estrogen and testosterone (T)], which interact with FSH (Dickey and Swanson, 2000; Yamato et al., 2010).

Estrogen is an essential steroid hormone in reproduction whose effects are mediated by nuclear estrogen receptors (Esr); it plays important roles in sexual maturation and differentiation, which include oogenesis, vitellogenesis, and testicular development (Ishibashi and Kawashima, 2001). The induction of vitellogenin (Vtg), a precursor yolk protein, in response to 17β-estradiol (E_2) by GTH is well documented in several oviparous (egg-laying) fish species (Bowman et al., 2002; Davis et al., 2009).

However, 11-ketotestosterone (11-KT) and T in males affect testes development, and KT is a more potent androgen than T (Stacey and Kobayashi, 1996). 11-KT is considered essential for LH release in response to pre-ovulatory steroid pheromones and male sexual behavior in response to the post-ovulatory pituitary–gonad axis (PG axis); in addition, pre-ovulatory LH release in males is stimulated by behavioral interactions with ovulating females (Sorensen et al., 1989).

Kiss has not been analyzed extensively, with studies being limited to determining its nucleotide sequence and tissue distribution (Kitahashi et al., 2007; Um et al., 2010; Yang et al., 2010). Changes in sex steroid hormones during the maturation process, including the effects of Kiss on the HPG axis, have been only minimally researched.

Cinnamon clownfish, *Amphiprion melanopus* typically live as part of a mated adult pair or an adult pair and an immature individual and social ranking in the group controls the sexes of the fish (Godwin and Thomas, 1993). In general, the female is the larger and dominant individual. If a dominant female dies or is absent, the male partner experiences a sex change to become a female, and an immature individual experiences a sex change to become a male (Godwin and Thomas, 1993).

Here, we have studied the relationship between HPG axis regulation and sexual maturation using Kiss. Our aim was to produce a pair (female and male) of cinnamon clownfish with artificially induced sexes and to investigate the effects of weekly injections of Kiss on the regulation mechanism of sexual maturation in pairs of cinnamon clownfish, as assessed by the effects of Kiss on GnRHs, GTHs, GTH receptors, Esrs, Vtg mRNA expression, and plasma concentrations of 17α-hydroxypregnenolone, E_2, and 11-KT.

2. Materials and methods

2.1. Experimental fish

The study was conducted with pairs of cinnamon clownfish (*A. melanopus*, Bleeker, 1852; Perciformes, Pomacentridae; fishbase.org: fire clownfish) artificially made [average length, 8.4 ± 0.5 cm, mass, 14.6 ± 0.5 g, gonadosomatic index (GSI; gonad weight/body weight × 100) = 0.15 ± 0.03; male, length, 6.5 ± 0.4 cm, mass, 11.5 ± 0.3 g, GSI = 0.11 ± 0.03]. The fish were purchased from CCORA (Center of Ornamental Reef & Aquarium, Jeju, Korea). The water temperature was 27 °C ± 1 °C, and the photoperiod was 12-h light:12-h dark (lights on from 07:00 to 19:00 h), respectively. The fish were fed a commercial feed twice daily (at 09:00 and 17:00 h). The fish were reared under these conditions for 6 weeks.

2.2. Kiss treatment and sampling

To investigate the effects of Kiss, the fish were reared in 100-L circulating filter tanks in the laboratory and were anesthetized with tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO, USA) prior to injection. Kiss (metastin 45-54 amide; Sigma; 80% similarity with fish species) was dissolved in 0.9% physiological saline, and each fish was injected with Kiss [0.1 and 0.5 μg/g, body mass (BM)] at a volume of 10 μL/g BM. A sham group of fish was injected with an equal volume of 0.9% NaCl (10 μL/g BM) once a week at 2 p.m. The control samples were removed prior to injection. After the injection, pituitary, brain, gonad, and liver samples were removed from the fish at 2, 4, and 6 weeks. During the experimental period, the water temperature and photoperiod were maintained at 27 °C ± 1 °C and 12L/12D, respectively.

All fish were anesthetized with tricaine methane sulfonate (Sigma) and decapitated prior to tissue collection. Pituitary, brain, gonad, and liver samples from the fish were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until the total RNA was extracted for analysis. The plasma samples were separated by centrifugation (4 °C, 10,000 *g*, 5 min) and stored at −80 °C until analysis. After being dissected and weighed, the gonads were fixed in Bouin's solution and subjected to histological observation. The GSI was calculated for each fish.

2.3. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression levels of 3 GnRH types (sGnRH, sbGnRH, and cGnRH-II), the GTH subunits (GTHα, FSHβ, and LHβ), GTH receptors (FSHR and LHR), Esr subtypes (Esr1, Esr2a1, and Esr2a2), and Vtg using the total RNA extracted from cinnamon clownfish tissues. The primers for QPCR are shown in Table 1. These primers were designed for each gene using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database to ensure the specificity of primers. QPCR amplification was conducted similar to previous studies using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad). QPCR was performed in the following manner: 1 cycle of denaturation at 95 °C for 5 min followed by 35 cycles of denaturation

Table 1
Primers used for amplification of QPCR.

Genes (GenBank accession no.)	Primer	DNA sequences
sGnRH (HQ883477)	Forward	5 -GAA GAG AAG TGT GGG AGA G-3
	Reverse	5 -CGA AAG GAC TGG AAT CAT CT-3
sbGnRH (HQ883476)	Forward	5 -CTG CTG GTG GTG ATG ATG-3
	Reverse	5 -GGT CTC TCT TCC CTC CTG-3
cGnRH-II (EU908057)	Forward	5 -AGC ACA TCA GAG ATT TCA GAG-3
	Reverse	5 -CCA AGG CAT CCA AAA GAA TG-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
FSHR (GU722648)	Forward	5 -CCT CTC ATT ACC GTG TCC GAC TC-3
	Reverse	5 -GGG TGA AGA AGG CAT ACA GGA AGG-3
LHR (GU722649)	Forward	5 -GGA AAC AGA AAT AGA GCC CAC TAC AG-3
	Reverse	5 -CAC TTG ACG AAG GGG TTG TTA AGA C-3
Esr1 (HM185179)	Forward	5 -CTG GGC TGG AGG TGC TGA TG-3
	Reverse	5 -TCA ACA CAG TCG CCT TCG TTC C-3
Esr2a1 (HM185180)	Forward	5 -GCT TCA GGC TAC CAC TAT-3
	Reverse	5 -CGG CGG TTC TTG TCT ATA-3
Esr2a2 (HM185178)	Forward	5 -GTC TCG GTT CCG TGA GCT GAA G-3
	Reverse	5 -GCA CAG ATT GGA GTT GAG GAG GAT C-3
Vtg (HM185181)	Forward	5 -CGA GAT TCT GAA ACA CCT G-3
	Reverse	5 -GCTGAA ATA ATT CCA CAA ACT T-3
β-actin (JF273495)	Forward	5 -GGA CCT GTA TGC CAA CAC TG-3
	Reverse	5 -TGA TCT CCT TCT GCA TCC TG-3

at 95 °C for 20 s and annealing at 55 °C for 20 s. Each reaction was run in triplicate to confirm consistency. The experiments were duplicated with β-actin as an internal control. The efficiencies of the reactions were determined by performing the QPCR. All data were expressed as changes 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 $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

2.4. Western blot analysis

The total protein isolated from the brain, gonads, and liver during sexual maturation was extracted using protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL PMSF, and 0.15 mg/mL leupeptin), sonicated, and quantified using the Bradford method (Bio-Rad, USA). The total protein (30 μg per lane) was loaded onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel, and a protein ladder (Bio-Rad) was used for reference. The samples were electrophoresed at 80 V through the stacking gel and at 150 V through the resolving gel until the bromophenol blue dye front had run off of the gel. The gels were then immediately transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 1.5 h at 4 °C. Thereafter, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min and then washed in TBS. The membranes were incubated with GnRH [LRH13; a monoclonal mouse antiserum that recognizes most vertebrate GnRH forms; dilution 1:5,000; courtesy of M.K. Park (Park and Wakabayashi, 1986)] and Vtg antibodies (dilution 1:4,000, ABIN326357, Antibodies-online, USA), followed by a horseradish peroxidase conjugated anti-mouse IgG secondary antibody (dilution 1:5,000, Bio-Rad, USA) for 60 min. In addition, the membranes were incubated with a polyclonal rabbit antibody to GTHα [anti-goldfish GTHα; a polyclonal rabbit antibody; dilution, 1:4,000; courtesy of M. Kobayashi (Kobayashi et al., 2006)] and Esr1 (dilution 1:1,000, E1528, Sigma) and Esr2 antibodies (dilution 1:4,000, sc-8974, Santa Cruz Biotech, CA, USA), followed by a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (dilution 1:5,000, Bio-Rad, USA) for 60 min. The internal control was a β-tubulin antibody (dilution, 1:5,000; ab6046, Abcam, UK), followed by a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5,000; Bio-Rad) for 60 min. The bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) with a 30-s exposure and a Molecular Imager® ChemiDoc™ XRS + System (Bio-Rad). The membrane images were scanned with a high-resolution scanner, and the band density was estimated using a computer program (Image Lab™ Software, version 3.0, Bio-Rad). The ratios of the internal control (β-tubulin) to the HPG axis genes (GnRH, GTHα, Esr1, Esr2, and Vtg) for each concentration were calculated and plotted against the concentration of the internal control.

2.5. Histological analysis

The gonadal tissues of each Kiss injection group (female and male; control, 2, 4, and 6 weeks) were fixed in Bouin's solution to analyze the gonads during sexual maturation. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin. Sections (5 μm thick) were selected and stained with hematoxylin-eosin for observation under a light microscope (DM 100; Leica, Wetzlar, Germany). The images were captured using a digital camera (DFC 290; Leica).

2.6. Plasma parameter analysis

The plasma GnRH (E08810f), FSH (E15790Fh), LH (E15791Fh), E_2 (E1317Fh), 17- hydroxypregnenolone (EQ027292FI), and 11-KT (E14106h) levels were analyzed using the immunoassay technique with the ELISA kit (Cusabio Biotech, Hubei, China).

An anti-antibody specific to the antibodies of hormones (GnRH, FSH, LH, 17- hydroxypregnenolone, E_2, and 11-KT) was pre-coated onto a microplate. There was 50 μL of plasma per well, and 50 μL of HRP-conjugate and 50 μL of an antibody was then added to each well. The samples were mixed well and then incubated for 2 h at 37 °C. After the final wash, any remaining wash buffer was removed by aspirating or decanting. Then, 50 μL of substrate A and 50 μL of substrate B were added to each well, and the samples were 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.

2.7. Statistical analysis

The gene-specific variation was calculated as the standard deviation of the log transformed expression of the gene. Data from duplicated cores in each plot were averaged prior to statistical analyses. In addition, for the ratio measurements of band intensity measurement (protein expression), data were transformed using the natural log.

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 test for significant differences in the data ($p < 0.05$). The values are expressed as the means ± standard error (SE).

3. Results

3.1. Time course and dose-related effects of Kiss on 3 types of GnRHs

The expression levels of 3 types of GnRH mRNA in the brains of paired cinnamon clownfish following Kiss injections are shown in Figs. 1 (female) and 2 (male).

In female cinnamon clownfish, treatment with 0.1 and 0.5 μg/g BW of Kiss significantly increased the mRNA levels of the 3 types of GnRH in the brain (Fig. 1). In particular, the level of sbGnRH mRNA was higher than that in the untreated controls and higher than the levels of the other GnRH forms at 6 weeks post-injection (Fig. 1C). In addition, following the Kiss injection, the plasma GnRH levels gradually increased to reach levels that were approximately 2.3-fold higher at 6 weeks than control (Fig. 1E). A western blot analysis revealed a protein with GnRH-specific immunoreactivity and a mass that corresponded to the predicted mass for cinnamon clownfish GnRH (52 kDa; Fig. 1A). The expression pattern of the protein resembled the pattern of the GnRH mRNA expressed in cinnamon clownfish brains.

Similar to the mRNA expression levels of the 3 types of GnRH in female cinnamon clownfish, the mRNA expression levels of the 3 types of GnRH in the brains of male cinnamon clownfish were significantly increased by treatment with 0.1 and 0.5 μg/g BW of Kiss (Fig. 2). The expression pattern of the protein resembled the pattern of the GnRH mRNA in cinnamon clownfish brains (Fig. 2A). In addition, following the Kiss injection, the plasma GnRH levels gradually increased to reach levels that were approximately 1.5-fold higher at 6 weeks than control (Fig. 2E).

3.2. Time course and dose-related effects of Kiss on GTH subtypes

The mRNA expression levels of GTH subtypes (GTHα, FSHβ, and LHβ) in the pituitary of paired cinnamon clownfish following a Kiss injection are shown in Figs. 3 (female) and 4 (male). Treatment with 0.1 and 0.5 μg/g BW of Kiss significantly increased the GTH mRNA levels in the pituitary glands of female and male cinnamon clownfish (Figs. 3 and 4). In addition, a western blot analysis detected a GTHα protein with a size that corresponded to the predicted size for cinnamon clownfish (approximately 35-kDa), which exhibited similar mRNA expression across GTHα (Figs. 3A and 4A). In addition, following the Kiss injection, the plasma FSH and LH levels significantly increased and reached levels that were approximately 3.1- and 2.8-fold (females;

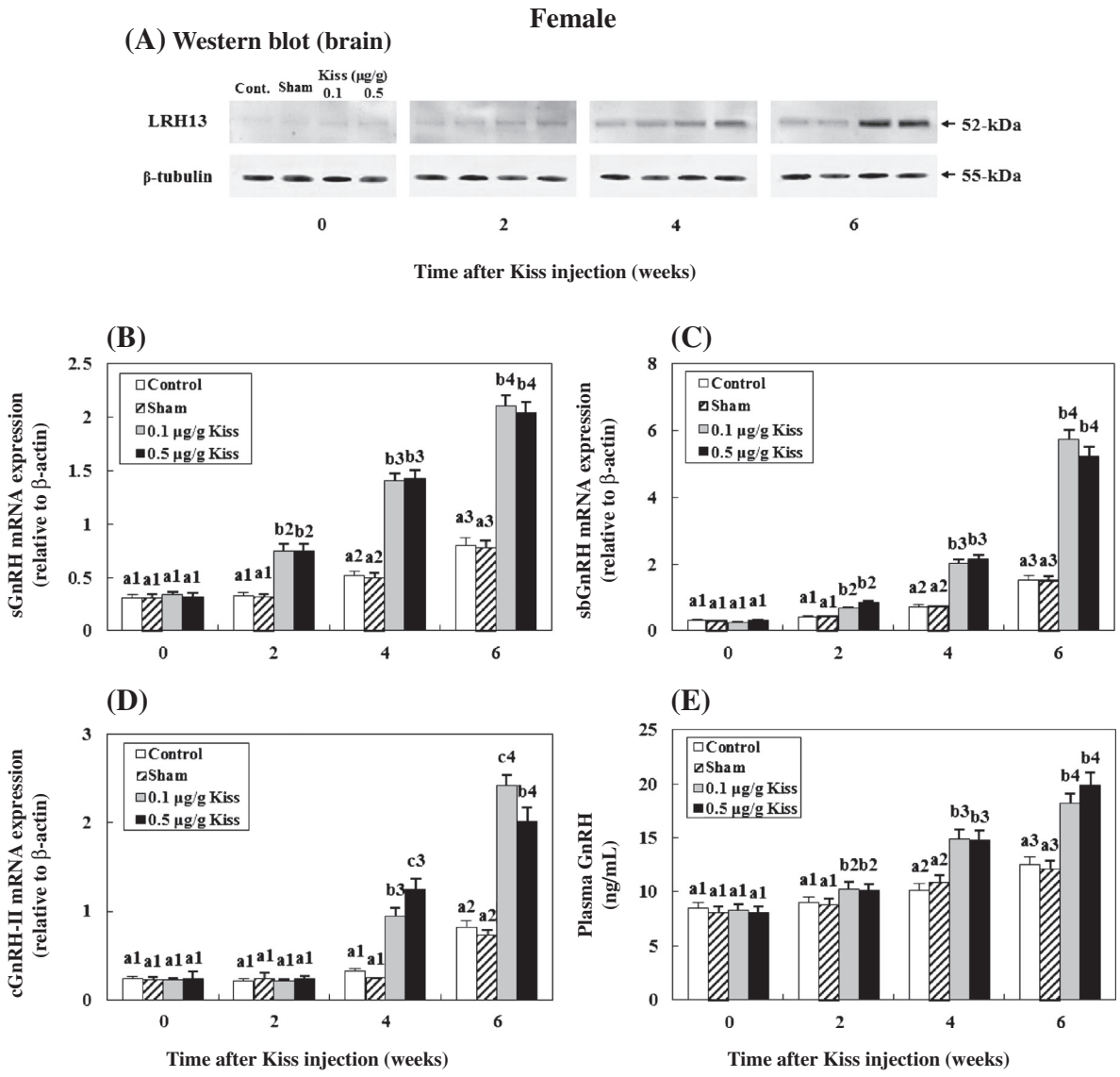

Fig. 1. Expression and activity of three types of GnRH in female cinnamon clownfish brains at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) Western blot using LRH13 (a monoclonal mouse antiserum that recognizes most vertebrate GnRH forms; dilution 1:5000; 52 kDa) to examine brain protein expression. β-Tubulin (55 kDa) was used as the internal control. (B) sGnRH, (C) sbGnRH, and (D) cGnRH-II mRNA levels relative to β-actin mRNA levels in the brain of cinnamon clownfish. (E) The activity of plasma GnRH of cinnamon clownfish at 2, 4, and 6 weeks after Kiss injection was also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

Fig. 3D and F) and 2.9- and 1.8-fold (males; Fig. 4D and F) higher at 6 weeks than their control, respectively.

3.3. Time course and dose-related effects of Kiss on GTH receptors

The mRNA expression levels of GTH receptors (FSHR and LHR) in the gonads following the Kiss injection are shown in Fig. 5 (females and males). Treatment with 0.1 and 0.5 μg/g BW of Kiss significantly increased the GTHR mRNA levels in the gonads of cinnamon clownfish.

3.4. Time course and dose-related effects of Kiss on Esrs in female cinnamon clownfish

The mRNA expression levels of Esrs (Esr1, Esr2a1, and Esr2a2) in the gonads following a Kiss injection are shown in Figs. 6 (female) and 7 (male). Treatment with 0.1 μg/g BW and 0.5 μg/g BW of Kiss significantly increased all Esr mRNA levels in the gonads of female and male cinnamon clownfish (Figs. 6 and 7). In addition, a Western blot analysis detected Esr1 and Esr2 proteins with sizes that corresponded to the predicted sizes for cinnamon clownfish (approximately 66 kDa; Esr1 and 56-kDa; Esr2, respectively), which exhibited similar mRNA expressions across Esrs.

3.5. Time course and dose-related effects of Kiss on Vtg in female cinnamon clownfish

The liver expression levels of Vtg were measured following treatment with Kiss (Fig. 8A and B). Treatments with both doses of Kiss significantly increased the Vtg mRNA levels, particularly at 6 weeks after treatment with Kiss.

3.6. Time course and dose-related effects of Kiss on circulating 17α-hydroxypregnenolone, E_2, and 11-KT concentrations

We measured the circulation level of 17α-hydroxypregnenolone in female cinnamon clownfish following injection with Kiss (Fig. 8C). The

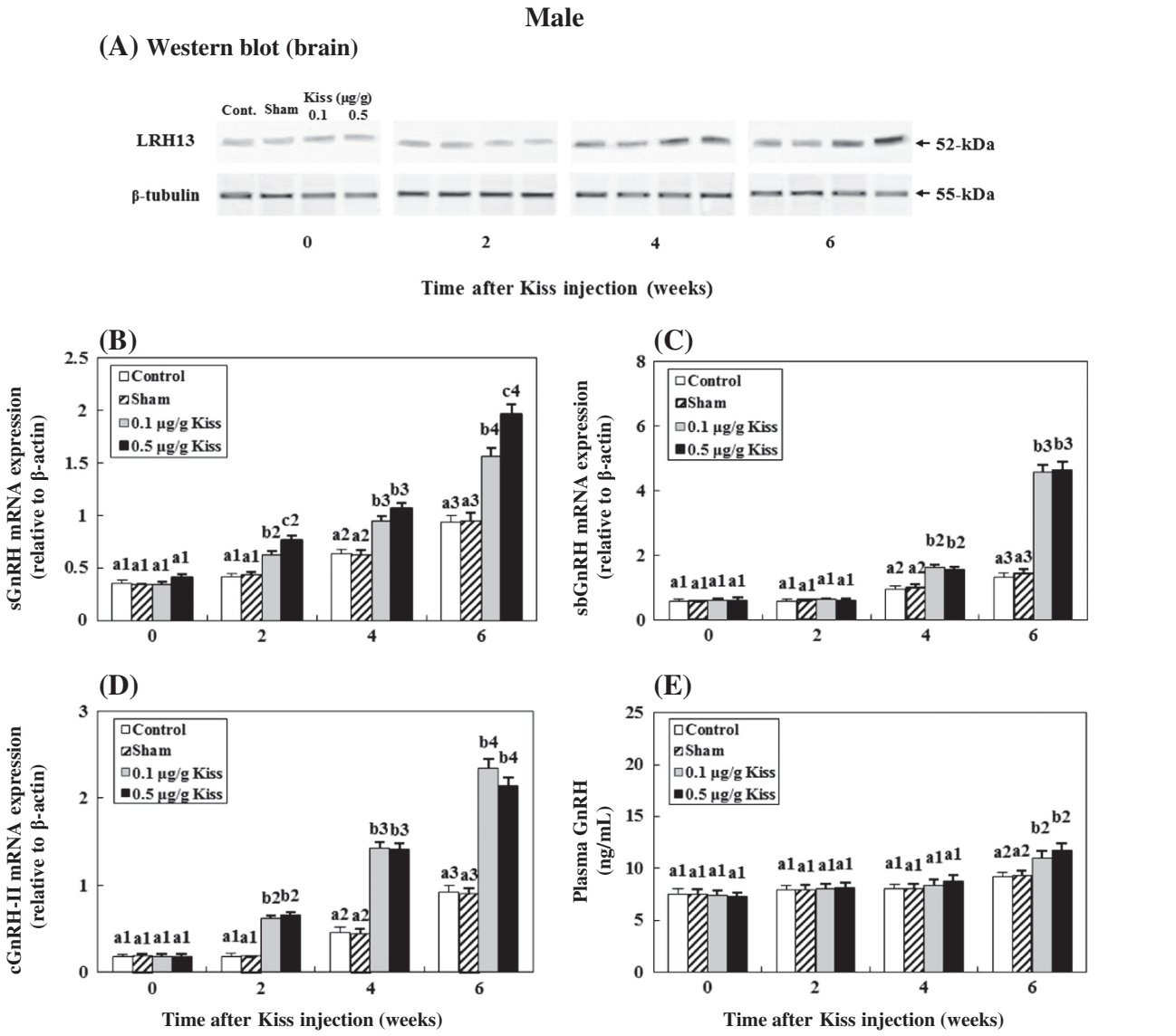

Fig. 2. Expression and activity of three types of GnRH in male cinnamon clownfish brains at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) Western blot using LRH13 (a monoclonal mouse antiserum that recognizes most vertebrate GnRH forms; dilution 1:5000; 52-kDa) to examine brain protein expression; β-tubulin (55 kDa) was used as the internal control. (B) sGnRH, (C) sbGnRH, and (D) cGnRH-II mRNA levels relative to β-actin mRNA levels in the brain of cinnamon clownfish. (E) The activity of plasma GnRH of cinnamon clownfish at 2, 4, and 6 weeks after Kiss injection was also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

plasma 17α-hydroxypregnenolone level was 22.4 ± 1.2 ng/mL at the beginning of the experiment. Most notably, the levels of 17α-hydroxypregnenolone levels increased to 58.2 ± 6.5 ng/mL at 6 weeks (0.5 μg/g of Kiss).

Following a Kiss injection in female, the plasma E_2 levels gradually increased to reach levels that were approximately 1.7-fold higher at 6 weeks than control (Fig. 9A). The plasma E_2 level was 393.2 ± 19.8 pg/mL at the beginning of the experiment. The levels of E_2 then increased to 781.1 ± 35.5 pg/mL at 6 weeks by treatment with Kiss.

The plasma E_2 levels in male cinnamon clownfish following an injection with Kiss are shown in Fig. 9B. The plasma E_2 level was 344.5 ± 18.8 pg/mL at the beginning of the experiment. Most notably, the 11-KT levels increased to 522.3 ± 30.5 pg/mL at 6 weeks by treatment with Kiss.

The 11-KT levels in male cinnamon clownfish following an injection with Kiss are shown in Fig. 9C. The plasma 11-KT level was 713.5 ± 39.8 pg/mL at the beginning of the experiment. Most notably, the 11-KT levels increased to 1111.1 ± 55.5 pg/mL at 6 weeks by treatment with Kiss.

3.7. GSI and histological observations

The GSI of female cinnamon clownfish injected with Kiss were significantly greater than those of the control fish (Table 2), and the greatest effect was observed at the end of the experiment (6 weeks), at which point the Kiss injection group (0.1 and 0.5 μg/g) exhibited the greatest GSI (2.13 ± 0.22 and 2.42 ± 0.18 cm, respectively, for the 2 doses). Similar to the GSI of female cinnamon clownfish, the GSI of male cinnamon clownfish were greater than those of control fish (Table 3). The GSI was 0.11 ± 0.03 at the beginning of the experiment. Following the Kiss injection, the GSI increased to approximately 4.3-fold (GSI = 0.50 ± 0.07, Table 3) higher at 6 weeks than control.

The gonads of females and males mainly consisted of testicular tissue with primary oocytes (Fig. 10A and E). In female cinnamon clownfish, the oocytes had developed and were largest in size

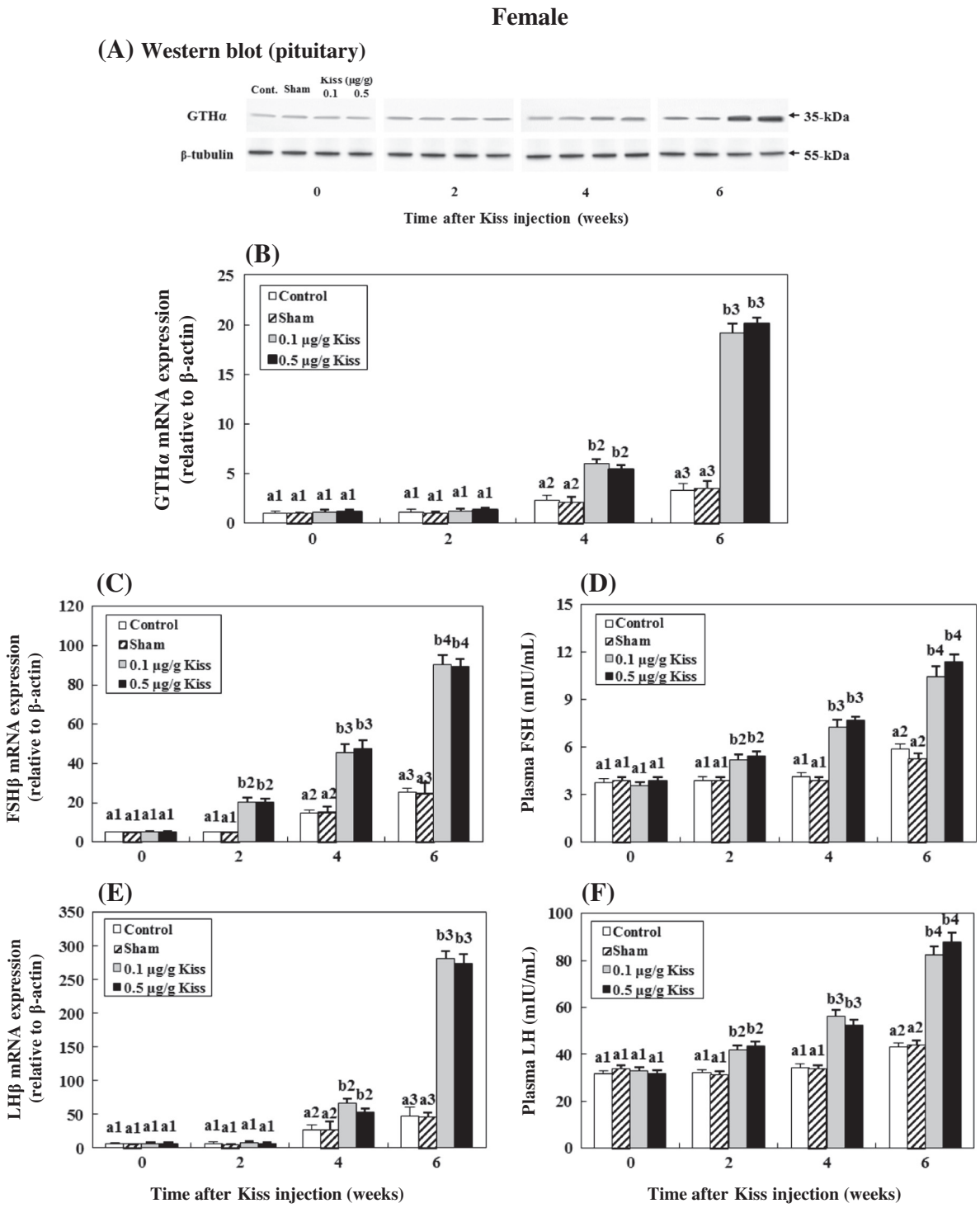

Fig. 3. Expression and activity of GTHs in female cinnamon clownfish pituitary glands at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) Western blot of GTHα (monoclonal rabbit antiserum; dilution, 1:4,000; 35 kDa) protein expression in the pituitary of cinnamon clownfish. β-Tubulin (55 kDa) was used as the internal control. (B) GTHα, (C) FSHβ, and (E) LHβ mRNA levels relative to β-actin mRNA levels in the pituitary glands of cinnamon clownfish. The plasma (D) FSH and (F) LH activities of cinnamon clownfish at 2, 4, and 6 weeks after the Kiss injections were also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

(Fig. 10C, from the Kiss injection to 4 weeks), the diameter of the oocytes increased, the testicular tissue regressed, and finally, the fish became female (Fig. 10D, from the Kiss injection to 6 weeks). In contrast, the size of the testicular tissue and the primary oocytes were maintained in the gonads of males (Fig. 10F, from the Kiss injection to 4 weeks). Much of the testicular tissue then increased and contained spermatids (Fig. 10H, from the Kiss injection to 6 weeks).

4. Discussion

We investigated the effects of 6 weekly injections of Kiss on the regulation mechanism of sexual maturation in pairs of cinnamon clownfish, as assessed by studying the effects of Kiss on GnRHs, GTHs, GTH receptors, Esrs, and Vtg mRNA expression, and the plasma concentrations of the precursor of gonadal steroid hormone, 17α-

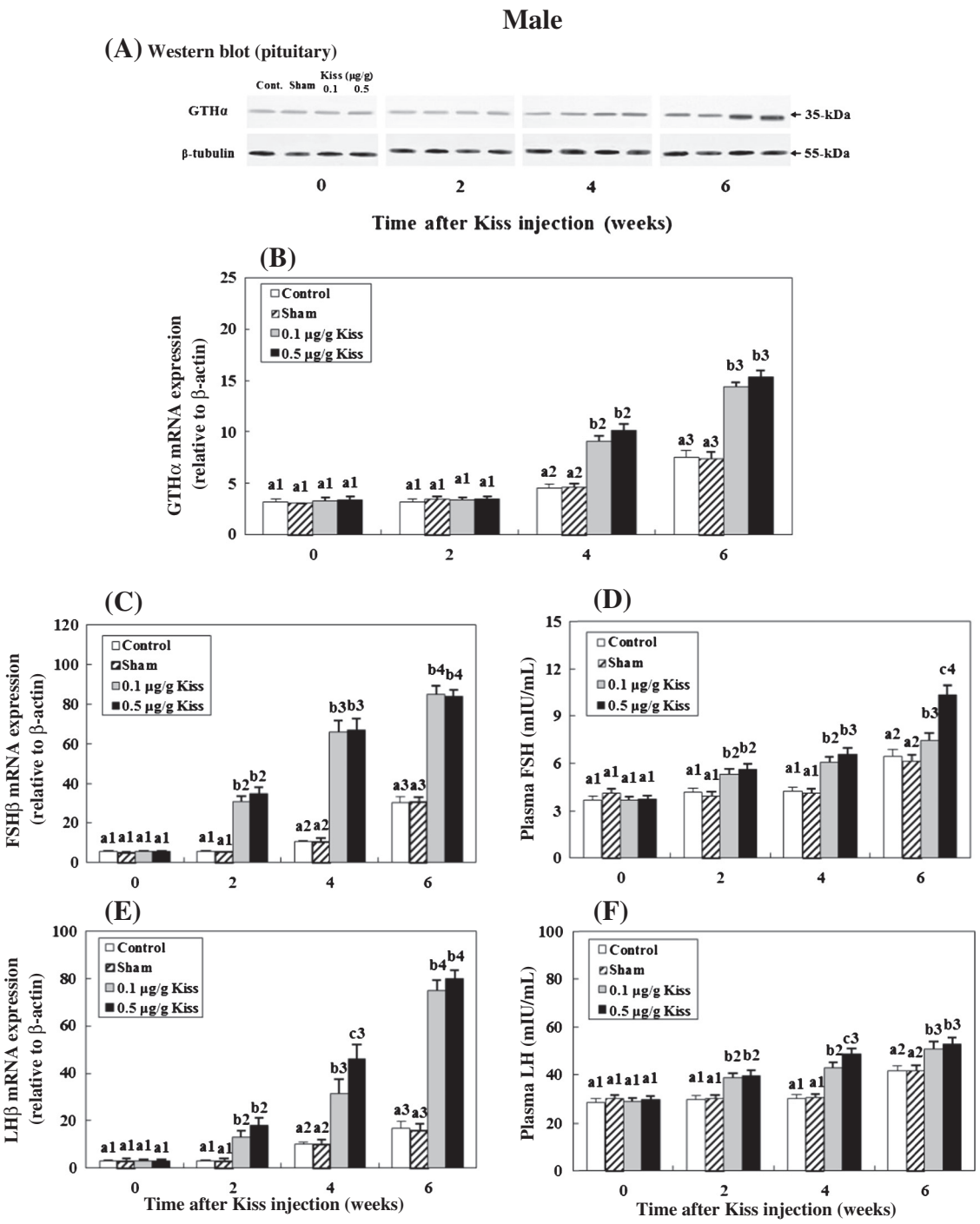

Fig. 4. Expression and activity of GTHs in male cinnamon clownfish pituitary glands at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) Western blot of GTHα (monoclonal rabbit antiserum; dilution, 1:4,000; 35 kDa) protein expression in the pituitary of cinnamon clownfish. β-Tubulin (55 kDa) was used as the internal control. (B) GTHα, (C) FSHβ, and (E) LHβ mRNA levels relative to β-actin mRNA levels in the pituitary of cinnamon clownfish. The plasma (D) FSH and (F) LH activities of cinnamon clownfish at 2, 4, and 6 weeks after the Kiss injections were also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

hydroxypregnenolone, E_2, and 11-KT. In addition, we also examined the maturity of female and male gonads using histological analyses after the injection of Kiss and investigated the effects of Kiss on the development of oocytes and spermatocytes.

We investigated the effects of HPG axis activity and the process of sexual maturation after weekly injections of Kiss (0.1 and 0.5 μg/g of body weight) in each pair of cinnamon clownfish over a 6-week period. We observed that the mRNA expression levels of the 3 types of GnRH (sGnRH, sbGnRH, and cGnRH-II) in the hypothalamus, as well as plasma GnRH concentrations, increased significantly in all experimental groups, but there were no significant differences between the different Kiss treatment concentrations (Figs. 1 and 2). In particular, the expression of GnRH mRNA and the plasma GnRH levels increased significantly after 2 or 4 weeks of Kiss treatment and showed the greatest increase at 6 weeks. According to the histological observations of Irwig et al. (2004), Kiss is located with GnRH neurons in the hypothalamus. The

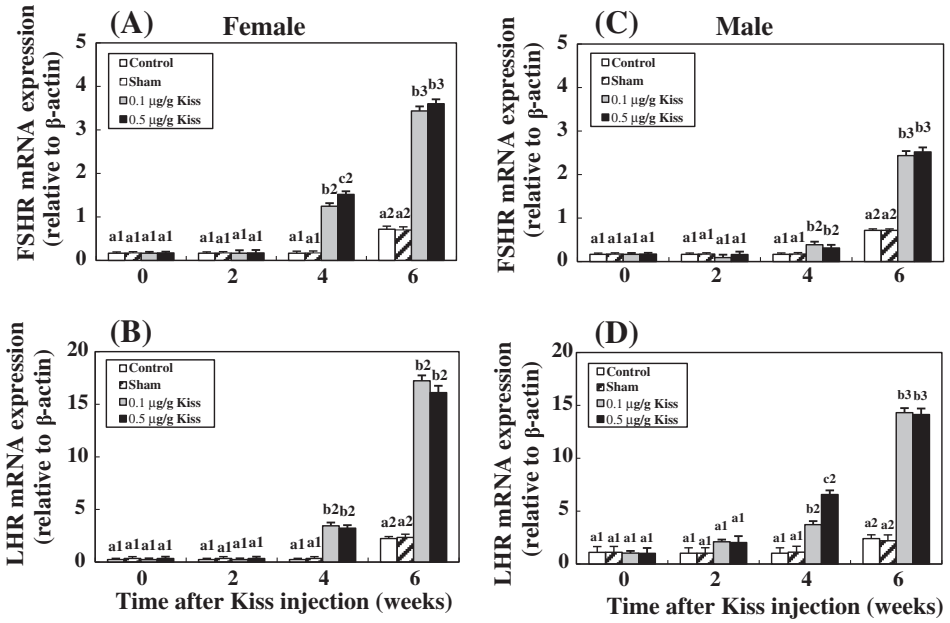

Fig. 5. Expression of GTHRs in cinnamon clownfish gonads [females (A and B) and males (C and D)] at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. FSHR (A, C) and LHR (B, D) mRNA levels relative to β-actin mRNA levels in the gonads of cinnamon clownfish. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

interaction between GnRH and Kiss is a function of the GnRH regulator, which regulates gonadal steroid hormones. These hormones interact with Kiss1 and are conveyed to the brain as a feedback mechanism. In addition, Kiss administration now offers promising and efficient stimulation of hypothalamic GnRH release, as observed in previous studies in fish, including the medaka, *Oryzias latipes* (Zhao and Wayne, 2012), and yellowtail kingfish, *Seriola lalandi* (Nocillado et al., 2012). Similar to the previous study, the results of this study demonstrated high GnRH expression as a result of Kiss treatment. We have considered the possibility that Kiss increases the expression of GnRH, and the activity of the HPG axis plays an important role in the sexual maturation of teleosts. In particular, this study observed higher mRNA expression level and secretion of GnRH in females (Fig. 1) than in males (Fig. 2) during Kiss treatment, which is consistent with a previous study indicating that Kiss influences GnRH secretion to a greater extent in females than that in males (Cheng et al., 2010; Pita et al., 2011). Kiss is distributed largely in the anteroventral periventricular nucleus (AVPV) of the POA, more so in females than that in males (Bleier et al., 1982), which is why—with respect to GnRH secretion—Kiss is believed to affect the maturation of females to a greater extent than that of males.

In this study, we investigated GTH changes according to neuron expression levels of GnRH, the secretion of which was in turn stimulated by Kiss. We observed that the levels of GTHs (GTHα, FSHβ, and LHβ) in the pituitary, plasma FSH and LH, and GTH receptors (FSHR and LHR) increased significantly in all experimental groups (Figs. 3–5). GTH levels in the pituitary increased with Kiss injections; increased GTH stimulates germ intracellular cAMP, which regulates the expression of GTH receptors (Vischer et al., 2003). In addition, FSH regulates the early phases of gametogenesis, such as vitellogenesis and spermatogenesis, and LH stimulates the final maturation stages, such as ovulation and spermatogenesis (Swanson et al., 2003). In the present study, Kiss treatment in cinnamon clownfish significantly increased FSH and LH levels at 6 weeks. In the present study, the FSH and LH levels of cinnamon clownfish after Kiss treatment increased significantly, with the highest value at 6 weeks. The results of this study were similar to previous reports that FSH and LH expression increased with Kiss injections in the adult female zebrafish, *Danio rerio* (Kitahashi et al., 2009), goldfish, *Carassius auratus* (Li et al., 2009), and male European sea bass, *Dicentrarchus labrax* (Felip et al., 2009), because of stimulation of the HPG axis. In addition, LH secretion in the pituitary cells of goldfish (Chang et al., 2012) increased after Kiss treatment (*in vitro*). In rodents, Kiss1 neurons have been found to express both estrogen and androgen receptors, which explains how gonadal steroids can have both stimulatory and inhibitory effects on Kiss1 expression (Smith et al., 2005; Kauffmann et al., 2007). Furthermore, in sheep, both positive and negative feedback of sex steroids are mediated by kisspeptin neurons (Estrada et al., 2006). It was proposed that Kiss1 neural sensitivity to estrogen not only provides a critical estradiol-dependent amplification mechanism to activate GnRH neurons and complete the onset of puberty, but also subsequently facilitates the regular preovulatory GnRH/LH surge in adult females (Clarkson et al., 2010). Recent work in female zebrafish has similarly shown that Kiss2 neurons of the ventral hypothalamus are estrogen-sensitive (Servili et al., 2011), with estradiol treatment causing a significant increase in both Kiss1 and Kiss2 mRNA expression in the brain of juvenile zebrafish (Servili et al., 2011). We hypothesized that Kiss directly influences pituitary cells.

Kiss interacts with various steroid hormones in the gonads (Colledge, 2009), and with E_2 and ERs to synthesize combined Vtg in the liver, thus affecting oocyte maturation (Bowman et al., 2002; Davis et al., 2009). In this study, the expression levels of Ers1, Ers2a1, Ers2a2, and Vtg mRNAs, and the plasma concentrations of 17α-hydroxypregnenolone and E_2, increased at 6 weeks (Figs. 6, 8, and 9A). Choi et al. (2007) reported that GnRH treatment increased gonadal ER and that plasma E_2 and E_2 regulated the development and maturation of oocytes. Similarly, in this study, we hypothesized that Kiss treatment regulated ER mRNA expression and the plasma E_2 level, thus playing an important role in the sexual maturation of cinnamon clownfish. Plasma 17α-hydroxypregnenolone levels were significantly higher in the Kiss treatment group than that in the other groups (Fig. 8B). 17α-Hydroxypregnenolone is generated from plasma steroids during steroidogenesis by stimulating LH and FSH (Hu et al., 2001), is involved in sexual maturation along with progesterone and 17-hydroxyprogesterone, and plays a role in the formation of the egg yolk (Canario and Scott, 1988). Thus, we hypothesized that increasing FSH

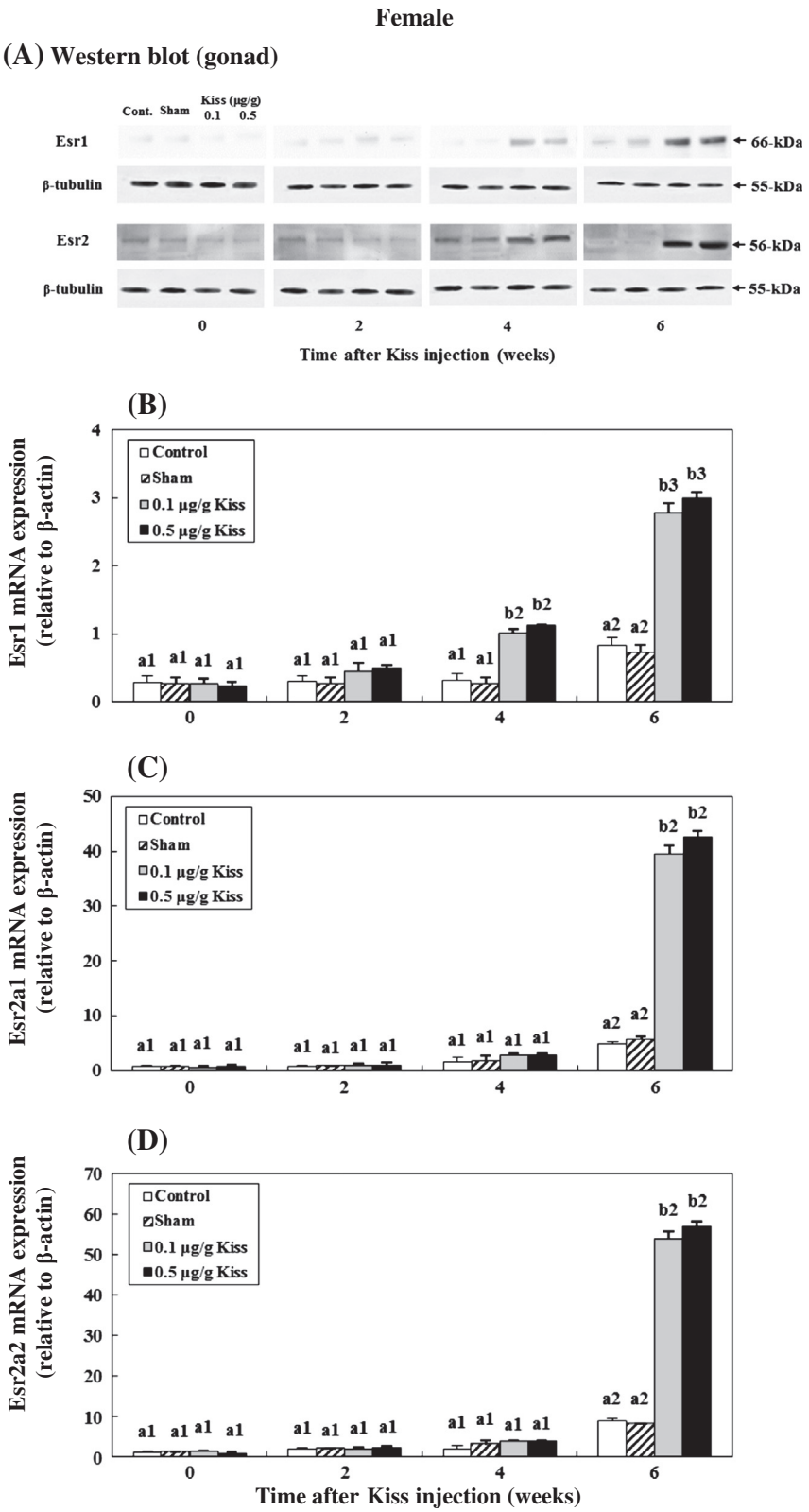

Fig. 6. Expression of Esrs in female cinnamon clownfish gonads at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) Western blot using Esr1 (66 kDa) and Esr2 (56 kDa) to examine protein expression in the gonads; β-tubulin (55 kDa) was the internal control. (B) Esr1, (C) Esr2a1, and (D) Esr2a2 mRNA levels relative to β-actin mRNA levels in the gonads of cinnamon clownfish. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

and LH with the concentrations of 17α-hydroxypregnenolone stimulates the formation of the yolk and affects final maturation. This result was supported by histological observations: mature oocyte cells were observed in many Kiss-treated female gonad tissues, in which we confirmed that sexual maturation was accelerated (Fig. 10D).

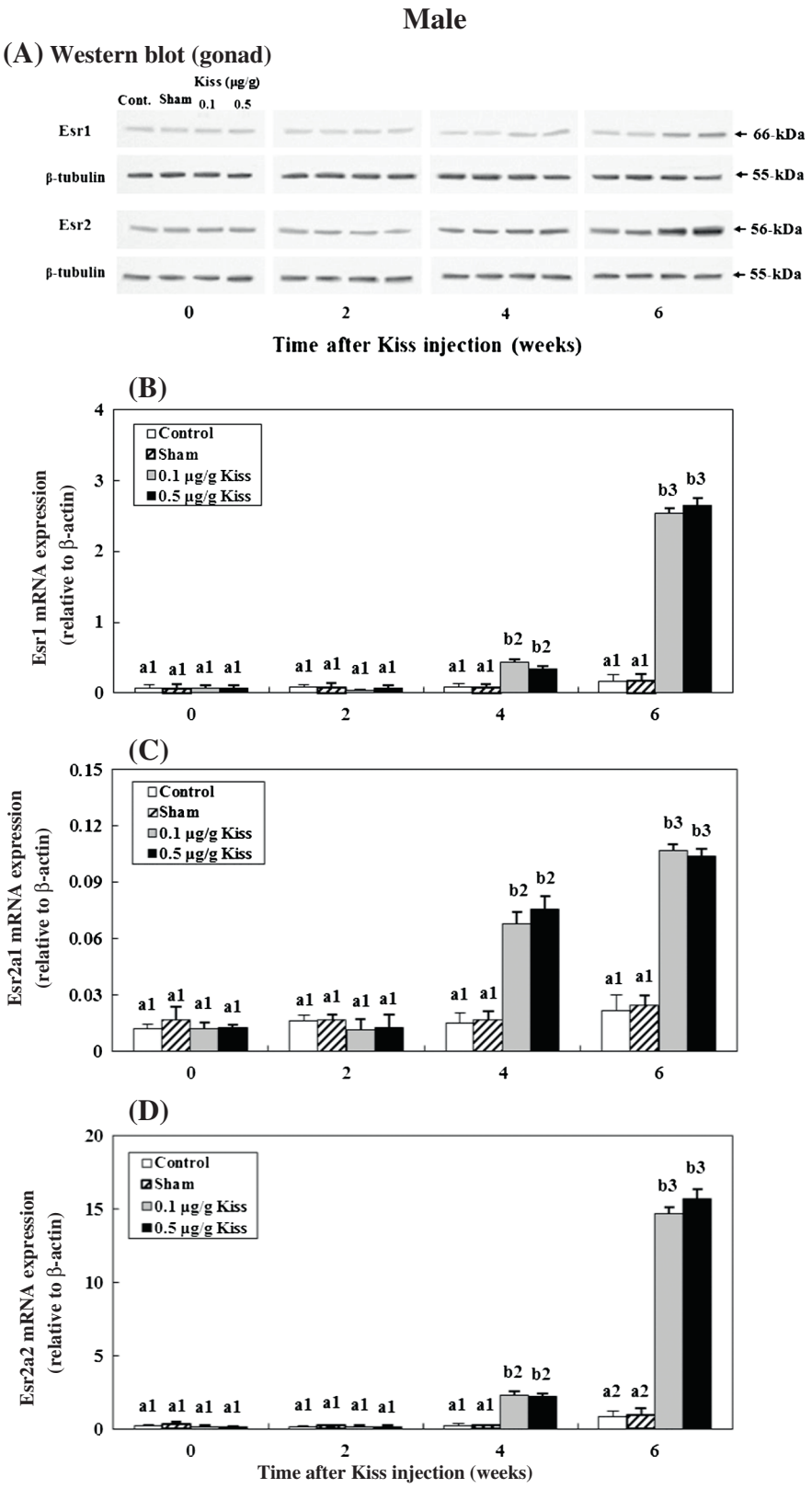

Fig. 7. Expression of Esrs in male cinnamon clownfish gonads at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) Western blot using Esr1 (66 kDa) and Esr2 (56 kDa) to examine protein expression in the gonads; β-tubulin (55 kDa) was the internal control. (B) Esr1, (C) Esr2a1, and (D) Esr2a2 mRNA levels relative to β-actin mRNA levels in the gonads of cinnamon clownfish. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

We also determined the expression levels of Ers1, Ers2a1, and Ers2a2 and the plasma concentrations of E_2 and 11-KT, which acts as an androgen and determines the degree of male maturation. In this study, the expression levels of Ers1, Ers2a1, Ers2a2, and plasma concentrations of E_2 increased at 6 weeks (Figs. 7 and 9B). The results of this study were similar to previous reports that E_2 promotes

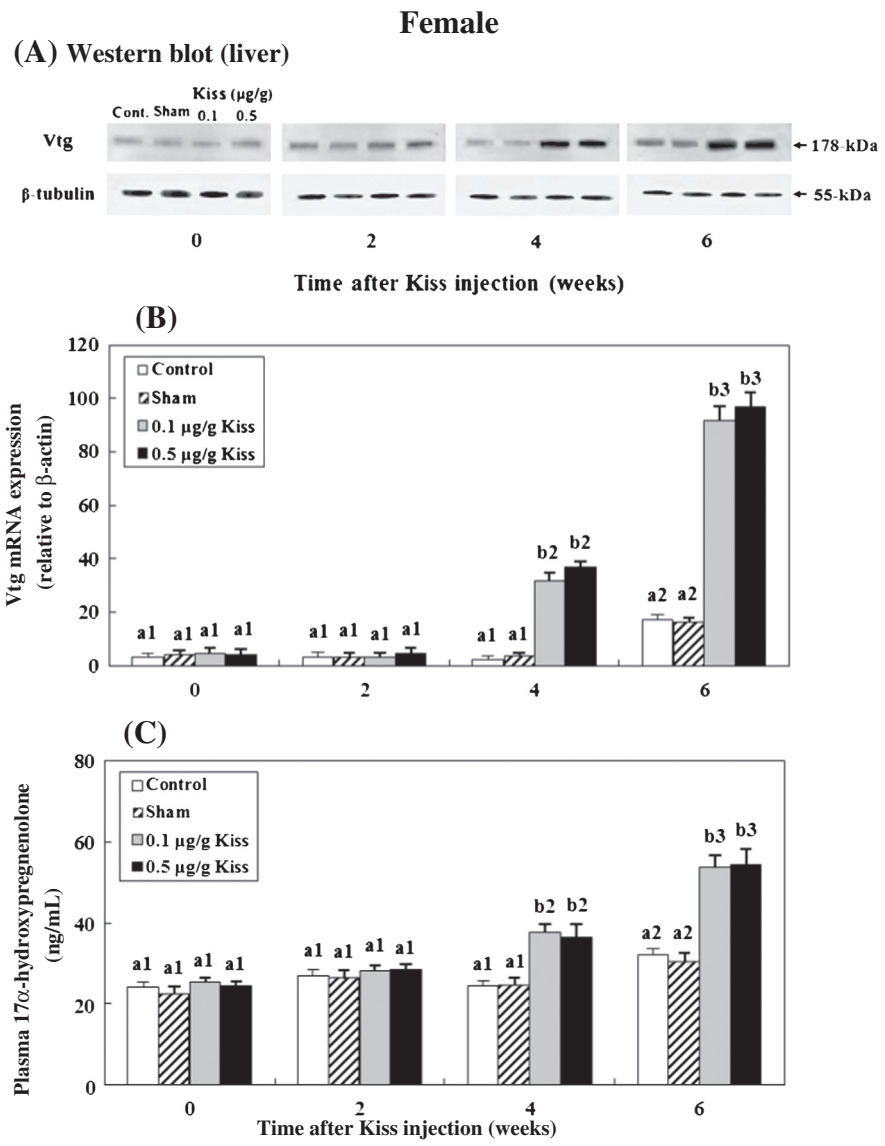

Fig. 8. Vtg expression in the liver and plasma 17α-hydroxypregnenolone levels of female cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection. (A) Western blot of Vtg (monoclonal rabbit antiserum; dilution 1:5000; 178 kDa) protein expression in the liver; β-tubulin (55 kDa) was the internal control. (B) Vtg mRNA levels relative to β-actin mRNA levels in the livers of cinnamon clownfish. (C) The plasma 17α-hydroxypregnenolone concentrations of cinnamon clownfish at 2, 4, and 6 weeks after Kiss injection was also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

early spermatogonia renewal, albeit in some species, such as medaka, *O. latipes* (Song and Gutzeit, 2003), and the gilthead sea bream, *Sparus auratus* (Kadmon et al., 1985). Similarly, in this study, we hypothesized that Kiss treatment regulated ER mRNA expression and plasma E_2 level, thus playing an important role in the sexual maturation of cinnamon clownfish. In addition, plasma 11-KT levels were increased by Kiss treatment in the male cinnamon clownfish (Fig. 9C). Sex steroids are known to be involved in the progression of spermatogenesis. In general, levels of androgens, particularly 11-KT, are shown to increase gradually as spermatogenesis progresses (Schulz et al., 2010). Selvaraj et al. (2013) reported that circulating levels of 11-KT in Kiss-treated fish increased in correlation with an increase in the gonadosomatic index (GSI) and progression of spermatogenesis, suggesting their involvement in the acceleration of spermatogenesis in Kiss-treated fish.

After processing, many mature spermatocytes were observed in the gonadal tissue of Kiss-treated male cinnamon clownfish at 6 weeks. Therefore, Kiss induced testes development in cinnamon clownfish (Fig. 10H).

In summary, Kiss, which is located in the POA cells of the hypothalamus, stimulated the secretion of GnRH in female and male cinnamon clownfish and played an important role in PG axis activity. Kiss also affected the sexual maturation of cinnamon clownfish, and we hypothesize that Kiss is widely distributed in the hypothalamus and plays an important role in the HPG axis activity and the final maturation of the gonads.

In this study, Kiss affected the activity of the HPG axis in the cinnamon clownfish, a teleost that can undergo sex changes. Therefore, we have considered the basic processes involved in teleost sexual maturation in this study.

Acknowledgments

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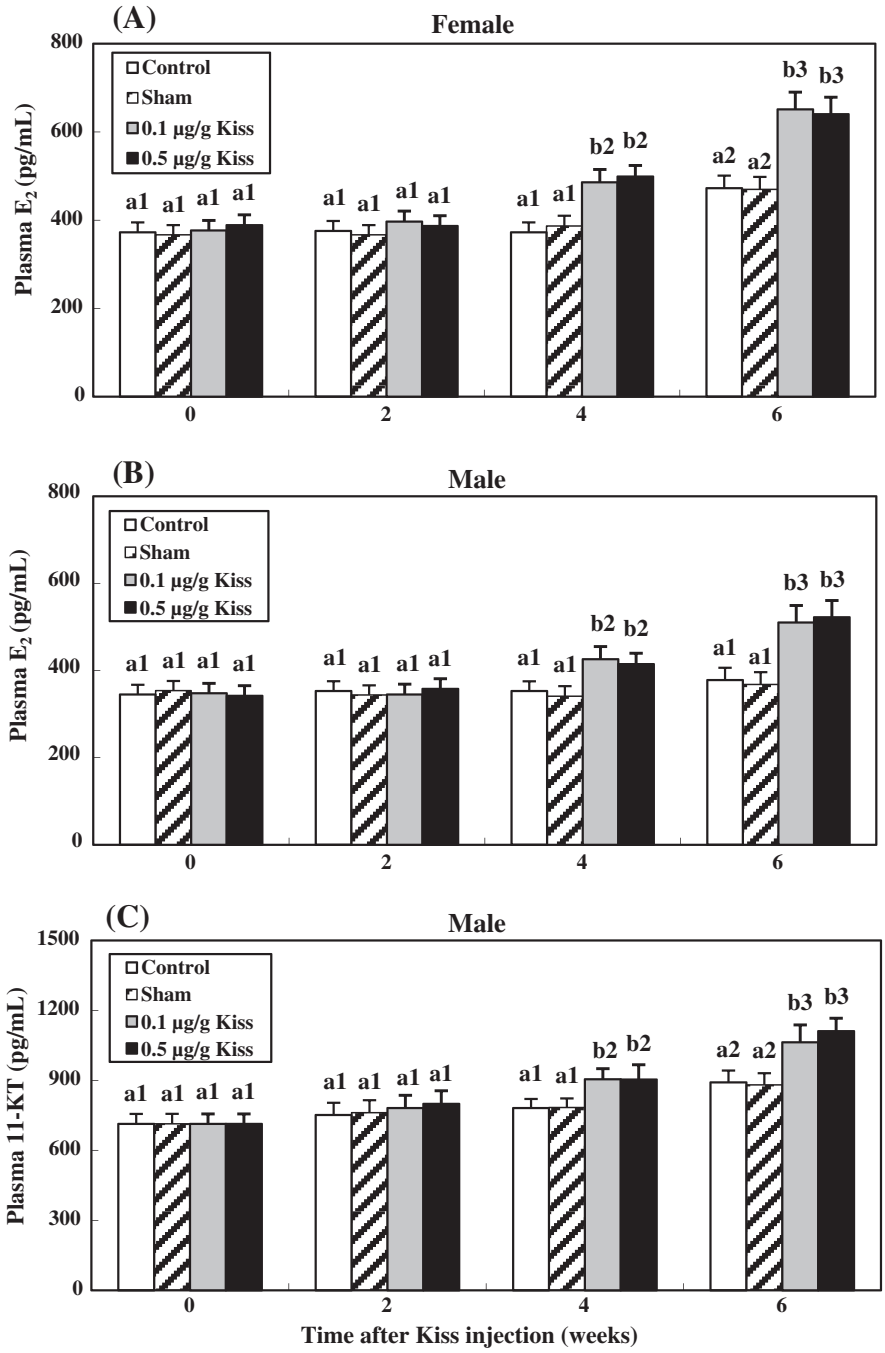

Fig. 9. Plasma E_2 (A, females; B, male) and 11- KT (C, males) levels of cinnamon clownfish at 2, 4, and 6 weeks after Kisspeptin (Kiss, 0.1 and 0.5 μg/g) injection were also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

Table 2
Changes of the GSI in female cinnamon clownfish.

Weeks	Control	Sham	Kiss	
			0.1 μg/g	0.5 μg/g
0	0.15 ± 0.03^{a1}	0.15 ± 0.03^{a1}	0.15 ± 0.03^{a1}	0.15 ± 0.03^{a1}
2	0.18 ± 0.02^{a1}	0.18 ± 0.04^{a1}	0.23 ± 0.14^{a1}	0.31 ± 0.14^{a1}
4	0.23 ± 0.03^{a1}	0.21 ± 0.04^{a1}	0.78 ± 0.12^{b2}	0.85 ± 0.23^{b2}
6	0.53 ± 0.04^{a2}	0.51 ± 0.03^{a2}	2.13 ± 0.22^{b3}	2.42 ± 0.18^{b3}

Numbers indicate significant differences between different time points at the same kisspeptin (Kiss) concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

Table 3
Changes of the GSI in male cinnamon clownfish.

Weeks	Control	Sham	Kiss	
			0.1 μg/g	0.5 μg/g
0	0.11 ± 0.03^{a1}	0.11 ± 0.03^{a1}	0.11 ± 0.03^{a1}	0.11 ± 0.03^{a1}
2	0.12 ± 0.02^{a1}	0.12 ± 0.03^{a1}	0.14 ± 0.04^{a1}	0.13 ± 0.03^{a1}
4	0.13 ± 0.03^{a1}	0.13 ± 0.04^{a1}	0.24 ± 0.02^{b2}	0.27 ± 0.03^{b2}
6	0.21 ± 0.04^{a2}	0.22 ± 0.03^{a2}	0.42 ± 0.06^{b3}	0.50 ± 0.07^{b3}

Numbers indicate significant differences between different time points at the same Kisspeptin (Kiss) concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are means ± SE ($n = 5$).

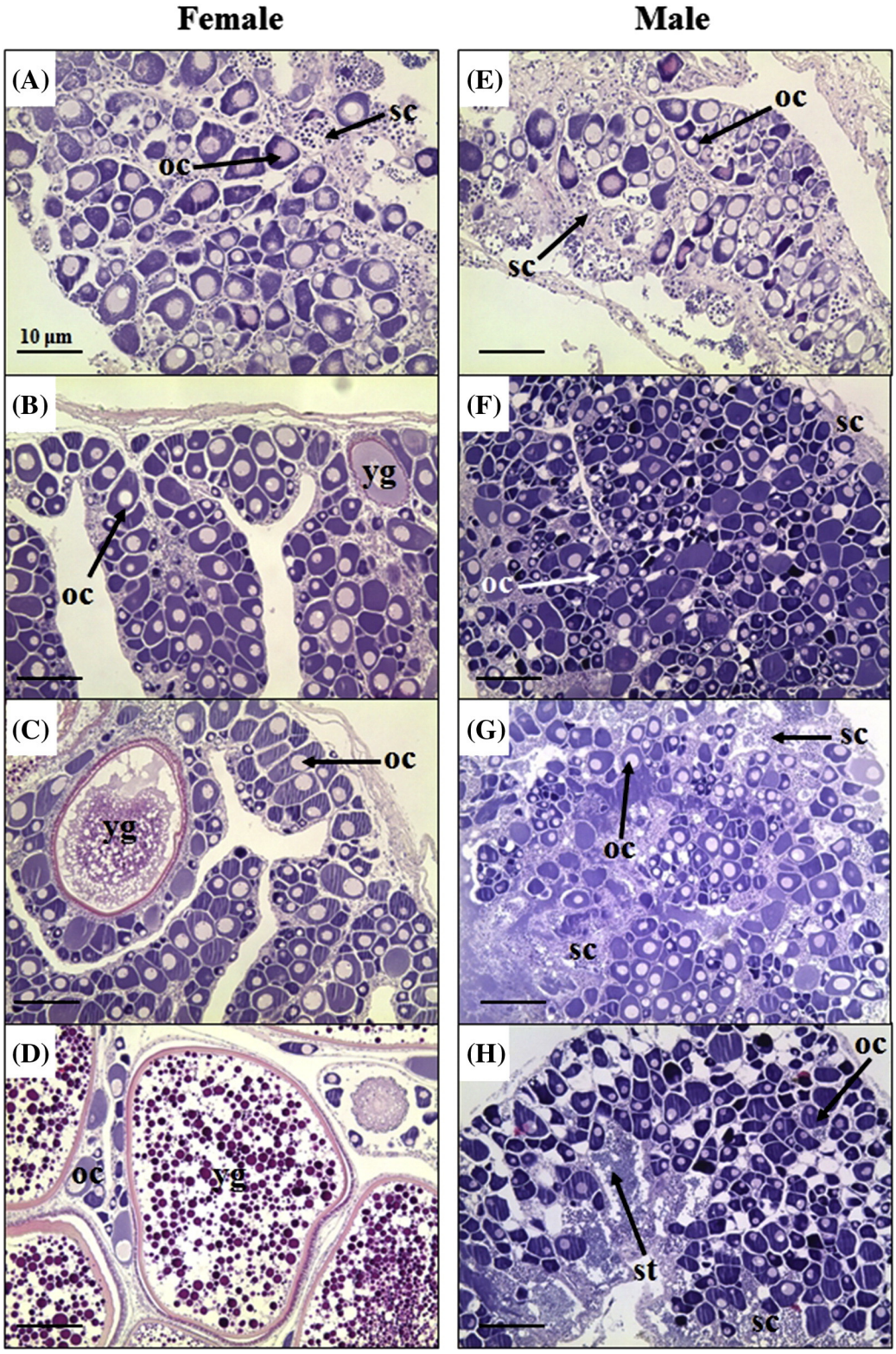

Fig. 10. Photomicrographs of cross sections of cinnamon clownfish after treatment with Kisspeptin (Kiss, 0.5 μg/g). Females: (A) controls (un-injected control), (B) 2 weeks after treatment with Kiss, (C) 4 weeks after treatment with Kiss, and (D) 6 weeks after treatment with Kiss. Males: (E) controls (uninjected control), (F) 2 weeks after treatment with Kiss, (G) 4 weeks after treatment with Kiss, and (H) 6 weeks after treatment with Kiss. sc: spermatocytes, st: spermatids, oc: oocytes, yg: yolk granules. Scale bar = 10 μm.

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