



Effect of cortisol on gonadotropin inhibitory hormone (GnIH) in the cinnamon clownfish, *Amphiprion melanopus*



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ABSTRACT

Hypothalamic peptides, gonadotropin-releasing hormone (GnRH) and gonadotropin inhibitory hormone (GnIH), play pivotal roles in the control of reproduction and gonadal maturation in fish. In the present study we tested the possibility that stress-mediated reproductive dysfunction in teleost may involve changes in GnRH and GnIH activity. We studied expression of brain GnIH, GnIH-R, seabream GnRH (sbGnRH), as well as circulating levels of follicle stimulating hormone (FSH), and luteinizing hormone (LH) in the cinnamon clownfish, *Amphiprion melanopus*. Treatment with cortisol increased GnIH mRNA level, but reduced sbGnRH mRNA and circulating levels of LH and FSH in cinnamon clownfish. Using double immunofluorescence staining, we found expression of both GnIH and GnRH in the diencephalon region of cinnamon clownfish brain. These findings support the hypothesis that cortisol, an indicator of stress, affects reproduction, in part, by increasing GnIH in cinnamon clownfish which contributes to hypothalamic suppression of reproductive function in *A. melanopus*, a protandrous hermaphroditic fish.

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1. Introduction

The hypothalamic GnRH is the main stimulator of gonadal maturation and function in fish by regulating release and synthesis of pituitary gonadotropins (follicle stimulating hormone [FSH] and luteinizing hormone [LH]) [1,2]. Multiple forms of GnRH are expressed in the brain of the same species and in some teleosts, there are three forms of GnRH, including salmon GnRH [sGnRH], chicken GnRH-II [cGnRH-II], and seabream GnRH [sbGnRH] [3,4]. There is evidence that, sbGnRH is produced as a third form in neuronal groups localized in the ventral forebrain along the terminal nerve, and controls gonadotropins (GTHs) secretion [1,5]. In fish, FSH is involved in control of ovarian follicular development and spermatogenesis, and LH promotes final follicular maturation, ovulation, and the synthesis of steroid hormones [6–8].

Until recently, GnRH was considered to be the only hormone in

the family of hypothalamic neuropeptides that controlled the synthesis and release of GTHs. However, Tsutsui et al. [9] discovered gonadotropin-inhibitory hormone (GnIH) in the brain of the Japanese quail, *Coturnix japonica*, in 2000. GnIH belong to the family of RF-amide peptides and plays a predominantly an inhibitory role in the control of reproduction in birds, mammals and number of other species vertebrate species [9–11]. GnIH acts on the anterior pituitary gland through a specific GnIH receptor (GnIH-R or GPR147) and inhibits the synthesis and release of GTHs, and inhibits the reproductive cycle [10,12,13]. Furthermore, GnIH affects the GnRH neuron of the hypothalamus and directly inhibits GnRH synthesis and secretion [14]. GnIH delays maturation by inhibiting the secretion of the anterior pituitary FSH and LH [9,15,16]. However in a number of gonochoristic teleost species studied, GnIH was shown to exert both stimulate and inhibit actions on the pituitary LH and growth hormone production, depending on the season and mode of administration [2,17,18]. More recent studies have demonstrated inhibitory actions of GnIH on the expression of reproductive hormones in hermaphroditic cinnamon clownfish [19]. An interesting recent finding is potential interaction of GnIH with the brain-pituitary-adrenal (HPA) axis and stress response which is the focus of

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the present study. A significant decrease in GnRH mRNA level was observed when there was an increase in the corticosterone concentration [20]. Corticosterone was found to directly inhibit the expression of GnIH in the mammalian cells [21,22]. This is important since glucocorticoids are known to be associated with stress response [23–25], and chronic stress often has negative effects on reproduction. In this regard, The hypothalamus–pituitary–interrenal (HPI) axis is activated to maintain homeostasis in organisms exposed to stress [23,25]. To regulate the hormones released in response to stress, the corticotropin-releasing hormone is secreted by the hypothalamus and it acts on the anterior pituitary to stimulate the secretion of the adrenocorticotropic hormone [23]. Adrenocorticotropic hormone, which is derived from the precursor protein pro-opiomelanocortin, stimulates the synthesis and release of cortisol in interrenal cells of the head kidney [23,25]. In vertebrates, glucocorticoid hormones secreted from the HPI axis include cortisol, and have a negative effect on the physiological aspects of vertebrate reproduction by inhibiting the activity of the hypothalamus–pituitary–gonad (HPG) axis [26,27].

In this study, we investigated the effect of cortisol, a stress response-related hormone, on the sexual maturation of the cinnamon clownfish, *Amphiprion melanopus* and investigated the effects of cortisol on the function of GnIH, which delays maturation. We investigated two concentrations of cortisol injection on cinnamon clownfish over the course of a 48 h short-term experiment, as well as by sampling the fish at 3 week intervals for a long-term experiment (9 weeks). We measured mRNA expression of GnIH, GnIH-R, and sbGnRH, as well as plasma levels of FSH and LH to determine cortisol effects. Additionally, we stained the GnIH and GnRH molecules in the hypothalamus cells after cortisol treatment using immunohistochemistry techniques.

2. Materials and methods

2.1. Experimental fish

The study was conducted with immature cinnamon clownfish ($n = 200$, length, 4.6 ± 0.5 cm, weight, 6.3 ± 0.4 g, gonadosomatic index [GSI; gonad weight/body weight $\times 100$] = 0.03 ± 0.03). The water temperature and photoperiod were 27 ± 1 °C and 12-h light:12-h dark cycle (lights were on from 07:00–19:00 h), respectively. The fish were fed a commercial feed twice daily (09:00 h and 17:00 h) and were reared under these conditions for 9 weeks.

2.2. Experimental design and sampling

To investigate the effects of cortisol on maturation during the course of a short- and long-term treatment, the cinnamon clownfish were anesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO, USA) prior to injection.

For short-term experiment, cortisol (hydrocortisone-21-hemisuccinate; Sigma, St Louis, MO, USA) was dissolved in 0.9% physiological saline, and each fish was given an intraperitoneal injection with cortisol (10 or 50 µg/g body mass (BM)) at a volume of 10 µL/g BM. The sham control group was injected with an equal volume of physiological saline (0.85% NaCl in distilled water, 10 µL/g BM). The control samples (untreated control groups) were removed prior to injection. Brain were collected from cinnamon clownfish at 0, 6, 12, 24, and 48 h, and immediately frozen in liquid nitrogen, and stored at -80 °C until the total RNA was extracted for analysis. Plasma samples were separated from blood of five fish by centrifugation (4 °C, 10,000g, 5 min) and stored at -80 °C until analysis.

For long-term experiment, each fish was injected with cortisol

(10 or 50 µg/g BM) once weekly at 14:00 h. The sham control group was injected with an equal volume of physiological saline once weekly at 14:00 h. The control samples (untreated control groups) were removed prior to injection. After injection, brain and blood samples were obtained from cinnamon clownfish at 3, 6, and 9 weeks, and immediately frozen in liquid nitrogen, and stored at -80 °C until the total RNA was extracted for further analysis. Plasma samples were separated from blood of five fish by centrifugation (4 °C, 10,000g, 5 min) and stored at -80 °C until analysis.

2.3. Quantitative PCR (qPCR)

qPCR was conducted to determine the relative expression levels of GnIH, GnIH-R, and sbGnRH mRNA using the total RNA extracted from the tissue of cinnamon clownfish using the Tri-Reagent® (MRC, Cincinnati, OH, USA), respectively. Reverse transcription (RT) of cDNA was performed using M-MLV reverse transcriptase (Promega, OH, USA) according to the manufacturer's instructions. The following qPCR primers were designed in reference to known sequences (GenBank accession numbers: GnIH, **KT455505**; GnIH-R, **KT455506**; sbGnRH, HQ883476; 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'); GnIH-R forward (5'-CTG GTG GAC AAC CTC ATC AC-3') and reverse (5'-TTG GAG AAG GCC AAC CAG TG-3'); sbGnRH forward (5'-CTG CTG GTG ATG ATG-3') and reverse (5'-GGT CTC TCT TCC CTC CTG-3'); and finally β -actin forward (5'-AGC ACG GTA TTG TGA CCA AC-3') and reverse (5'-ACG GAA CCT CTC ATT GCC AG-3'). PCR 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. The qPCR program was as follows: one cycle of denaturation at 95 °C for 5 min, 35 cycles each consisting of denaturation at 95 °C for 20 s and annealing and extension at 55 °C for 20 s. Each experimental group was run in triplicate. Transcript levels of genes mRNAs 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 (Δ Ct) levels. The calibrated Δ Ct value ($\Delta\Delta$ Ct) for each sample and internal control (β -actin) was calculated as $\Delta\Delta$ Ct = $2^{-(\Delta$ Ct_{sample} - Δ Ct_{internal control})}.

2.4. Plasma parameters analysis

Plasma FSH and LH levels were analyzed using the immunoassay technique with the ELISA kit (FSH (catalog no. MBS035576; Mybiosource, USA) and LH (catalog no. MBS283097; Mybiosource, USA). An anti-antibody specific to the antibody of the hormones tested (FSH and LH) was pre-coated onto a microplate. A total of 50 µL of plasma was added to each well, in addition to 50 µL of HRP-conjugate, followed by 50 µL of the antibody. All wells were thoroughly mixed then incubated for 2 h at 37 °C. After the final wash, remaining wash buffer was removed by aspirating or decanting. At this point, 50 µL of Substrate A and Substrate B was added to each well, and incubated for 15 min at 37 °C in the dark. After incubation, add 50 µL of stop solution was added to each well. Finally, in order to determine the optical density of each well after 10-min intervals, using a microplate reader set to 450 nm.

2.5. Double immunofluorescence staining and visualization

The diencephalon tissue of cinnamon clownfish collected 9 weeks after cortisol treatment were fixed in Bouin's solution, dehydrated in increasing ethanol concentrations, clarified in xylene, embedded in paraffin, cut into 5 µm thick sections, and prepared on individual microscope slides. For the double

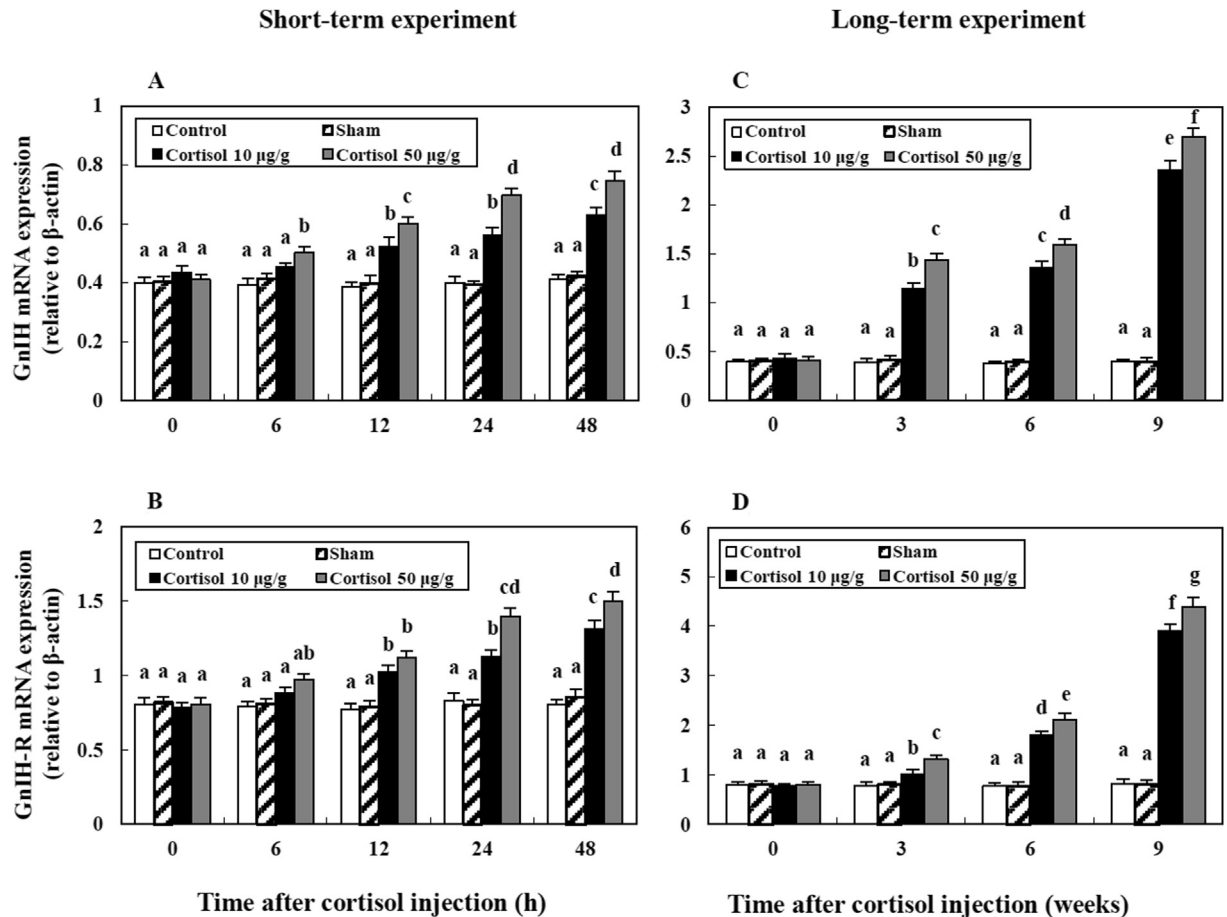


Fig. 1. mRNA expression levels of GnIH (A, C) and GnIH-R (B, D) in brains of cinnamon clownfish during 48 h (A, B) and 9 weeks (C, D) after cortisol injection (10 μ g/g and 50 μ g/g). mRNA levels are relative to the β -actin mRNA levels in the cinnamon clownfish. Values with different letters indicate significant differences ($P < 0.05$). All values are means \pm SE ($n = 5$).

immunofluorescence staining of selected sections, both primary antibodies (rabbit GnIH [1:1000] and mouse GnRH [1:5000; courtesy of M.K. Park [28]) and, subsequently, both secondary antibodies were combined. Secondary anti-rabbit antibodies conjugated to Texas Red (Vector Laboratories, Burlingame, CA, USA) were used to visualize *anti*-GnIH, and secondary anti-mouse antibody conjugated to fluorescein (Vector Laboratories) was used to visualize *anti*-GnRH. Both secondary fluorescent antibodies were used at a 1:100 dilution in PBS with 0.5% BSA. The slides were incubated in a humidified chamber at 37 $^{\circ}$ C for 1.5 h, washed three times in PBS for 10 min, dipped in ddH₂O, and coverslipped with Vectashield (Vector Laboratories). The fluorescently labeled material was then visualized, using an epifluorescence microscope (Nikon ES800; Tokyo, Japan) and a double-band pass cube (FITC-Texas Red) to allow for simultaneous visualization of both antibodies.

2.6. Statistical analysis

All data were analyzed using Two-way analysis of variance followed by Tukey's post hoc test. Differences were considered significant when $p < 0.05$. Values are expressed as mean \pm standard error (SE). We used a SPSS statistical package to perform data analysis (version 10.0; SPSS Inc., USA).

3. Results

3.1. Time-related effects of cortisol on GnIH and GnIH-R mRNA levels

In the short-term experiment, treatment with cortisol increased GnIH mRNA levels in a time and dose-related manner, reaching levels that were approximately 1.8-fold higher after 6 h of treatment (Fig. 1A). Similar trend was observed for GnIH-R mRNA level, although the differences became significant after 12 h of treatment. Both concentrations of cortisol tested at 10 and 50 μ g/g exerted significant effects, although the higher dose elicited the greatest response (Fig. 1B).

In the long-term experiment, treatments with cortisol at both doses tested (10 and 50 μ g/g) resulted in significantly higher levels of GnIH and GnIH-R mRNA levels after 3 weeks. The effect of cortisol became significantly greater after 6 and 9 weeks in a clear time-related manner. There was also a pattern of dose-related effect, although the differences between 10 and 50 μ g/g were not large, but statistically significant (Fig. 1C and D).

3.2. Time-related effects of cortisol on sbGnRH mRNA levels

In the same experiments, we also measured sbGnRH mRNA levels. Treatment with cortisol had the opposite effect compared to GnIH. In this case we only measured sbGnRH following treatment with cortisol following short-term treatment (up to 48 h) and long-

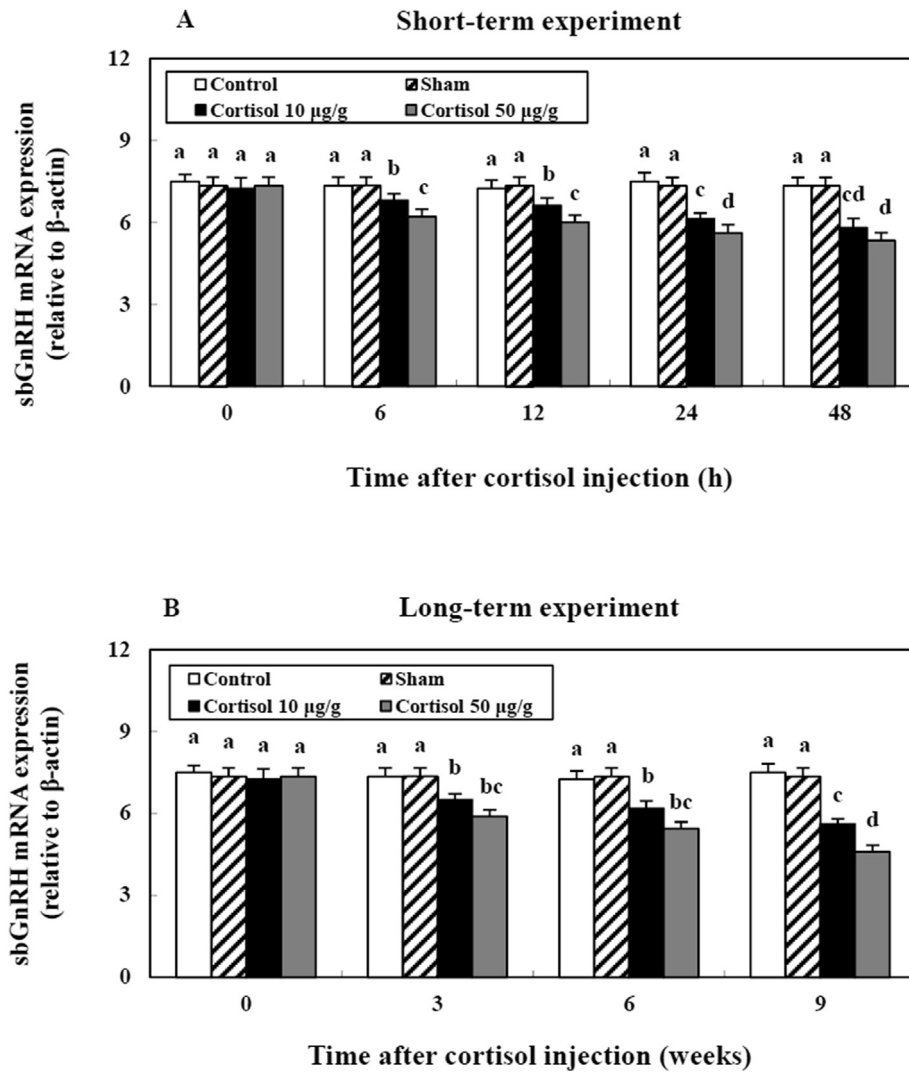


Fig. 2. Expression levels of sbGnRH mRNA in brains of cinnamon clownfish during 48 h (A) and 9 weeks (B) after cortisol injection (10 µg/g and 50 µg/g). Values with different letters indicate significant differences ($P < 0.05$). All values are means \pm SE ($n = 5$).

term treatment (up to 9 weeks). Here we also see a dose and time related reduction in sbGnRH mRNA level following treatment with cortisol (Fig. 2A and B). The inhibitory action of cortisol was significant after 6 h of treatment (Fig. 2A). Long-term treatment also resulted in a dose-, and time-related reduction in sbGnRH mRNA level (Fig. 2B).

3.3. Immunofluorescence staining of GnIH and sbGnRH following cortisol treatment

Double immunofluorescence staining demonstrated that the GnIH and GnRH proteins were expressed in the same region of diencephalon in the cinnamon clownfish brain (Fig. 3). The results are very consistent with the mRNA levels measured for both GnIH and GnRH. Similar to the expression of GnIH mRNA (Fig. 1C), GnIH protein level was more abundant after 9 weeks of treatment with cortisol (Fig. 3G). Also the GnRH protein levels decreased after 9 weeks of treatment with cortisol (Fig. 3H).

3.4. Time-course effects of hormone on GTHs

In the present study, we measured the circulating concentrations of LH and FSH levels, using a heterologous immunoassay. The levels and trends observed for both LH and FSH were very similar, and consistent with the results observed for GnIH and sbGnRH mRNA level and GnRH protein above. The effects of cortisol were significantly greater in the long-term study than the acute effect observed in the short-term experiment. Cortisol treatment resulted in a dose- and time-related reduction in both LH and FSH concentrations (Fig. 4). The results are shown are relative and are not absolute.

4. Discussion

It is generally accepted that long-term stress response may impair normal reproduction. The aim of this study was to investigate potential role of GnIH as a contributing factor that suppress reproduction in fish, using a proandrous hermaphrodite species, cinnamon clownfish, as model organism. The present study provides strong evidence that treatment with cortisol results in

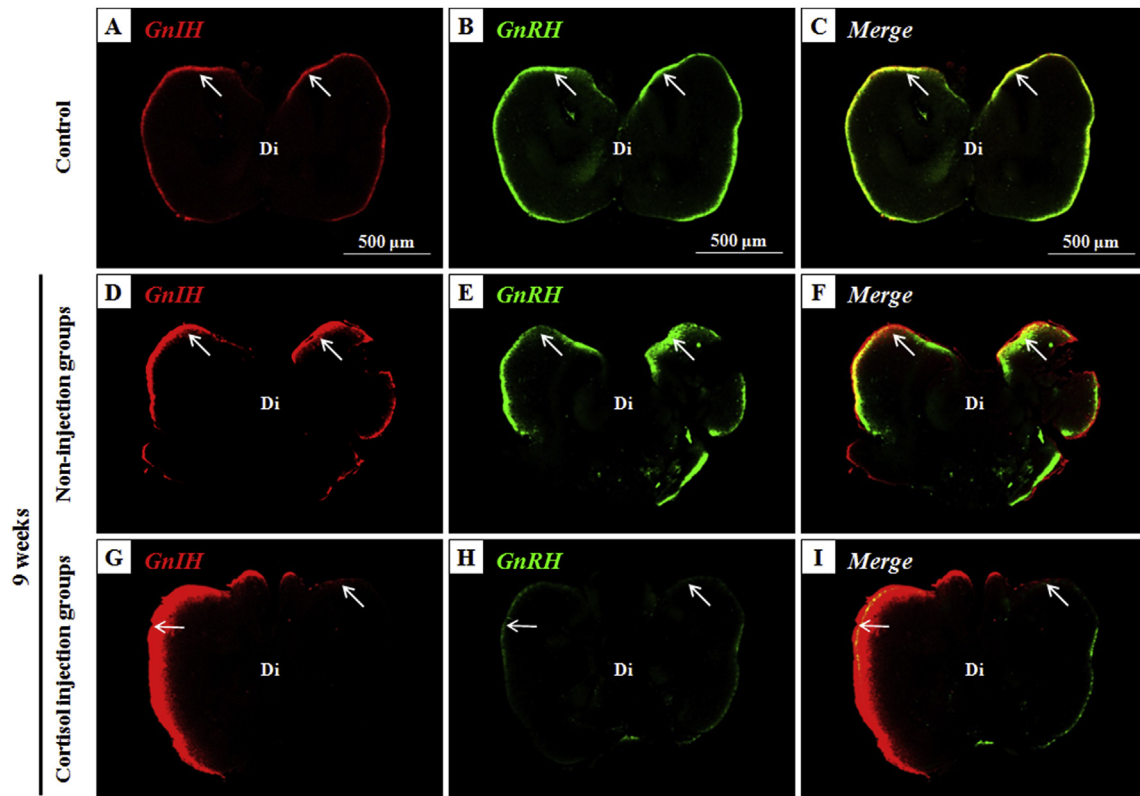


Fig. 3. Co-localization of GnIH and GnRH proteins in the diencephalon of cinnamon clownfish 9 weeks after cortisol injection (50 $\mu\text{g/g}$), revealed by double immunofluorescence staining. (A–C) controls (non-injection groups), (D–F) 9 weeks after non-injection groups, (G–I) 9 weeks after cortisol injection. Diencephalons (Di) were stained with Texas Red (red; A,D,G) or FITC (green; B,E,H) with anti- GnIH and GnRH conjugated secondary antibodies. The overlap of GnIH-immunoreactive (IR)- and GnRH-IR-stained cells with the red and green images are shown in yellow in the merged images (C, F, I) and also indicated by arrows. Scale bars = 500 μm .

suppression of GnRH production which is a stimulator of reproduction, and increase GnIH which is an inhibitory factor in cinnamon clownfish. The observed results are very consistent and demonstrate inhibitory actions of cortisol following both acute and chronic treatments at multiple levels which can lead to impairment of reproduction. The consequence would be different in gonochoristic and hermaphroditic teleost species. In the gonochoristic species the stress response could potentially range from delayed gonadal maturation and development if the stress is not severe to significant impairment of reproduction. The present results suggest that in hermaphroditic species such as cinnamon clownfish, chronic severe stress may impair sex change. The stress response in gonochoristic species may be different and dependent on the season based on the results observed in goldfish and salmonid [2,17]. One limitation of the present study is the use of heterologous immunoassay to measure circulating levels of LH and FSH. The results obtained for LH and FSH are very similar and as a result, we cannot rule out cross reactivity of the antibodies used for LH and FSH. However, the results are consistent with the observed effects on GnRH. We used the heterologous assay since homologous immunoassay since homologous assay for cinnamon clownfish LH and FSH are not currently available.

The results obtained following treatment with cortisol are very similar in terms of increase in GnIH and GnIH-R provide a strong support for the hypothesis that the effect of cortisol is direct at the level of neuro secretory cells. However, the results do not rule out indirect action mediated by other hormones or biochemical factors affected by cortisol.

The results obtained following immunostaining for GnIH and

GnRH provide direct support for the observed actions on GnIH and sbGnRH mRNA levels. Double staining of GnIH and GnRH proteins in the diencephalon, revealed that both peptides are expressed in the same brain regions in cinnamon clownfish. The immunostaining study also verify that cortisol differentially regulate expression of GnIH and GnRH leading to increased inhibitory and reduced stimulatory factors controlling reproduction. Our results are consistent with a previous finding by Tsutsui et al. [29] demonstrating the presence of GnIH-R in the GnRH neurons, located in the hypothalamus of the brain. Also, Kirby et al. [30] reported that exposure to acute and chronic stress in the mature rat male, changes the concentration of plasma corticosterone, which is similar to the function identified of cortisol. This also caused increased GnIH mRNA expression, which was suggested to occur due to the activity of the HPA axis that increases the levels of glucocorticoids, and decreases the activity of HPG. These studies reported that, when removed, the adrenal gland that is the location for the synthesis of glucocorticoids, corticosterone levels and mRNA expression of GnIH do not change even when the HPA axis is activated. This suggests that the glucocorticoids, including corticosterone, are factors that directly increase the expression of the GnIH [30]. The results of Kirby et al. [30] indicated that GRs are located in the 53% of GnIH (RFPR) cells of the hypothalamus, suggesting that glucocorticoids act directly on the RFPRs cells to increase GnIH, so inhibit the HPG axis. We did not investigate GRs in the present study.

Thus, similar to previous studies, here mRNA expression levels of GnIH and GnIH-R increased during cortisol treatment. This was the case with hormones secreted mainly by the HPI axis, indicating

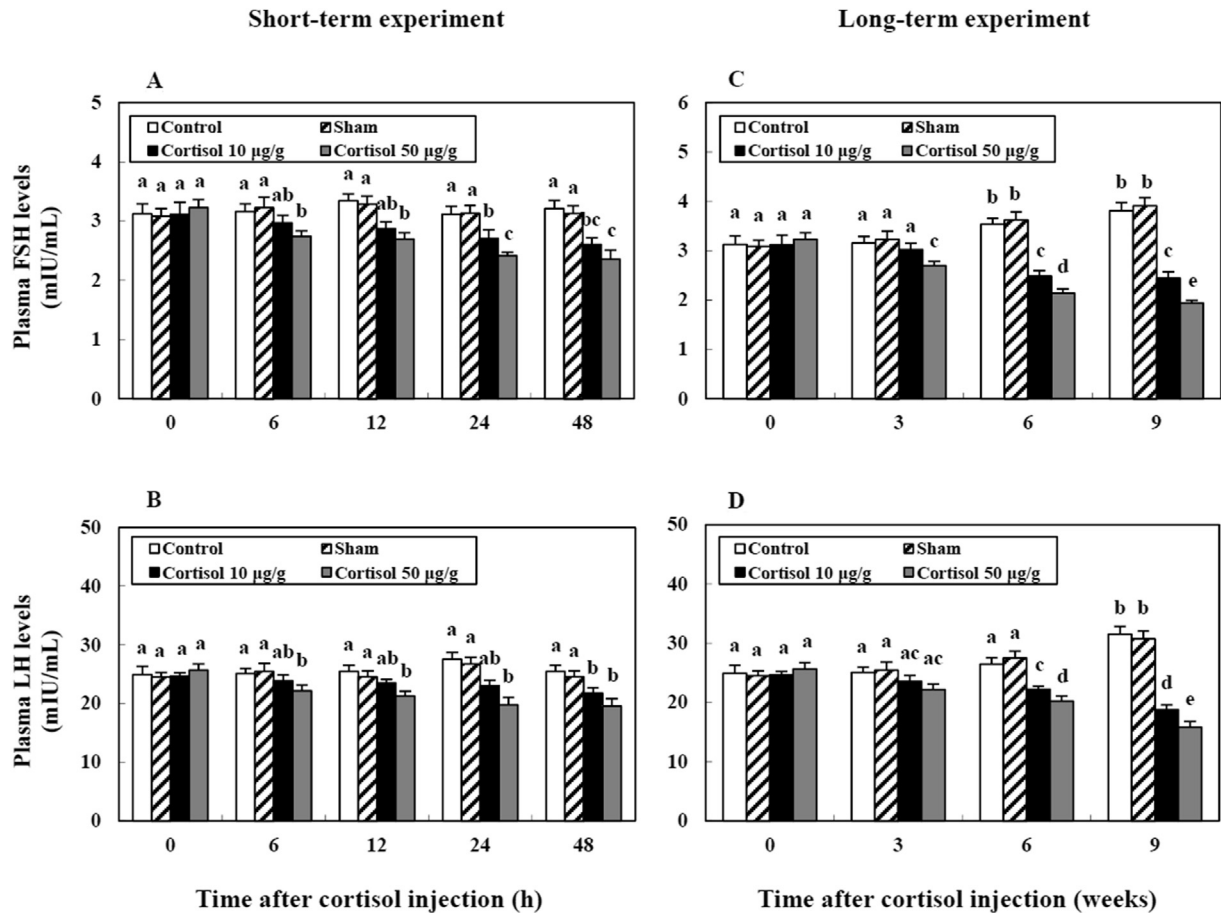


Fig. 4. The activity of plasma FSH (A, C) and LH (B, D) of cinnamon clownfish during 48 h (A, C) and 9 weeks (B, D) after cortisol injection (10 µg/g and 50 µg/g) was analyzed with a plate reader. Values with different letters indicate significant differences ($P < 0.05$). All values are means \pm SE ($n = 5$).

that interactions of GnIH and cortisol have a positive feedback.

In this study, mRNA expression levels of sbGnRH and plasma levels of GTHs significantly decreased after cortisol treatment (short-term experiment). However, mRNA expression of GnIH and GnIH-R significantly increased after cortisol treatment (short-term experiment). Additionally, in the long-term experiment, similar to what was found in the short-term experiment, mRNA expression levels of sbGnRH and plasma levels of FSH and LH decreased.

Qi et al. [31] reported that mRNA expression of GnRH, FSH, and LH significantly decreased in goldfish, *Carassius auratus* after GnIH treatment. Additionally, Kirby et al. [30] confirmed that as cortisol levels increase, GnIH mRNA expression are also enhanced proportionally, suggesting that an increase in concentrations of cortisol and GnIH reduce the levels of plasma LH.

Overall, the present results provide a strong support for the hypothesis that cortisol which is a mediator of stress response can impair reproduction by directly increase GnIH and reduce GnRH, and lead to impairment of reproduction. The findings provide a framework for better understanding of adverse stress response in teleosts and other vertebrates, in general.

Conflict of interest

The authors declare no conflict of interest.

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