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Profiles of gonadotropin-inhibitory hormone and melatonin during the sex change and maturation of cinnamon clownfish, *Amphiprion melanopus*

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ABSTRACT

The present study aimed to determine the relationship between melatonin and gonadotropin-inhibitory hormone (GnIH) and their effect on reproduction in cinnamon clownfish, *Amphiprion melanopus*. Accordingly, we investigated the expression pattern of GnIH, GnIH receptor (GnIH-R), and melatonin receptor (MT-R1) mRNA and protein, as well as the plasma levels of melatonin, during sex change in cinnamon clownfish. We found that GnIH and MT-R1 mRNA and melatonin activity were higher in fish with mature brain than in fish with developing gonads, and using double immunofluorescence staining, we found that both GnIH and MT-R1 proteins were co-expressed in the hypothalamus of cinnamon clownfish. These findings support the hypothesis that melatonin plays an important role in the negative regulation of maturation and GnIH regulation during reproduction.

1. Introduction

Until recently, gonadotropin-releasing hormone (GnRH) has been the only known hormone to control the synthesis and release of gonadotropin hormones (GTHs) from neuropeptides present in the hypothalamus. However, Tsutsui et al. [1] discovered gonadotropin-inhibitory hormone (GnIH) in the brain of Japanese quail, *Coturnix japonica*, and a subsequent study reported that GnRH is not the only hormone that controls vertebrate reproduction, since it interacts with GnIH [2,3]. Studies in birds have also 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 GTHs, thereby influencing the hypothalamic-pituitary-gonadal (HPG) axis and controlling the reproductive cycle [3–6].

The regulation of GTH synthesis and its secretion is also affected by light exposure, including photoperiod [1], and photoperiod-mediated changes in reproductive hormone production are mainly mediated by melatonin, which is released from the pineal gland and retina [7]. Melatonin levels increase during the night and decrease during the day, thereby functioning as a neuroendocrine signal that is closely associated with the regulation of circadian rhythms [7]. The effect of melatonin is mediated by melatonin receptors (MT-Rs), which promote growth and inhibit sexual maturation by reducing the release of both follicle stimulating hormone (FSH) and luteinizing hormone (LH) [8,9]. Teleost fish have three subtypes of MTs: MT-R1, MT-R2, and MT-R3 [10,11]. Previous study reported the function of MT-R1 that modulate arterial vasoconstriction, cell proliferation in cancer cells, and reproductive and metabolic functions [12]. In addition, melatonin also affects the neurogenic function of GnIH, interacts with other hypothalamic peptides in the reproduction control system, *via* its responses to light levels and photoperiod, and stimulates the synthesis and secretion of GnIH [9,13].

Therefore, we examined the effect of GnIH and melatonin during the sex change and sexual maturation of cinnamon clownfish, *Amphiprion melanopus*. This species of clownfish typically lives as adult mated pairs or as an adult pair with an immature individual, and social ranking in the group determines the sexes of the fish. In general, the female is the larger and dominant individual. However, if a dominant female dies or becomes absent, the male partner will undergo a sex change to become female, and the immature individual will undergo a sex change to become male [14].

Abbreviations: GnIH, gonadotropin-inhibitory hormone; GnIH-R, gonadotropin-inhibitory hormone receptor; MT, melatonin; MT-R, melatonin receptor.

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In the present study, we aimed to investigate the relationship of GnIH and melatonin during sex change; for this purpose, we measured the mRNA expression of GnIH, GnIH-R, and MT-R1, as well as plasma levels of melatonin, using double staining for GnIH and GnIH-R and immunohistochemistry techniques for MT-R1 and plasma melatonin.

2. Materials and methods

2.1. Fish husbandry

The present study was conducted with male (10.5 ± 1.2 g), males at 90 days after removing female (15.2 ± 0.9 g) and female (22.2 ± 2.1 g) cinnamon clownfish, which were purchased from the Corea Center of Ornamental Reef and Aquarium (Jeju, Korea). Sexual maturity was determined by the presence of mature ova and sperm, and mated pairs (male and dominant female) were established prior to the experiments in 100-L tanks with circular filtration at 27 ± 1 °C and with a photoperiod of 12 h light:12 h dark (lights on 07:00–19:00 h). In addition, the fish were fed twice daily (09:00 h and 17:00 h) with a commercial marine aquarium fish feed (Jeilfeed Company, Kyoungnam, Korea).

The technique for inducing sex change was modified from the methods described by An et al. [15]. After mated pairs (male and dominant female) were established, sex change was induced in male fish by removing the female from each group and adding an immature individual. At 90 days after female removal, male fish underwent sex changes from males to females. We divided the sex change process into three developmental stages (i.e., maturity stages: I, mature male; II, male at 90 days after female removal; III, mature female) and sampled fish from each stage. All fish were anesthetized in 2-phenoxyethanol (Sigma, St. Louis, MO, USA) and decapitated prior to tissue collection, during which brain tissue was removed, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. The plasma samples were separated from blood using centrifugation (4 °C, 10,000 × *g*, 5 min) and stored at −80 °C until analysis.

2.2. Quantitative PCR (qPCR)

QPCR was conducted to determine the relative expression levels of GnIH, GnIH-R, and MT-R1 mRNA using cDNA reverse-transcribed from the total RNA extracted from the brains during the sex change. Total RNA was extracted from each sample using the TRI reagent® (Molecular Research Center, Inc., Ohio, USA) according to the manufacturer's instructions using DNase treated total RNA. Total RNA (2 μg) was reverse-transcribed in a total volume of 20 μL, using an oligo-d(T)$_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4 °C for use in qPCR. The following qPCR primers were designed with reference to the known sequences of the cinnamon clownfish (GenBank accession numbers: GnIH, KT455505; GnIH-R, KT455506; MT-R1, HM107821; β-actin, JF273495): GnIH forward (5′-CCC TCT TCG CTT CGG GCG GGA TG-3′) and reverse (5′-GAA TCG CTG AGG GAG GTT GAT A-3′) primers; GnIH-R forward (5′-AAC CAC AGC GGC TCA GTG TGT CC-3′) and reverse (5′-ACC AGA CAG AGG AAG ACA AA-3′) primers; MT-R1 forward (5′-CTG CTG GTG GTG ATG ATG-3′) and reverse (5′-GGT CTC TCT TCC CTC CTG-3′) primers; β-actin forward (5′-GGA CCT GTA TGC CAA CAC TG-3′) and reverse (5′-TGA TCT CCT TCT GCA TCC TG-3′) primers. qPCR amplification was conducted, using a Bio-Rad CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions, with initial denaturation at 95 °C for 5 min, then followed by 35 cycles of denaturation (95 °C for 20 s), annealing (55 °C for 20 s), and extension (72 °C for 10 s), followed a final extension at 72 °C for 10 min. Specific amplification was confirmed by melting curve analysis. Each experimental group was run in triplicate in 5 different experiments, and β-actin was used as an internal control. The efficiencies of the reactions were determined by analyzing the amplification curves and 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 the internal control (β-actin) was calculated using: $\Delta\Delta Ct = 2^{\wedge}-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

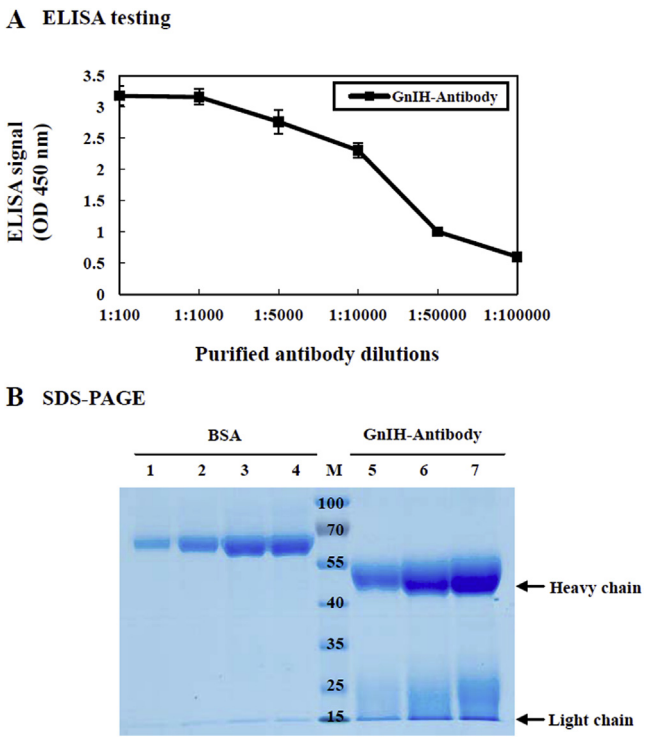

Fig. 1. Titration of the purified anti-GnIH antibody with ELISA and SDS-PAGE. (A) The ELISA curve exhibits a hyperbolic shape in the range of 1:100–1:100,000 dilution on the logarithmic dilution scale. (B) Analysis of purified GnIH polyclonal antibody using SDS-PAGE and Coomassie Brilliant Blue staining. Lane 1: 1 mg/mL bovine serum albumin (BSA), Lane 2: 2 mg/mL BSA, Lane 3: 3 mg/mL BSA, Lane 4: 4 mg/mL BSA, M: molecular weight marker, Lane 5: 1 μL of purified GnIH antibody, Lane 6: 2 μL of purified GnIH antibody, Lane 7: 3 μL of purified GnIH antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Production of GnIH polyclonal antibody

To obtain the antigen of the cinnamon clownfish GnIH antibody, a synthetic peptide was designed from highly conserved regions of GnIH amino acid sequences of cinnamon clownfish and other teleosts (N-ter-TLNVAPTSGRVSSPTILRLH-C-ter), synthesized by Cosmo Genetech (Seoul, Korea), and coupled to BSA. A rabbit was injected with 100 μg of the BSA-conjugated synthetic peptide in Freund's complete adjuvant and boosted at 2-week intervals using subcutaneous injections of 200 μg BSA-conjugated synthetic peptide in Freund's incomplete adjuvant. The rabbit was bled at 1 week after the fifth injection, and antiserum was purified *via* affinity peptide column coupling using the BSA-conjugated peptide. The purified antibody recognized full-length (23 kDa) cinnamon clownfish GnIH.

The serum antibody titer and purity of the purified antibody were determined using enzyme-linked immunosorbent assay

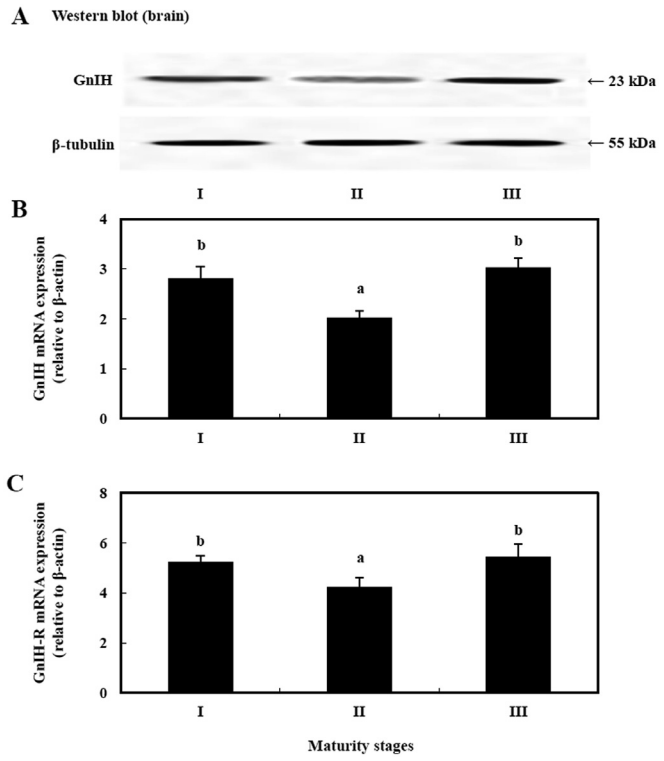

Fig. 2. Expression of GnIH and GnIH-R in the brain of cinnamon clownfish during sex change. (A) Western blot using GnIH (dilution 1:5,000; 23 kDa) to examine protein expression and β-tubulin (55 kDa) as the internal control. (B) GnIH and (C) GnIH-R mRNA levels, relative to β-actin mRNA levels, were examined using real-time PCR. I, mature male; II, male at 90 days after removing female; III, mature female. Values with dissimilar letters are significantly different ($P < 0.05$), and all values are means ± SE ($n = 5$).

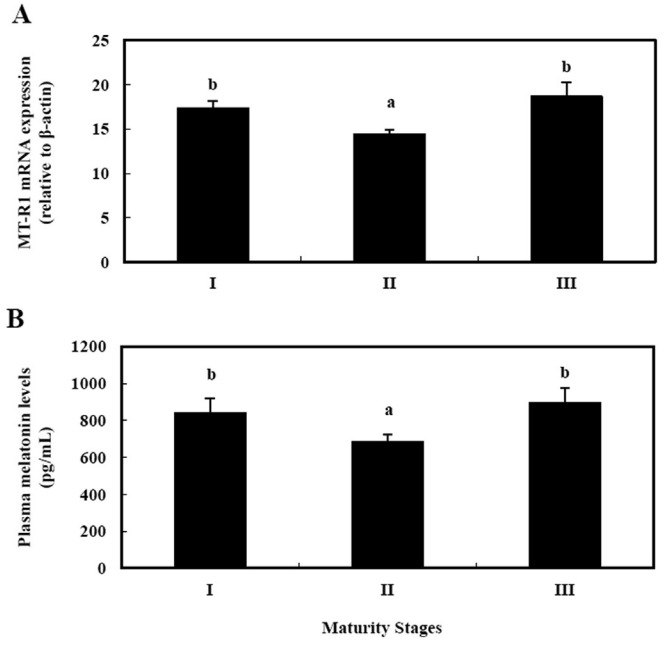

Fig. 3. Change in MT-R1 mRNA expression and plasma melatonin activity in cinnamon clownfish during the sex change. (A) MT-R1 mRNA levels were analyzed, relative to β-actin mRNA levels, using real-time PCR, and (B) the activity of melatonin in the plasma of cinnamon clownfish was analyzed using a plate reader. I, mature male; II, male at 90 days after removing female; III, mature female. Values with dissimilar letters are significantly different ($P < 0.05$), and all values are means ± SE ($n = 5$).

(ELISA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. ELISA was conducted using the peptide antigen as the standard, was found to be 1:100 to 1:100,000 after 6 weeks of immunization. Briefly, 20 μL of each sample was added to pre-coated wells, 200 μL of enzyme-linked conjugate was dispensed into each well, and the samples were incubated for 60 min at 37 °C. Subsequently, 200 μL of freshly prepared substrate solution was added to the wells and incubated for 20 min at 37 °C, and between each step, the wells were washed three times with washing buffer (Tris-buffered saline with Tween (TTBS)). After blocking the reaction (5% milk in TTBS for 45 min), absorbance was measured at 450 nm with a microplate reader.

Purified protein was separated using SDS-PAGE on a 10–15% gradient gel, alongside a protein marker (Fermentas, Ontario, Canada). The gels were stained with 0.05% Coomassie Brilliant Blue R-250.

2.4. Western blot analysis

Total protein was isolated from the brains of clownfish from the sex change, using T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. A total of 30 μg protein was loaded onto each lane of Mini-PROTEAN® TGX™ Gels (Bio-Rad), and a protein ladder (Bio-Rad) was used for reference. The samples were electrophoresed at 180 V, and the gels were immediately transferred to a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min, using the Trans-Blot Turbo Transfer System (Bio-Rad). Subsequently, the membranes were blocked with 5% milk in TTBS for 45 min and subsequently washed in TTBS for 10 min. The membranes were incubated with anti-GnIH polyclonal rabbit antibody (1:5,000 dilution) for 120 min or, as an internal control, incubated with β-tubulin (dilution 1:5,000 ab6046; Abcam, Cambridge, UK) for 120 min and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (dilution 1:2,000, Bio-Rad) for 60 min. Bands were detected using WesternBright ECL (Advansta, Menlo Park, CA, USA) and exposure for 30 s with a Molecular Imager ChemiDoc XRS$^+$ System (Bio-Rad). The membrane images were then scanned using a high-resolution scanner, and band density was estimated using Image Lab Software (version 3.0; Bio-Rad).

2.5. Analysis of plasma parameters

The levels of melatonin was analyzed using the immunoassay technique with ELISA kits for melatonin (MyBioSource, San Diego, CA, USA). Briefly, anti-antibody that was specific to antibodies against hormones (melatonin) was pre-coated onto a microplate, and 50 μL of plasma, 50 μL of HRP-conjugate, and 50 μL antibody was added to each well and incubated at 37 °C for 2 h and washed the plate at three times. After the last wash, the remaining wash buffer was removed by aspirating or decanting. Next, 50 μL of substrate solution were added to each well, incubated at 37 °C for 15 min in the dark, and terminated with 50 μL stop solution. Finally, within 10 min after reaction termination, the optical density of each well was determined, using a microplate reader at 450 nm.

2.6. Double immunofluorescence staining and visualization

The brain tissues collected during the sex change and maturation processes were fixed in Bouin's solution, dehydrated in increasing ethanol concentrations, clarified in xylene, embedded in paraffin, cut into 1-μm thick sections, and prepared with on each microscope slide. For double immunofluorescence staining of selected sections, both primary antibodies (rabbit GnIH [1:1,000]

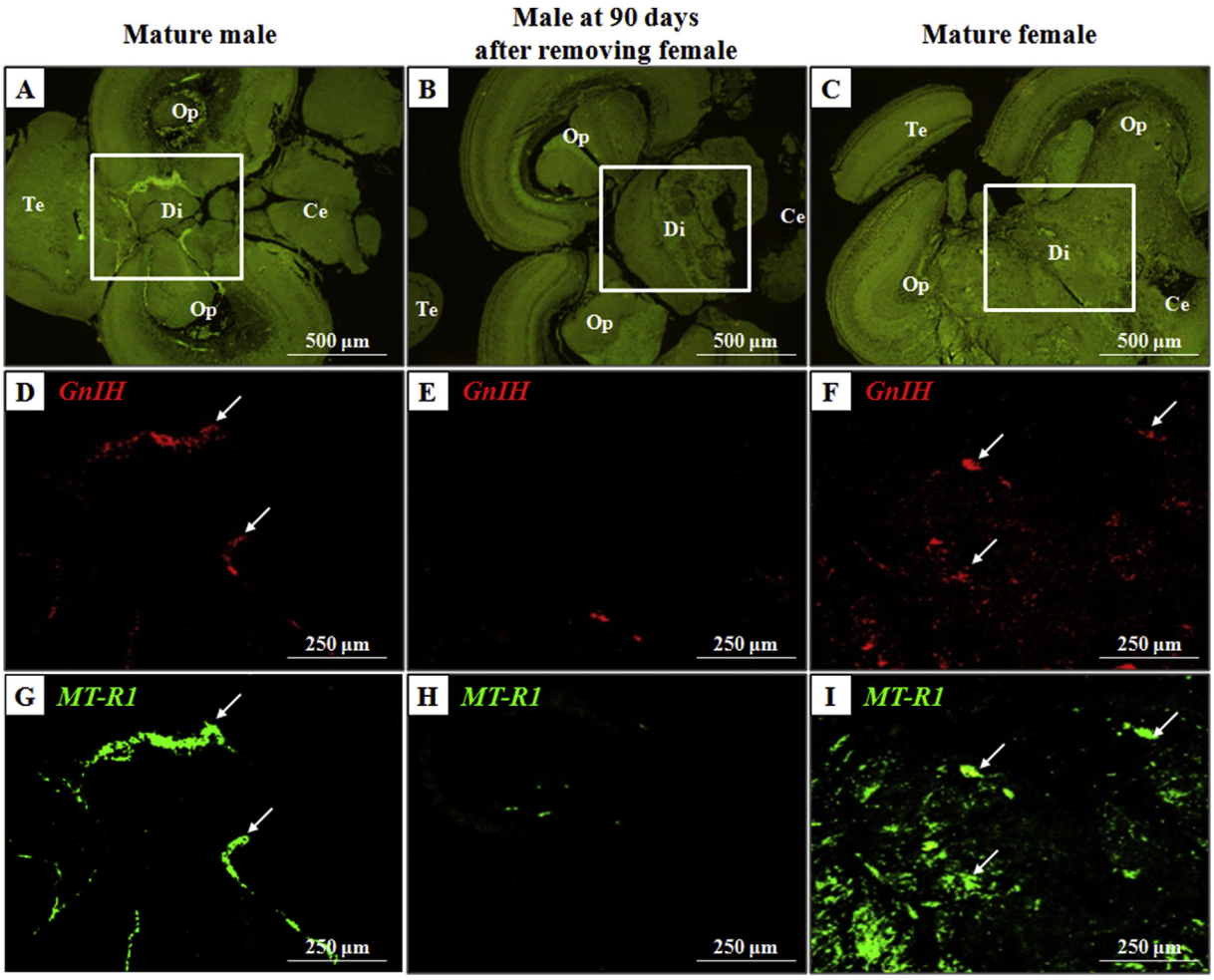

Fig. 4. Co-localization of GnIH and MT-R1 proteins in cinnamon clownfish hypothalamus, during sex change, as indicated by double immunofluorescence staining. I, mature male (A, D, G); II, male at 90 days after removing female (B, E, H); III, mature female (C, F, I). Whole brains (A–C) and diencephalons stained with Texas Red (red; D–F) or FITC (green; G–I). Arrows indicate the overlap of GnIH-immunoreactive (IR)- and MT-R1-IR-stained cells. Te, telencephalon; Op, optic tectum; Ce, cerebellum; Di, diencephalons. Scale bars = 500 μm (A–C) and 250 μm (D–I). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and anti-MT-R1 [1:100; Melatonin Receptor 1A Antibody; Novus Biologicals LLC, Littleton, CO, USA]) and, subsequently, both secondary antibodies were combined. Secondary anti-rabbit antibody conjugated to fluorescein (Vector Laboratories, Burlingame, CA, USA) was used to visualize anti-GnIH, and secondary anti-goat antibody conjugated to Texas Red (Vector Laboratories) was used to visualize anti-MT-R1. Both secondary fluorescence antibodies were used at 1:100 dilution in PBS with 0.5% BSA. The Slides were incubated in a humidified chamber at 37 °C for 1.5 h, washed three times in PBS for 10 min each, dipped in ddH_2O, and coverslipped with Vectashield (Vector Laboratories). The fluorescently labeled material was then visualized, using an epifluorescent microscope (Nikon ES800; Tokyo, Japan) outfitted and a double-band pass cube (FITC-Texas Red) to allow for simultaneous visualization of both antibodies. Photographs were taken using a fluorescence microscope (excitation filter 465–495 nm; Eclipse *Ci*, Nikon).

2.7. Statistical analysis

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

3. Results

3.1. Production of GnIH polyclonal antibody

The results of ELISA analysis indicated that the anti-GnIH antibodies collected at 2, 4, and 6 weeks cross-reacted with synthetic GnIH and that the signal decreased proportionally to the dilution of the anti-GnIH antibodies from 1:100 to 1:100,000 (Fig. 1A). Furthermore, competitive ELISA development demonstrated the availability of a standard and an antibody specific of the protein. We also confirmed the molecular weight of the GnIH antibody (~23 kDa) by Western blot analysis (Fig. 2A). SDS-PAGE analysis of purified GnIH and staining with anti-GnIH polyclonal antibody showed a major band corresponding to GnIH (Fig. 1B). Under non-reducing conditions, the apparent molecular sizes of the GnIH IgG antibodies were between 55 and 75 kDa, and under reducing conditions, the protein bands corresponding to the heavy (40–45 kDa) and light (15 kDa) chains, which suggests that the antibodies were properly folded and glycosylated.

3.2. Expression of GnIH and GnIH-R mRNA and GnIH protein during the sex change process

Western blot analysis revealed a protein with GnIH

immunoreactivity and with a size similar to the predicted size for cinnamon clownfish GnIH (23 kDa; Fig. 2A). The protein expression pattern also resembled the pattern of GnIH and GnIH-R mRNA expression in cinnamon clownfish brains, since the levels of GnIH and GnIH-R mRNA were higher in mature males (approximately 1.4-fold) and females (approximately 1.3-fold) than in individuals undergoing sex change (Fig. 2B and C, respectively).

3.3. Change of MT-R1 and melatonin during the sex change process

Similarly, we found that MT-R1 mRNA was higher in mature males (approximately 1.2-fold) and females (approximately 1.3-fold) than in individuals undergoing sex change (Fig. 3A), and the plasma levels of melatonin were 842.1 ± 60.2 pg/mL in males with a female present, 682.1 ± 52.2 pg/mL in males at 90 days after female removal, and 896.7 ± 71.3 pg/mL in mature females (after sex change; Fig. 3B).

3.4. Double immunofluorescence staining during the sex change process

Double immunofluorescence staining demonstrated that the GnIH and MT-R1 proteins were co-expressed in the cinnamon clownfish diencephalon (Fig. 4). Furthermore, like the expression of GnIH and MT-R1 mRNA, both GnIH and MT-R1 proteins were more abundant in mature fish (male, Fig. 4D and 4G; female, Fig. 4F and 4I) than in individuals undergoing sex change (Fig. 4E and 4H).

4. Discussion

In the present study, we found that the expression levels of GnIH, GnIH-R, and MT-R1 mRNA was significantly lower in males at 90 days after female removal than in mature males and females, as were the expression levels of GnIH protein and levels of melatonin. In addition, in the results of GnIH and MT-R1 expression on diencephalon by IHC double staining, we observed the pattern that expression of GnIH and MT-R1 protein in the sex change groups reduced significantly in contrast to the mature male and female.

In contrast, a previous study [16] reported that the mRNA expression of GnRHs (sGnRH, sbGnRH, and cGnRH-II), which activate the HPG axis and promote sex change, significantly increased during gender transition, suggesting that GnRH increased in response to the inhibition of GnIH. In this study, GnIH expression of sex change stage decreased, we suggested that the increased GnRH due to the inhibition of the GnIH activate the HPG asix and promote sex change process. In fact, Tsutsui et al. [17] also reported that GnIH controls HPG axis activity by acting on the hypothalamus through its interaction with GnRH and directs the synthesis and release of GTHs (GTH, FSH, and LH) by acting on the pituitary gland. In Japanese quail, GnIH levels increased with increasing plasma levels of melatonin and they found that the expression of GnIH mRNA in brain cell cultures is increased by melatonin treatment (1, 10, and 100 nM), which suggests that melatonin directly controls GnIH expression and hormone levels [18]. In addition, Ubuka et al. [13] found that, after removing the pineal gland and retina (eye), which are the main components of melatonin biosynthesis in Japanese quail, both GnIH mRNA and hormone levels decreased in response to treatment with increasing concentrations of melatonin (2.5, 10, and 40 mg), whereas melatonin levels increased. Since the present study also found that levels of GnIH, GnIH-R, and MT-R1 mRNA, GnIH proteins, and plasma melatonin were lower during sex change, we suggest that their reduced expression activates the HPG axis and does not differ in the sex-determined condition (i.e. between mature males and females).

To date, research associated with GnIH has been actively conducted in mammals and birds [13,17,18], but studies in fish are limited. In particular, no studies have addressed the effects of GnIH during sexual maturation and sex change in hermaphroditic fish. Therefore, our results could be used as useful baseline data for identifying the mechanism of sex conversion and delayed maturation in hermaphroditic fish, such as the cinnamon clownfish.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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