Thesis for the Degree of Doctor of Philosophy

Molecular and Endocrinological Studies on Gonadotropin-Inhibitory Hormone (GnIH) in the Protandrous Cinnamon Clownfish *Amphiprion melanopus*

Young Jae Choi

Department of Marine BioScience and Environment

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Advisor: Prof. Cheol Young Choi

by

Young Jae Choi

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CONTENTS

List of Table

List of Figures

List of Abbreviations

ACTH	adrenocorticotropic hormone
BM	body mass
BSA	bovine serum albumin
CCORA	Center of Ornamental Reef and Aquarium
cDNAs	complementary DNAs
cGnRH-II	chicken GnRH-II
CRH	corticotropin-releasing hormone
Ct	cycle threshold
ELISA	enzyme-linked immunosorbent assay
FSH	follicle-stimulating hormone
GnIH	gonadotropin-inhibitory hormone
GnIH-R	gonadotropin-inhibitory hormone receptor
GnRH	gonadotropin-releasing hormone
GPR54	G protein coupled receptor 54
GR	glucocorticoid receptor
GSI	gonadosomatic index
GTH	gonadotropin
HPG	hypothalamus–pituitary–gonad
HPA	hypothalamus–pituitary–adrenal
HPI	hypothalamus–pituitary–interrenal
IF	immunofluorescence
Kiss	kisspeptin
LH	luteinizing hormone
MT-R	melatonin receptor
PCR	polymerase chain reaction
PVDF	polyvinylidene diflouride
qPCR	quantitative real-time PCR

RF-amide	Arg-Phe-amide
RFRPs	RF-amide-related peptides
RT	reverse transcription
sGnRH	salmon GnRH
sbGnRH	seabream GnRH
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
TBS	tris-buffered saline

웅성선숙형 성 전환 어류 시나몬 *Amphiprion melanopus*의 생식소자극호르몬억제호르몬(GnIH) 분비 조절에 관한 분자내분비학적 연구

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요 약

어류의 성 성숙 및 성 발달은 시상하부-뇌하수체-생식소 축(HPG 축)을 중심으로 생식소자극호르몬방출호르몬(GnRH), 생식소자극호르몬(GTH) 그리고 스테로이드 호르몬 이외에도 신경내분비 물질 및 생식소 호르몬과 같은 다양한 성 호르몬에 의해 조절된다. 최근까지 시상하부에 존재하는 여러 신경단백질 중 GnRH만이 GTH의 합성과 방출을 조절하는 것으로 알려져 있었으나, 2000년에 들어서 메추라기 *Coturnix japonica*의 뇌에서 생식소자극호르몬억제호르몬(GnIH)가 최초로 발견된 이후 척추동물에서는 GnRH만이 번식을 조절하는 것이 아니라, GnIH와 GnRH의 상호작용으로 인하여 번식이 조절되는 것이 밝혀졌다.

본 연구에서 실험어로 사용된 시나몬 *Amphiprion melanopus*는 수컷에서 암컷으로 성 전환하는 웅성선숙형 어류이다. 일반적으로 2-4 마리가 그룹을 형성해서 생활하고 그룹 내에서는 암컷이 가장 크고 가장 높은 서열을 가지는데, 만약 암컷이 죽거나 제거되는 경우에는 수컷이 그룹 내에게 가장 큰 개체로 성장하면서 암컷으로 성 전환을 하게 되고, 미성숙 개체는 수컷의 성을 갖게 된다. 따라서 본 연구는 GnIH가 시나몬의 성 성숙 및 성 전환 과정에 미치는 영향과 HPG 축을 중심으로 합성과 분비를 조절하는 유전자의 발현 및 성 스테로이드호르몬의 내분비학적 조절 메커니즘을 규명하기 위하여 수행되었다.

1. 시나몬의 성 전환 단계별 GnIH 발현에 미치는 재조합 GnIH 및 sbGnRH의 영향

GnIH는 번식과 성숙 메커니즘을 조절한다고 알려져 있는 RF-amide peptide 그룹에 포함되어 있으며, 어류를 포함한 척추동물의 시상하부에서 GnRHs (sGnRH, sbGnRH 및 cGnRH-II)와의 상호작용을 통해 번식을 조절할 뿐만 아니라, 뇌하수체에도 작용하여 GTHs (GTHα, FSHβ 및 LHβ)의 합성과 방출을 억제하는 역할을 한다.

본 연구에서는 GnIH가 시나몬의 성 전환에 미치는 영향과 HPG 축을 중심으로 합성과 분비가 조절되는 유전자 및 호르몬과의 상호 관련성을 파악하고자, 성 전환 단계(미성숙, 수컷 및 암컷)별 개체에 재조합 GnIH, sbGnRH 및 GnIH+ sbGnRH를 각각 0.1 μg/g의 농도로 주사한 후 24시간 동안 6시간 간격으로 sbGnRH, GTHs (GTHα, FSHβ 및 LHβ), GnIH 그리고 GnIH 수용체(GnIH-R) 유전자의 발현량 변화를 조사하였다. GnIH를 주사한 경우에는 sbGnRH와 GTHs 유전자 발현량은 유의하게 감소하였으나, GnIH 및 GnIH-R 유전자의 발현량은 유의하게 증가하였다($P < 0.05$). 그러나 sbGnRH를 주사한 경우에는 GnIH를 주사한 결과와는 반대되는 경향이 관찰되었다. 특히, 미성숙 개체에 비하여 성숙한 개체(수컷 및 암컷)에서 유전자 발현량이 높게 나타난 점으로 보아, 재조합 GnIH는 시나몬의 성 성숙 및 성 전환 조절을 억제하는 역할을 하며, 미성숙 개체에 비하여 성숙한 개체에 더 큰 영향을 미치는 것으로 판단되었다.

2. 시나몬의 성 전환 과정 동안 GnIH와 멜라토닌 수용체 유전자의 발현 및 멜라토닌의 농도 변화

어류의 성 성숙 및 성 발달은 시상하부-뇌하수체-생식소 축(HPG 축)을 중심으로 GnRH, GTH 및 스테로이드호르몬 이외에도 신경내분비 물질, 생식소호르몬과 같은 다양한 성 호르몬에 의해 조절된다. 특히, 멜라토닌은 송과체와 망막에서 분비되는 호르몬으로, 밤에 증가하고 낮에 감소하는 일주기성 생체리듬 조절과 밀접한 관련이 있는 신경내분비적 신호로 작용한다. 멜라토닌은 멜라토닌 수용체(MT-R)와 결합하여 개체의 성장을 촉진시키는 역할도 수행하지만, FSH와 LH의 분비를 감소시켜 성 성숙을 억제시키기도 한다. 뿐만 아니라, 뇌의 시상하부에 위치하는 GnIH 신경세포에 MT-R이 존재

하는 점으로 보아, 송과체와 망막에서 분비되는 멜라토닌은 GnIH 유전자의 발현 조절과 밀접한 연관이 있는 것으로 판단된다.

따라서 본 연구에서는 시나몬의 성 전환 과정 동안 GnIH와 멜라토닌과의 상호 관련성에 대하여 조사하기 위하여, 성 전환 단계별(성숙한 수컷 개체, 암컷 제거 후 90일째의 성 전환 중인 개체, 성숙한 암컷 개체) 시나몬을 대상으로 GnIH, GnIH-R 및 MT-R mRNA 발현량 변화 및 혈중 멜라토닌 농도를 측정하였다. 뿐만 아니라 간뇌의 시상하부에서 주로 합성되어 분비되는 GnIH 및 MT-R을 면역형광기법으로 이중 형광 염색하여 성 전환 단계별로 발현량 차이를 관찰하였다. 그 결과, 성 전환 중인 개체에서는 GnIH, GnIH-R 및 MT-R 유전자 발현량이 모두 감소하였다. 또한, 간뇌에서 GnIH 및 MT-R을 이중 형광 염색한 결과, GnIH 및 MT-R은 서로 동일한 부위에서 발현되었으며, 성 전환 중인 개체에서는 발현량이 유의하게 감소하였다($P < 0.05$). 따라서 멜라토닌은 GnIH와의 상호작용을 통하여 시상하부-뇌하수체-생식소 축의 활성을 억제시킴으로서 시나몬의 성 전환에 관여하고 있는 것으로 판단된다.

3. 코르티솔을 처리한 시나몬에서 GnIH 및 sbGnRH의 발현 변화

다양한 요인에 의해 발생하는 스트레스는 성 성숙을 억제시키는 원인으로 작용하는데, 시상하부-뇌하수체-신간체 축(HPI 축)을 중심으로 분비되는 호르몬인 코르티솔은 당질코르티코이드 수용체(GR)와의 결합을 통하여 시상하부-뇌하수체-생식소 축(HPG 축)의 활성을 억제시켜 척추동물의 번식생리학적 측면에 부정적인 영향을 미친다. GnIH는 GnRH 신경세포에 존재하는 GnIH-R과 결합을 통하여 GnRH의 합성과 방출을 직접적으로 억제시킬 뿐만 아니라, 뇌하수체 전엽에도 작용하여 FSH와 LH의 분비를 억제시킴으로써 척추동물의 성숙을 지연시킨다. 또한, GnIH 신경세포에는 GR이 존재하기 때문에 HPI 축의 활성 변화는 GnIH의 조절에 직접적인 영향을 미치는 것으로 알려져 있다.

따라서 본 연구에서는 성 성숙 지연 메커니즘과 관련하여 코르티솔과 GnIH와의 상호작용을 파악하고자, 시나몬에 코르티솔을 농도별(10, 50 μg/g)로 주사하여 성 성숙에 영향을 미치는 유전자의 발현과 호르몬의 농도 변화를 조사하였다. 그 결과, 코르티솔 농도가 증가할수록 GnIH, GnIH-R 유전자의 발현은 유의하게 증가하였으나, sbGnRH 유전자의 발현과 혈장 내 FSH 및 LH 농도는 유의적으로 감소하였다($P < 0.05$). 또한, 면역형광기법을 활용하여 간뇌의 시상하부 부위에서 GnIH 및 GnRH의 발현을

관찰한 결과, 동일한 부위에서 발현되었으며, 코르티솔을 주사한 실험군에서는 GnIH 발현이 유의적으로 증가하였으나, GnRH 발현은 유의적으로 감소하였다($P < 0.05$). 따라서 코르티솔은 GnIH와의 상호작용을 통하여 시나몬의 성숙을 억제시키는 역할을 하는 것으로 판단된다.

Molecular and Endocrinological Studies on Gonadotropin-Inhibitory Hormone (GnIH) in the Protandrous Cinnamon Clownfish *Amphiprion melanopus*

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Abstract

Sexual maturation and development in fish are regulated by various sex hormones in the hypothalamus–pituitary–gonad (HPG) axis, including gonadotropin-releasing hormone (GnRH), gonadotropin (GTH), sex steroid hormones, and other neurohormones. Until recently, GnRH has been the only known hormone to control the synthesis and release of GTHs from neuropeptides present in the hypothalamus. However, the discovery of 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. The purpose of this study was to evaluate the mechanism underlying the effects of GnIH in cinnamon clownfish *Amphiprion melanopus*, a protandrous hermaphroditic fish, during the period of sex reversal and its role in the regulation of the HPG axis, sexual maturation, melatonin, and cortisol. This is the first study focus on the role of GnIH in sex reversal of teleost fish, specifically, the cinnamon clownfish.

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

Hypothalamic peptide neurohormones, such as GnRHs and GnIH, play pivotal roles in the control of reproduction and gonadal maturation in teleost fish. To study the effects of GnIH on fish reproduction, I 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-R], sbGnRH, and GTHs) during different stages of gonadal maturation in male, female, and immature cinnamon clownfish. As the results, the expression levels of GnIH, GnIH-R, and MT-R 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-R mRNA levels of GnIH treatment alone. However, the expression levels of the HPG 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 ($P < 0.05$). 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 cinnamon clownfish, a protandrous hermaphroditic fish.

2. Profile of gonadotropin-inhibitory hormone and melatonin during the sex reversal and maturation of cinnamon clownfish *Amphiprion melanopus*

The present study aimed to determine the relationship between melatonin and GnIH and their effect on reproduction in cinnamon clownfish. Accordingly, this study investigated the expression pattern of GnIH, GnIH-R, and MT-R mRNA and protein, as well as the plasma levels of melatonin, during sex reversal in cinnamon clownfish. The results of present study found that GnIH and MT-R mRNA and

melatonin activity were higher in fish with mature brain than in fish with developing gonads, and using double immunofluorescence staining, present study found that both GnIH and MT-R 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.

3. Effect of cortisol on gonadotropin-inhibitory hormone in the cinnamon clownfish *Amphiprion melanopus*

Hypothalamic peptides, GnRH and GnIH, play pivotal roles in the control of reproduction and gonadal maturation in fish. In the present study I tested the possibility that stress-mediated reproductive dysfunction in teleost may involve changes in GnRH and GnIH activity. Also, this study measured expression of brain GnIH, GnIH-R, sbGnRH, as well as circulating levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) and cortisol in the cinnamon clownfish. Increased level of cortisol is associated with stress response in most vertebrate species studied. 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, this study found expression of both GnIH and GnRH in the diencephalon region of cinnamon clownfish brain. Circulating cortisol level was significantly increased following injection with GnIH, and reduced after treatment with a sbGnRH ($P < 0.05$). 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.

Chapter 1.

General Introduction

Sexual maturation and development in fish are regulated by various sex hormones in the hypothalamus–pituitary–gonad (HPG) axis, including gonadotropin-releasing hormone (GnRH), gonadotropin (GTH), sex steroid hormones, and other neurohormones (Lee et al., 2001; Moussavi et al., 2012). Many studies suggest that GnRH-expressing neurons in teleosts are distributed among three distinct GnRH populations (salmon GnRH [sGnRH], chicken GnRH-II [cGnRH-II], and seabream GnRH [sbGnRH]) within the brain, most likely reflecting distinct phylogenetic relationships and functions (Andersson et al., 2001; Kim et al., 2012). Another key regulator of this system is GnRH, which activates the GnRH receptor and stimulates the synthesis and release of pituitary hormones, most notably, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Andrews et al., 1988). FSH and LH are members of the glycoprotein hormone family and are synthesized in the pituitary gland. In general, FSH regulates both estrogen for vitellogenesis and spermatogenesis, whereas LH promotes follicular maturation, ovulation, and the synthesis of sex steroid hormones in teleosts (Nagahama et al., 1995; Ando and Urano, 2005; Kobayashi et al., 2006).

Until recently, GnRH has been the only known hormone to control the synthesis and release of GTHs from neuropeptides present in the hypothalamus. However, Tsutsui et al. (2000) 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 (Tsutsui et al., 2000; Tsutsui et al., 2006; Tsutsui et al., 2012; Tsutsui and Ubuka, 2014). In most species tested, GnIH appears to impair the gonadotropic axis and works in opposition to 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, 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-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH_2, SIKPSAYLP LRF-amide]) (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 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 HPG axis (Bentley et al., 2006; Tsutsui et al., 2006; Ubuka et al., 2008).

Also, 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 (MT-R) mediate the actions of melatonin, which in some species leads to the impairment of sexual maturation by inhibiting FSH and LH release (Sébert et al., 2008; McGuire et al., 2011). Also, melatonin acts via MT-R 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 (Ubuka et al., 2005; McGuire et al., 2011).

A significant decrease in GnRH mRNA expression was observed when there was an increase in the corticosterone concentration of glucocorticoid types (McGuire et al., 2013), due to the presence of the glucocorticoid receptor (GR) in the GnIH neuron. It has also been reported that corticosterone directly controls the expression of GnIH (Son et al., 2012, 2014).

Cortisol, a type of glucocorticoid hormone, is also associated with the stress response and is secreted by the hypothalamus–pituitary–interrenal (HPI) axis (Wendelaar Bonga, 1997; Flik et al., 2006). The HPI axis is activated to maintain homeostasis in organisms exposed to stress (Wendelaar Bonga, 1997; Flik et al., 2006). To regulate the hormones released in response to stress, the corticotropin-releasing hormone (CRH) is secreted by the hypothalamus and it acts on the anterior pituitary to stimulate the secretion of the adrenocorticotropic hormone (ACTH) (Wendelaar Bonga, 1997). ACTH, which is derived from the precursor protein pro-opiomelanocortin, stimulates the synthesis and release of cortisol in interrenal cells of the head kidney (Wendelaar Bonga, 1997; Filk et al., 2006). In vertebrates, glucocorticoid hormones, secreted from the center of the HPI axis include cortisol, and have a negative effect on the physiological aspects of vertebrate reproduction by inhibiting the activity of the HPG axis (Rivier and Rivest, 1991; Moore and Jessop, 2003).

Recent research on the correlation between various maturation-related hormones and GnIH, controlled by the HPG axis has mainly focused on mammals and birds (Ubuka et al., 2008; Kirby et al., 2009; Chowdhury et al., 2010; Tsutsui et al., 2010), insufficiently exploring patterns in fish. In particular, research on the relationship between sex reversal and GnIH targeting sex reversal in fish has not been explored. An ideal model system to explore this relationship is the cinnamon clownfish *Amphiprion melanopus*. Cinnamon clownfish 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 reversal to become female, and the immature fish develops into a male: protandrous fish species (Godwin and Thomas, 1993).

The purpose of this study was to evaluate the mechanism underlying the effects of GnIH in cinnamon clownfish, a protandrous hermaphroditic fish, during the period of sex reversal and its role in the regulation of the HPG axis, sexual maturation, melatonin, and cortisol. This is the first study focus on the role of GnIH in sex reversal of teleost fish, specifically, the cinnamon clownfish.

Chapter 2.

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

1. Introduction

The discovery of GnIH in the brain of Japanese quail *C. 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 GnRH. However, in some species, especially gonochoristic fishes such as goldfish *C. auratus* and salmonid, GnIH has both stimulatory and inhibitory effects on reproduction. Thus, 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 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-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH_2, SIKPSAYLP LRF-amide]) (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 hypophysiotrophic actions and GnIH was shown to inhibit the release of 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).

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 *Oncorhynchus nerka*, all three goldfish LPXRF-amide 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, 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 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, cGnRH-II, and 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 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 MT-Rs mediate the actions of melatonin, which in some species leads to the impairment of sexual maturation by inhibiting FSH and LH release (Sébert et al., 2008; McGuire et al., 2011). Teleost fish have three subtypes of MT-Rs: MT-R1, MT-R2, and MT-R3 (Ikegami et al., 2009; Shin et al., 2011). A previous study found that the melatonin-mediated responses elicited by the activation of MT-R1–3 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-R1 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 *A. 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 reversal 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 reversal and the relationship between HPG axis regulation and sexual maturation. This study 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. This study was 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. So, I measured the mRNA expression of GnIH, GnIH-R, GnRH, GTHs, and MT-R, as well as plasma GnRH, GTHs, and melatonin. In particular, this study is the first of its kind to focus on the sex reversal of teleost fish.

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, total length, 4.6 ± 0.5 cm, body mass, 6.3 ± 0.4 g, gonadosomatic index [GSI; gonad mass / body mass × 100] = 0.03 ± 0.03; male, n = 100, total length, 6.5 ± 0.4 cm, body mass, 11.5 ± 0.3 g, GSI = 0.11 ± 0.03; female, n = 100, total length, 8.4 ± 0.5 cm, body mass, 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. Kisspeptin (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 cinnamon 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-Trp^{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 complementary DNA (cDNA) using M-MLV reverse transcriptase (Promega, OH, USA), according to the instructions of the manufacturer. The following RT-polymerase chain reaction (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^{TM}, 1kb 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-R, 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. qPCR anaysis was measured the followed mehtods; 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-R = 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 ΔΔCt = 2^ – (ΔCt_{sample} – $\Delta Ct_{internal\ control}$). Based on qPCR assays, the intra- and inter-assay coefficients of variation (CV) for GnIH, GnIH-R, MT-R, 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	Forward	5′-CCC TCT TCG CTT CGG GCG GGA TG-3′
(**KT455505**)	Reverse	5′-GAA TCG CTG AGG GAG GTT GAT A-3′
GnIH-R	Forward	5′-AAC CAC AGC GGC TCA GTG TGT CC-3′
(**KT455506**)	Reverse	5′- ACC AGA CAG AGG AAG ACA AA-3′
MT-R	Forward	5′-CTG CTG GTG GTG ATG ATG-3′
(**HM107821**)	Reverse	5′-GGT CTC TCT TCC CTC CTG-3′
sbGnRH	Forward	5′-CTG CTG GTG GTG ATG ATG-3′
(**HQ883476**)	Reverse	5′-GGT CTC TCT TCC CTC CTG-3′
GTHα	Forward	5′-AAT GTT CCC GCC AGA GAA-3′
(**EU908056**)	Reverse	5′-AGA GGT TGG AGA AGG CAG-3′
FSHβ	Forward	5′-CTC ATC CTG TCC GCA CTT-3′
(**FJ868867**)	Reverse	5′-GAG AAG CAG CAG CCT GTA-3′
LHβ	Forward	5′-ACC ATC ATC GTG GAG AGA G-3′
(**FJ868868**)	Reverse	5′-GAT AGT TCA GGT CCG TTG TTT C-3′
β-actin	Forward	5′-GGA CCT GTA TGC CAA CAC TG-3′
(**JF273495**)	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 enzyme-linked immunosorbent assay (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, the standard curve was compared 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.

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; 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 cinnamon 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, 2E, and 2F), and decreased with GnRH treatment. The *P* values of all of the experimental groups were less than 0.05.

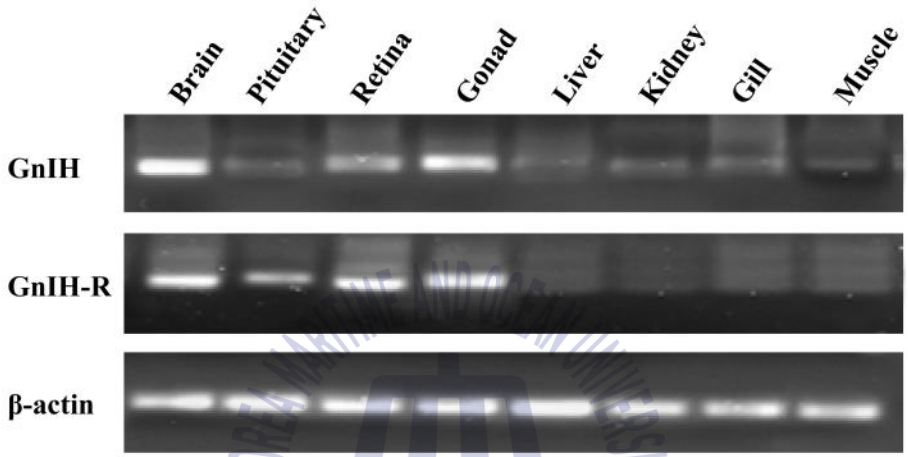

Fig. 1. Tissue distribution of cinnamon clownfish GnIH and GnIH-R. Reverse transcriptase-PCR analysis of GnIH and GnIH-R transcripts in different tissues, as shown in a 1.0% agarose electrophoresis gel with ethidium bromide. The sizes of the RT-PCR products were 84 bp for GnIH, 701 bp for GnIH-R, and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

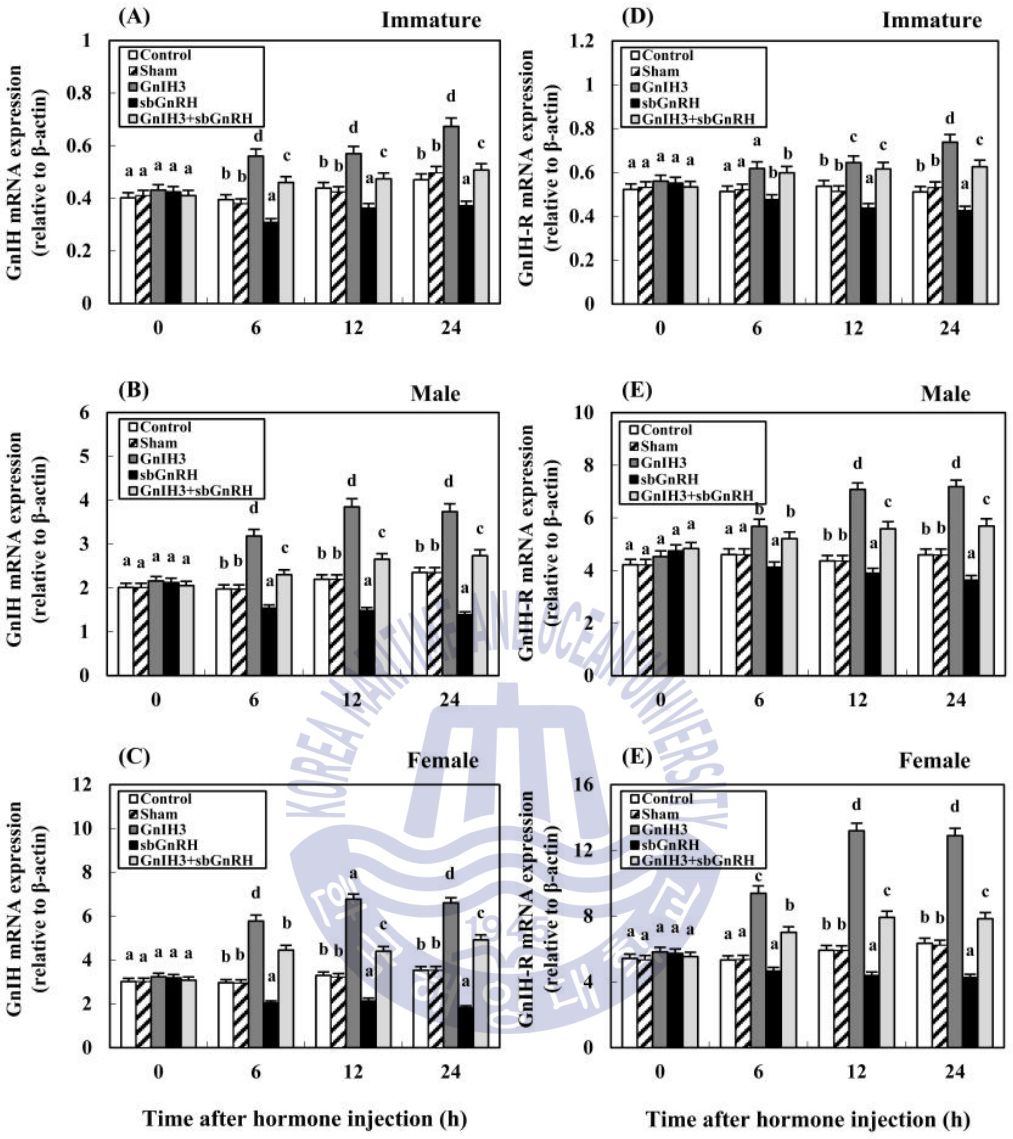

Fig. 2. Expression of GnIH (A, B, and C) and GnIH-R (D, E, and F) mRNA in cinnamon clownfish (immature fish, A and D; male, B and E; female, C and F) after GnIH3 (0.1 μg/g BM), sbGnRH (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH). mRNA levels are relative to the β-actin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

3.3. Time-related changes in MT-R mRNA and melatonin

In all cinnamon clownfish groups, MT-R 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-R 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-R mRNA levels significantly decreased at 24 h after GnRH treatment in all fish (Fig. 4). In addition, in all groups, MT-R mRNA levels gradually decreased after GnIH3 and sbGnRH combination treatment, as compared to the MT-R mRNA levels of GnIH treatment alone (Fig. 3). The *P* values of all of the experimental groups were less than 0.05.

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 GnIH3 treatment alone. The *P* values of all of the experimental groups were less than 0.05.

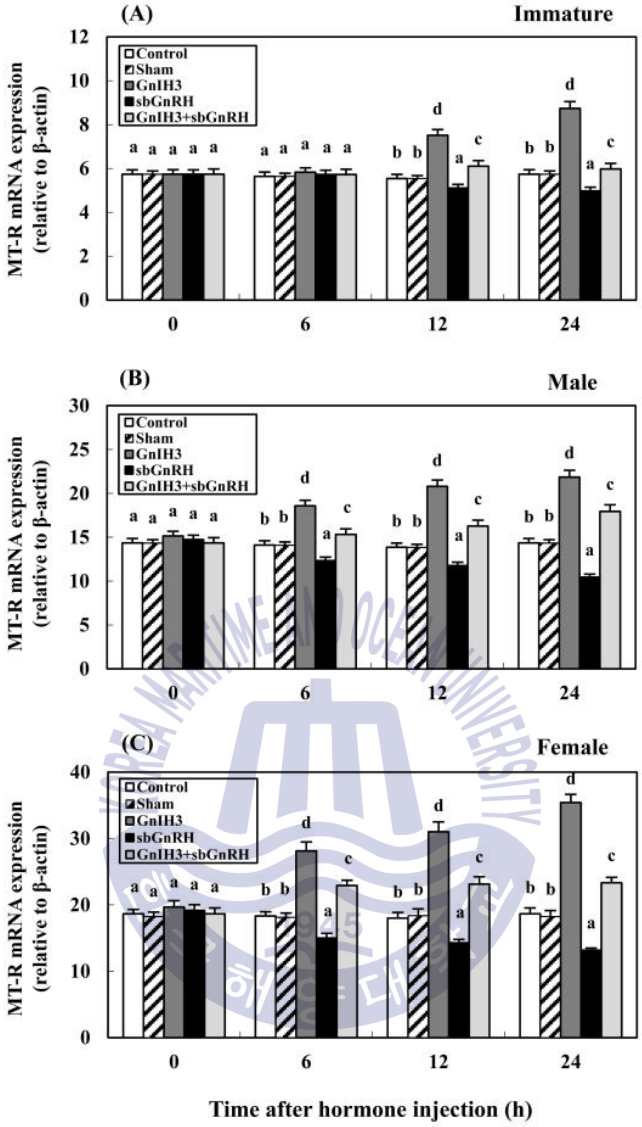

Fig. 3. Expression of MT-R mRNA in the brain (A, B, and C) of cinnamon clownfish (immature fish, A; male, B; female, C) after GnIH3 (0.1 μg/g BM), sbGnRH (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH). mRNA levels are relative to the β-actin mRNA levels in the brain of cinnamon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

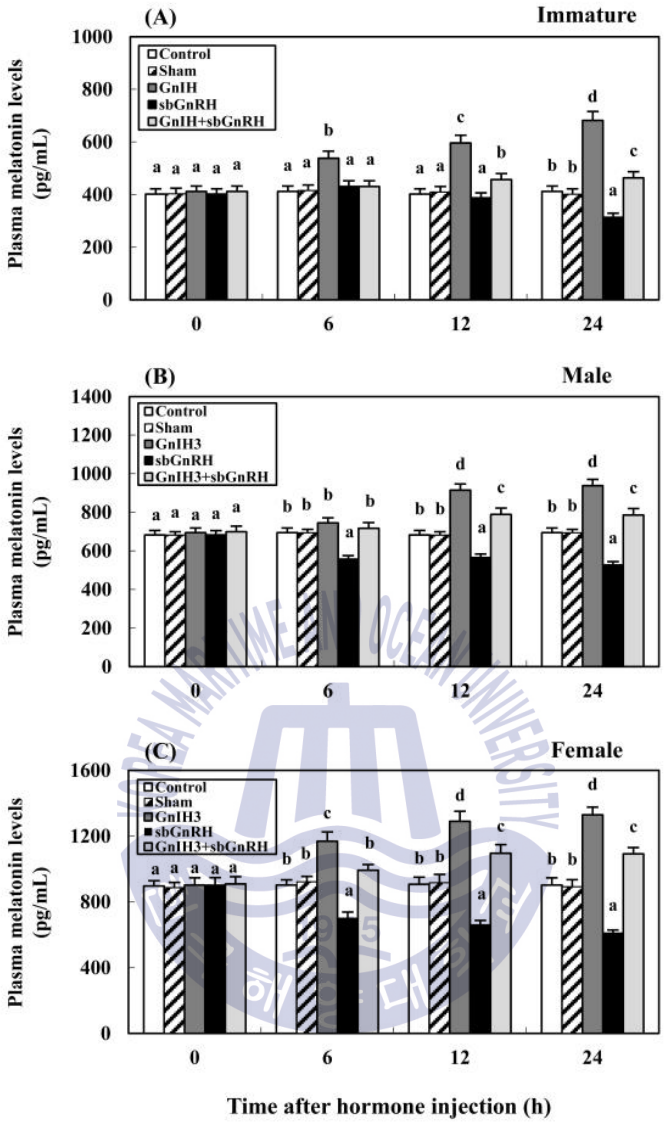

Fig. 4. The levels of plasma melatonin of cinnamon clownfish (immature fish, A; male, B; female, C) after GnIH3 (0.1 μg/g BM), sbGnRH (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH) treatment as analyzed with a plate reader. Values with letters indicate significant differences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

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, 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 cinnamon 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, 5E, and 5F), and decreased with GnIH treatment. The *P* values of all of the experimental groups were less than 0.05.

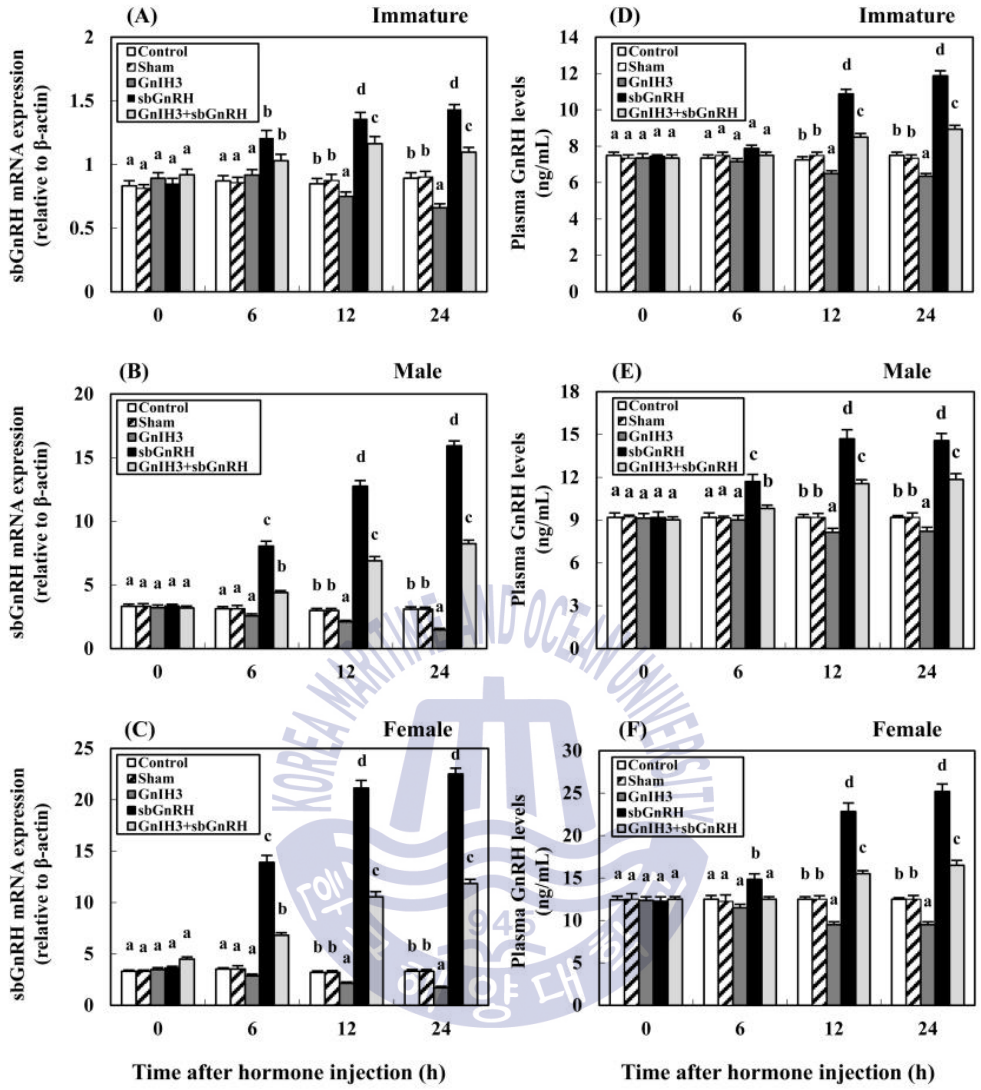

Fig. 5. Expression (A, B, and C) and activity (D, E, and F) of GnRH in cinnamon clownfish (immature fish, A and D; male, B and E; female, C and F) after GnIH3 (0.1 μg/g BM), sbGnRH (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH). sbGnRH mRNA levels are relative to the β-actin 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 ($n = 5$).

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 (Fig. 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 sbGnRH 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 (Fig. 6 and 7). The *P* values of all of the experimental groups were less than 0.05.

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.

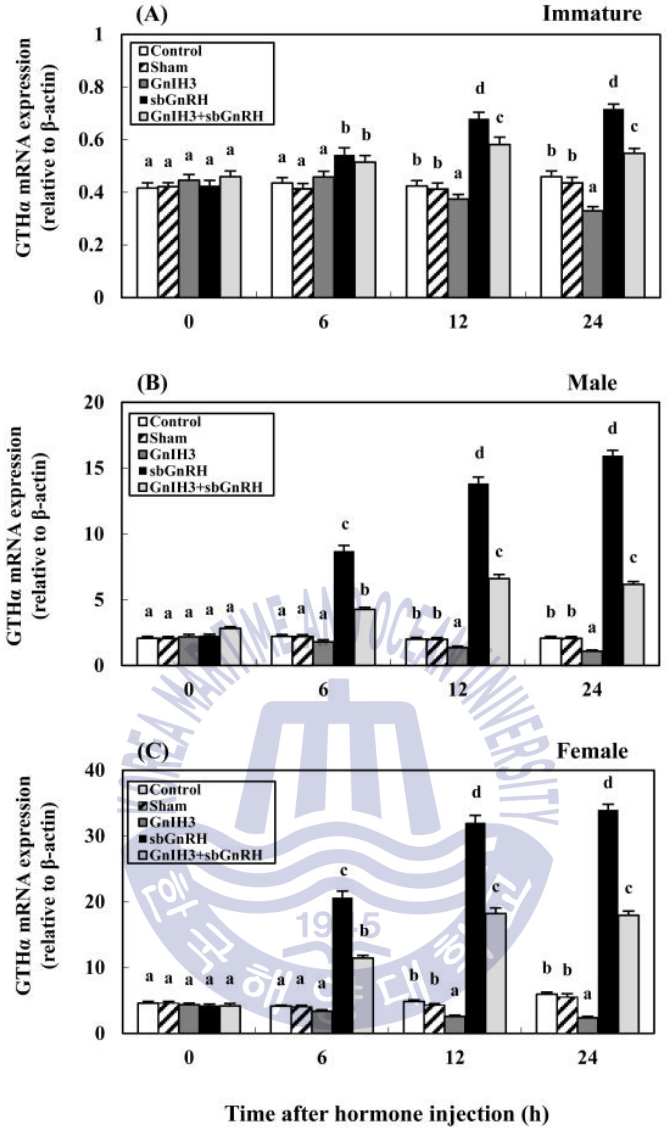

Fig. 6. Expression of GTHα mRNA in the pituitary gland of cinnamon clownfish (immature fish, A; male, B; female, C) after GnIH3 (0.1 μg/g BM), sbGnRH (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH) treatment. mRNA levels are relative to the β-actin mRNA levels in the pituitary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

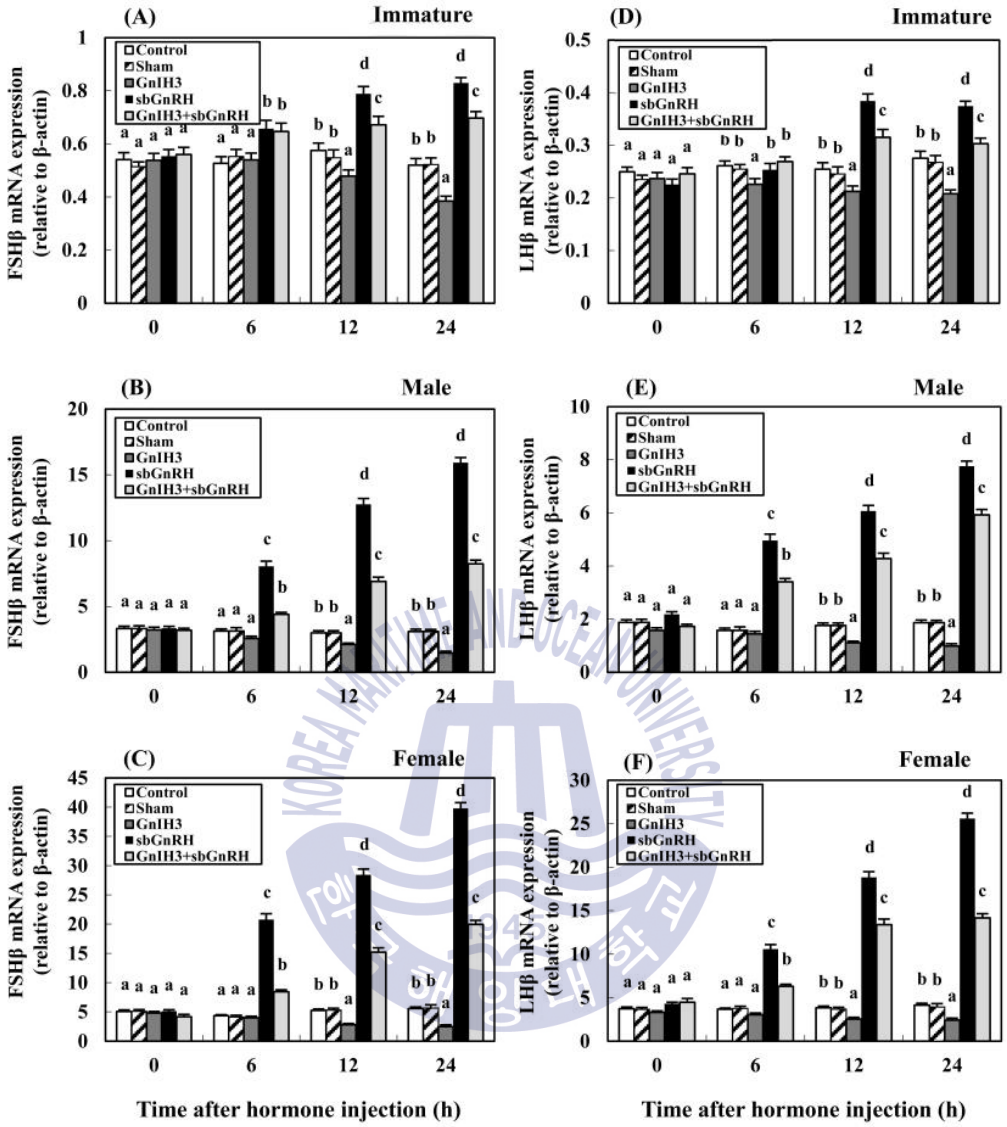

Fig. 7. Expression of FSHβ (A, B, and C) and LHβ (D, E, and F) in cinnamon clownfish (immature fish, A and D; male, B and E; female, C and F) after GnIH3 (0.1 μg/g BM), sbGnRH (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH) treatment. mRNA levels are relative to the β-actin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

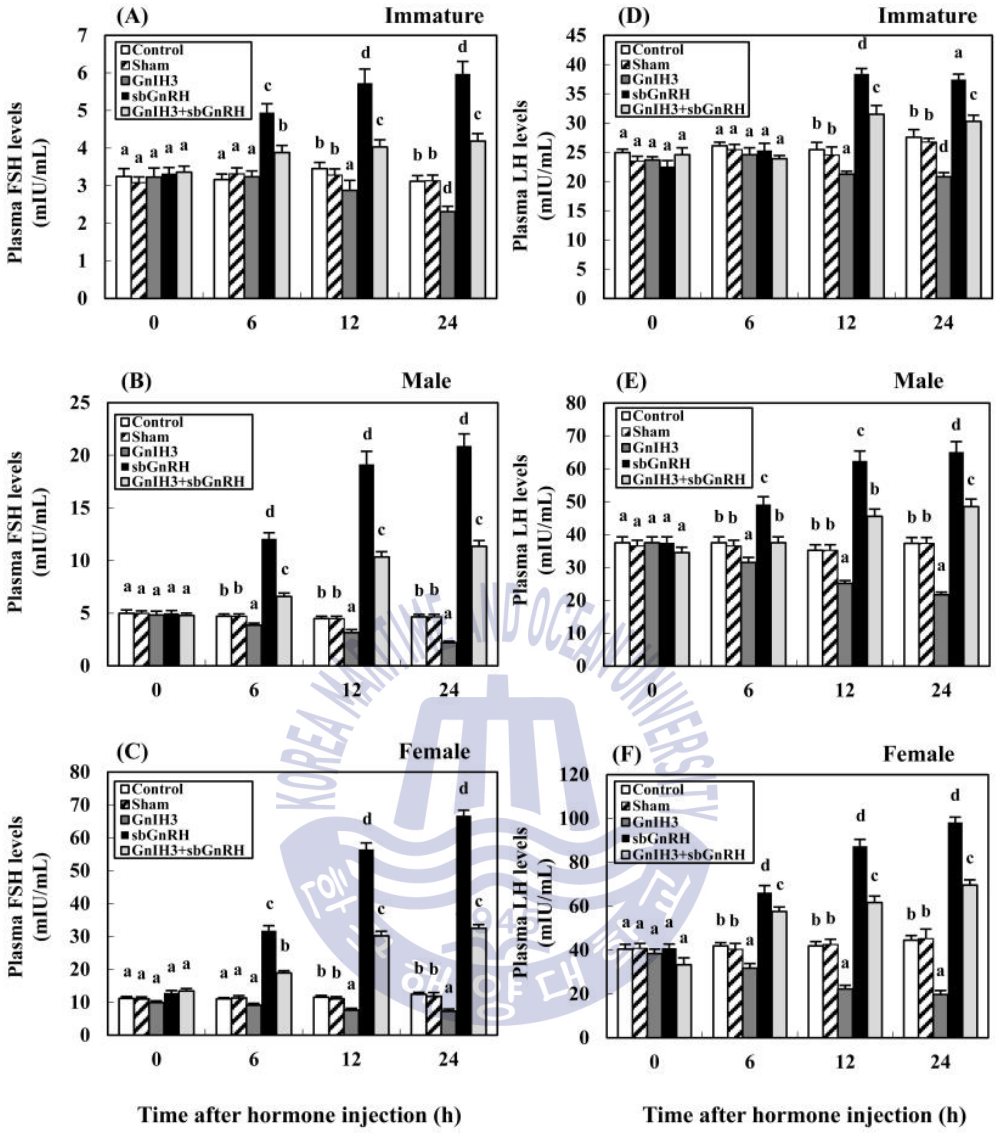

Fig. 8. The activity of plasma FSH (A, B, and C) and LH (D, E, and F) of cinnamon clownfish (immature fish, A and D; male, B and E; female, C and F) after GnIH3 (0.1 μg/g BM), sbGnRH (0.1 μg/g BM), and mixed GnIH3 and sbGnRH (0.1 μg/g GnIH3 + 0.1 μg/g sbGnRH) treatment as analyzed with a plate reader.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

4. Discussion

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

Cinnamon 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 reversal 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.

This study 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 (immature, male, and female). Also, I 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 reversal 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 (Holland et al., 1998; Amano et al., 2008). 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 treatment with progesterone, progesterone + estradiol, and estradiol, which stimulate gonadal maturation and development (Maddineni et al., 2008).

Collectively, these studies support findings that GnIH treatment reduced the expression of sbGnRH 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.

The present study investigated that MT-R mRNA level and the plasma concentrations of melatonin are important factors in the regulation of GnIH production. In this study, plasma melatonin and MT-R 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, The results of this study observed that the plasma melatonin and MT-R 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-R 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-R 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 reversal in cinnamon clownfish.

Chapter 3.

Profile of gonadotropin-inhibitory hormone and melatonin during the sex reversal and maturation of cinnamon clownfish *Amphiprion melanopus*

1. Introduction

Until recently, GnRH has been the only known hormone to control the synthesis and release of GTHs from neuropeptides present in the hypothalamus. However, Tsutsui et al. (2000) discovered GnIH in the brain of Japanese quail *C. japonica* and a subsequent study reported that GnRH is not the only hormone that controls vertebrate reproduction, since it interacts with GnIH (Tsutsui et al., 2012; Tsutsui and Ubuka, 2014). 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 HPG axis and controlling the reproductive cycle (Bentley et al., 2006; Tsutsui et al., 2006; Ubuka et al., 2008; Tsutsui and Ubuka, 2014).

The regulation of GTH synthesis and its secretion is also affected by light exposure, including photoperiod (Tsutsui et al., 2000), and photoperiod-mediated changes in reproductive hormone production are mainly mediated by melatonin, which is released from the pineal gland and retina (Falcón et al., 2007). 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 (Falcón et al., 2007). The effect of melatonin is mediated by MT-Rs, which promote growth and inhibit sexual maturation by reducing the release of both FSH and LH (Sébert et al., 2008; McGuire et al., 2011). Teleost fish have

three to four subtypes of MT-Rs: MT-R1, MT-R2, and MT-R3 (Ikegami et al., 2009; Shin et al., 2011). Previous study reported the function of MT-Rs that modulate arterial vasoconstriction, cell proliferation in cancer cells, and reproductive and metabolic functions (Dubocovich and Markowsk, 2005). 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 (Ubuka et al., 2005; McGuire et al., 2011).

Therefore, present study examined the effect of GnIH and melatonin during the sex reversal and sexual maturation of cinnamon clownfish *A. melanopus*. This species of cinnamon 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 reversal to become female, and the immature individual will undergo a sex reversal to become male (Godwin and Thomas, 1993).

The present study aimed to investigate the relationship of GnIH and melatonin during sex reversal; for this purpose, I measured the mRNA expression of GnIH, GnIH-R, and MT-R, as well as plasma levels of melatonin, using double staining for GnIH and GnIH-R and immunofluorescence techniques for MT-R and plasma melatonin.

2. Materials and methods

2.1. Fish husbandry

The present study was conducted with male (body mass: 10.5 ± 1.2 g), males at 90 days after removing female (body mass: 15.2 ± 0.9 g) and female (body mass: 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 reversal was modified from the methods described by An et al. (2010). After mated pairs (male and dominant female) were established, sex reversal 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 reversal from males to females. fish was divided the sex reversal 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-R mRNA using cDNA reverse-transcribed from the total RNA

extracted from the brains during the sex reversal. 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-R, **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-R 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: $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

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 bovine serum albumin (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 (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 an 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 cinnamon clownfish from the sex reversal and melatonin-injected experiments, 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 (PVDF; 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 reversal 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] and anti-MT-R [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-R. 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 dH_2O, and coverslipped with Vectashield (Vector Laboratories). The fluorescently labeled material was then visualized, using a 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). Two-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 ± 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. 9A). Furthermore, competitive ELISA development demonstrated the availability of a standard and an antibody specific of the protein. The molecular weight of the GnIH antibody (23 kDa) was also confirmed by Western blot analysis (Fig. 10A). SDS-PAGE analysis of purified GnIH and staining with anti-GnIH polyclonal antibody showed a major band corresponding to GnIH (Fig. 9B). Under non-reducing conditions, the apparent molecular sizes of the GnIH IgG antibodies were between 55−75 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.

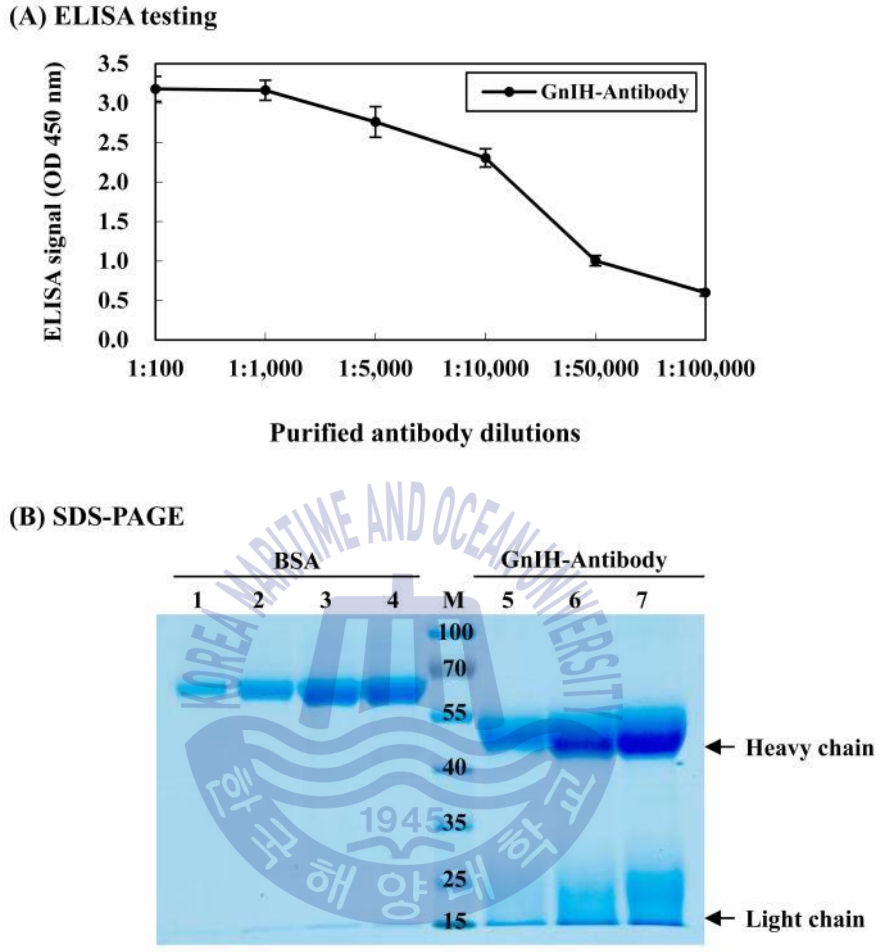

Fig. 9. Titration of the purified anti-GnIH antibody with ELISA and SDS-PAGE. (A) The ELISA curve exhibits a hyperbolic shape in the range of 1:100 to 1:100,000 dilution on the logarithmic dilution scale. (B) Analysis of purified GnIH polyclonal antibody using SDS-PAGE and Coomassie brilliant blue staining. 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.

3.2. Expression of GnIH, GnIH-R mRNA, and GnIH protein during the sex reversal 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. 10A). 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 reversal (Fig. 10B and 10C, respectively). The *P* values of all of the experimental groups were less than 0.05.

3.3. Change of MT-R and melatonin during the sex reversal process

Similarly, expression of MT-R mRNA was higher in mature males (approximately 1.2-fold) and females (approximately 1.3-fold) than in individuals undergoing sex reversal (Fig. 11A), 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 reversal; Fig. 11B). The *P* values of all of the experimental groups were less than 0.05.

3.4. Double immunofluorescence staining during the sex reversal process

Double immunofluorescence staining demonstrated that the GnIH and MT-R proteins were co-expressed in the cinnamon clownfish diencephalon (Fig. 12). Furthermore, like the expression of GnIH and MT-R mRNA, both GnIH and MT-R proteins were more abundant in mature fish (male, Fig. 12D and 12G; female, Fig. 12F and 12I) than in individuals undergoing sex reversal (Fig. 12E and 12H).

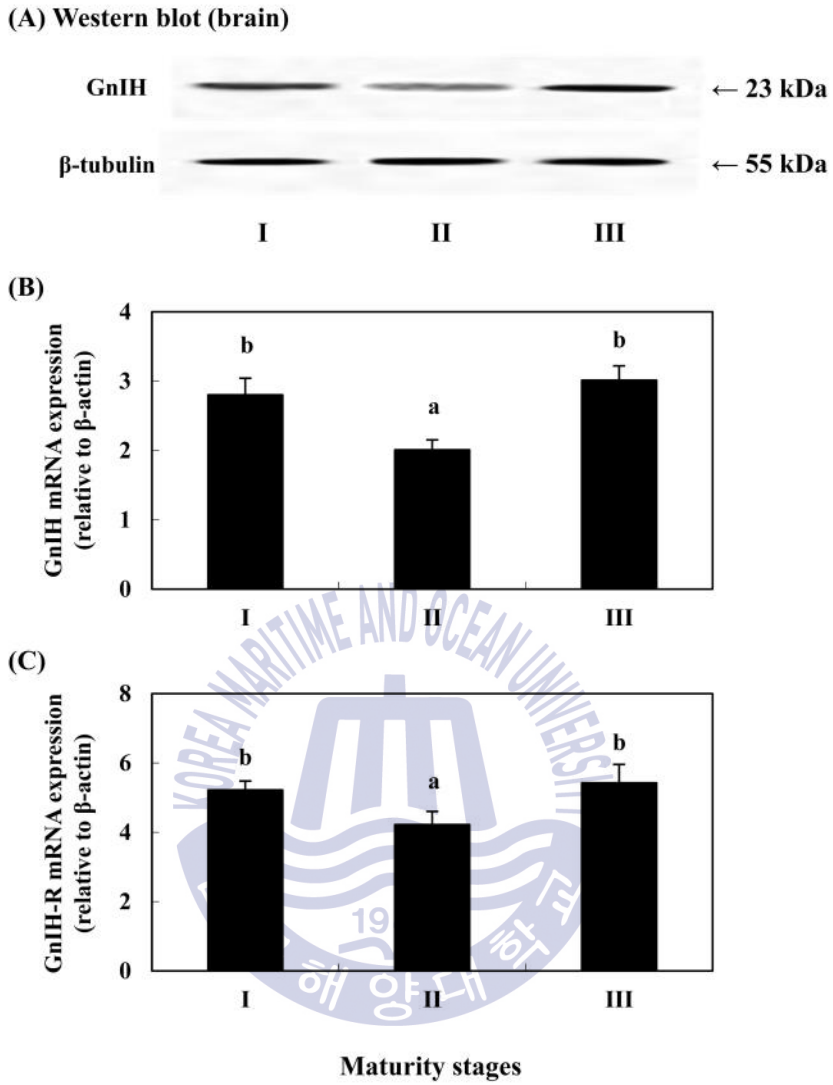

Fig. 10. Expression of GnIH and GnIH-R in the brain of cinnamon clownfish during sex reversal. (A) Western blot using GnIH (dilution 1:5,000; 23 kDa) to examine protein expression and β-tubulin (55 kDa) as the internal control. (B) GnIH and (C) GnIH-R mRNA levels, relative to β-actin mRNA levels, were 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 ($n = 5$).

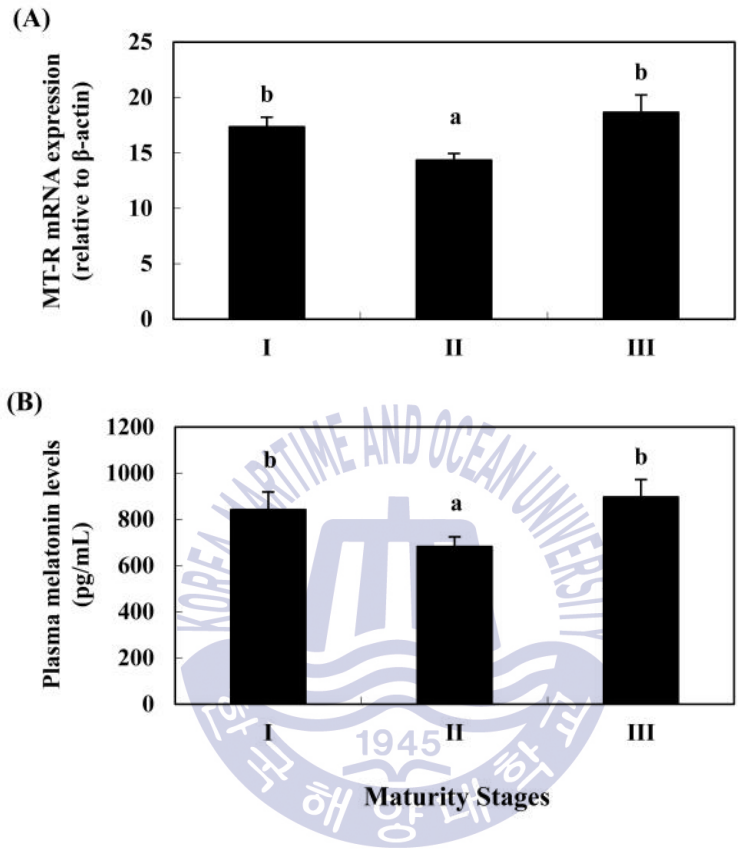

Fig. 11. Change in MT-R mRNA expression and plasma melatonin activity in cinnamon clownfish during the sex reversal. (A) MT-R mRNA levels were analyzed, relative to β-actin mRNA levels, using real-time PCR, and (B) the activity of melatonin in the plasma of cinnamon clownfish was analyzed using a plate 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 ($n = 5$).

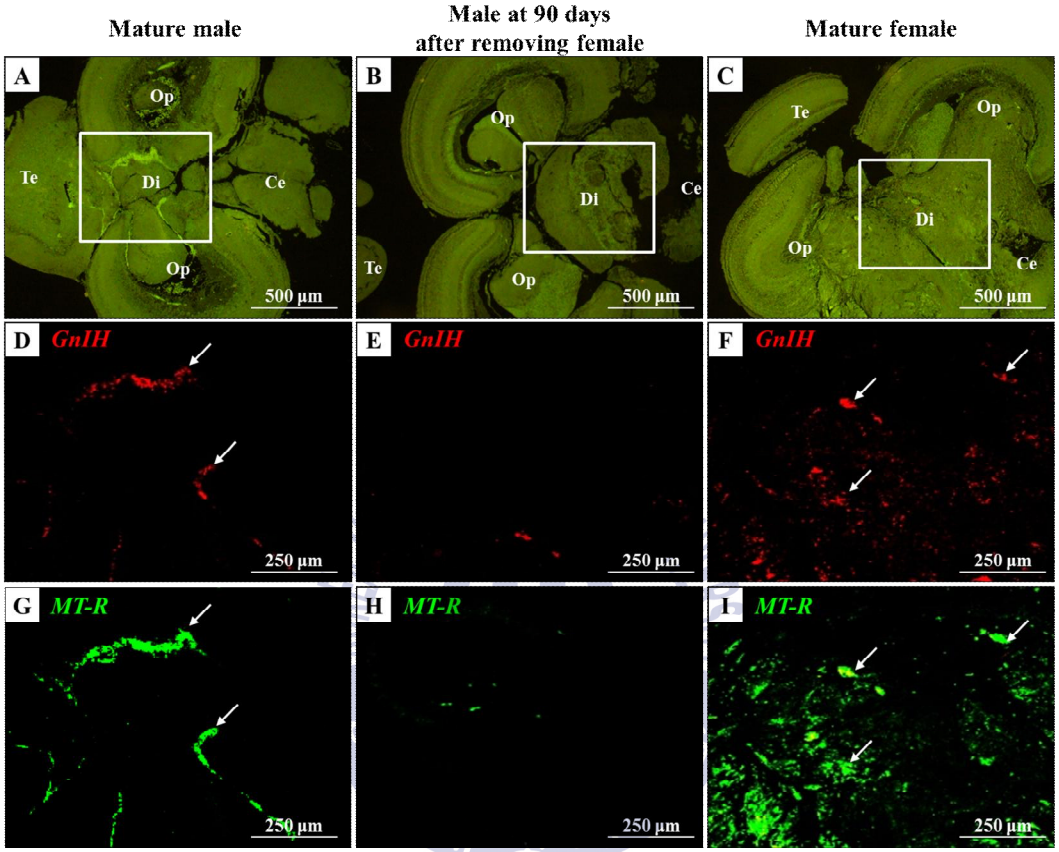

Fig. 12. Co-localization of GnIH and MT-R proteins in cinnamon clownfish hypothalamus, during sex reversal, as indicated by double immunofluorescence staining. I, mature male (A, D, and G); II, male at 90 days after removing female (B, E, and H); III, mature female (C, F, and I). Whole brains (A–C) and diencephalons stained with Texas Red (red; D–F) or FITC (green; G-I). Arrows indicate the overlap of GnIH-immunoreactive (IR)- and MT-R-IR-stained cells. Te, telencephalon; Op, optic tectum; Ce, cerebellum; Di, diencephalons. Scale bars = 500 μm (A–C) and 250 μm (D–I).

4. Discussion

In the present study, I found that the expression levels of GnIH, GnIH-R, and MT-R 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 melatoin. In addition, in the results of GnIH and MT-R expression on diencephalon by IF double staining, this study observed the pattern that expression of GnIH and MT-R protein in the sex reversal groups reduced significantly in contrast to the mature male and female.

In contrast, a previous study (Kim et al. 2012) reported that the mRNA expression of GnRHs (sGnRH, sbGnRH, and cGnRH-II), which activate the HPG axis and promote sex reversal, significantly increased during gender transition, suggesting that GnRH increased in response to the inhibition of GnIH. In this study, GnIH expression of sex reversal stage decreased, the results of this study suggested that the increased GnRH due to the inhibition of the GnIH activate the HPG asix and promote sex reversal process. In fact, Tsutsui et al. (2010) 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 (Chowdhury et al., 2010). In addition, Ubuka et al. (2005) 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-R mRNA, GnIH proteins, and plasma melatonin were lower during sex

reversal, present study 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 (Ubuka et al., 2005; Chowdhury et al., 2010; Tsutsui et al., 2010), but studies in fish are limited. In particular, no studies have addressed the effects of GnIH during sexual maturation and sex reversal in hermaphroditic fish. Therefore, this 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.

Chapter 4.

Effect of cortisol on gonadotropin-inhibitory hormone in the cinnamon clownfish *Amphiprion melanopus*

1. Introduction

The hypothalamic GnRH is the main stimulator of gonadal maturation and function in fish by regulating release and synthesis of pituitary gonadotropins (FSH and LH) (Lee et al., 2001; Chang et al., 2009; Moussavi et al., 2012). Multiple forms of GnRH are expressed in the brain of the same species and in some teleosts, there are three forms of GnRH, including sGnRH, cGnRH-II, and sbGnRH) (Andersson et al., 2001; Zohar et al., 2010). There is evidence that, sGnRH is produced as a third form in neuronal groups localized in the ventral forebrain along the terminal nerve, and controls GTH secretion (Okuzawa et al., 1997; Senthilkumaran et al., 1999; Chang et al., 2009). 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 (Nagahama et al., 1995; Ando and Urano, 2005; Kobayashi et al., 2006; Schulz et al., 2010).

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. (2000) discovered GnIH in the brain of the Japanese quail *C. 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 (Tsutsui et al., 2000; Tsutsui et al., 2006; Tsutsui and Ubuka, 2014). 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 (Bentley et al., 2006; Tsutsui et al., 2006; Ubuka et al., 2008). Furthermore, GnIH affects the GnRH neuron of the hypothalamus and directly inhibits GnRH synthesis and secretion (Ducret et al., 2009). GnIH delays maturation by inhibiting the secretion of the anterior pituitary FSH and LH (Tsutsui et al., 2000; Ciccone et al., 2004; Sari et al., 2009). 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 (Moussavi et al., 2012, 2013, 2014). More recent studies have demonstrated inhibitory actions of GnIH on the expression of reproductive hormones in hermaphroditic cinnamon clownfish (Choi et al., 2016). An interesting recent finding is potential interaction of GnIH with the brain-pituitary glucocorticoid axis and stress response which is the focus of the present study. A significant decrease in GnRH mRNA level was observed when there was an increase in the corticosterone concentration (McGuire et al., 2013). Corticosterone was found to directly inhibit the expression of GnIH in the mammalian cells (Son et al., 2012, 2014). This is important since glucocorticoids are known to be associated with stress response (Vijayan et al., 1990; Wendelaar Bonga, 1997; Aluru and Vijayan, 2006; Flik et al., 2006), and chronic stress often has negative effects on reproduction. In this regard, The HPI axis is activated to maintain homeostasis in organisms exposed to stress (Wendelaar Bonga, 1997; Flik et al., 2006). To regulate the hormones released in response to stress, the CRH is secreted by the hypothalamus and it acts on the anterior pituitary to stimulate the secretion of the ACTH (Wendelaar Bonga, 1997). ACTH, which is derived from the precursor protein pro-opiomelanocortin, stimulates the synthesis and release of cortisol in interrenal cells of the head kidney (Wendelaar Bonga, 1997; Filk et al., 2006). 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 HPG axis (Rivier and Rivest, 1991; Moore and Jessop, 2003).

This study investigated the effect of cortisol, a stress response-related hormone,

on the sexual maturation of the cinnamon clownfish *A. melanopus* and investigated the effects of cortisol on the function of GnIH, which delays maturation. I investigated two concentrations of cortisol injection on immature cinnamon clownfish over the course of a 48 h short-term experiment, as well as by sampling the fish at 3 weeks intervals for a long-term experiment (9 weeks). I measured mRNA expression of GnIH, GnIH-R, and sbGnRH, as well as plasma levels of FSH and LH to determine cortisol effects. Additionally, this study 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 cinnamon clownfish (immature fish, n = 260, total length, 4.6 ± 0.5 cm, body mass, 6.3 ± 0.4 g, gonadosomatic index [GSI; gonad mass / body mass × 100] = 0.03 ± 0.03). Fish were purchased from Corea Center of Ornamental Reef & Aquarium (CCORA) in Jeju, Korea). 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

2.2.1. Short-term experiment during hormone treatment (*in vivo*)

To investigate the effects of hormones (cortisol, GnIH, and sbGnRH) on maturation and cortisol regulation, the cinnamon clownfish were anesthetized with 2-phenoxyethanol prior to injection. Cortisol (hydrocortisone-21-hemisuccinate; Sigma, St Louis, MO, USA), GnIH3 (goldfish LPXRF-3 [SGTGLSATLPQRF- NH_2] and sbGnRHa (des Gly10-[D-Trp^6], H4284; Bachem, Torrance, CA, 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)), GnIH3 (0.1 μg/g BM), and sbGnRH (0.1 μg/g 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. After 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 by centrifugation (4°C, 10,000 ×g, 5 min) and stored at –80°C until analysis. During the experimental period, the fish were maintained at a water temperature and

photoperiod of 27 ± 1°C and 12 h light:12 h dark, respectively.

2.2.2. Long-term experiment during cortisol treatment (*in vivo*)

To investigate the effects of cortisol on sex maturation during the course of a long-term treatment, the cinnamon clownfish were reared in 100 L circulating filter tanks in the laboratory and then anesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO, USA) prior to injection. Cortisol was dissolved in 0.9% physiological saline, and each fish was injected with cortisol (10 or 50 μg/g BM) at a volume of 10 μL/g BM once weekly at 14:00 h. The control group was injected with an equal volume of physiological saline (0.85% NaCl in distilled water, 10 μL/g BM) once weekly at 14:00 h. The control samples (non-treatment groupts) 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 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.

2.2.3. Brain cell culture experiment after cortisol treatment (*in vitro*)

After the cinnamon clownfish were anesthetized, their brains were dissected and placed in an ice-cold medium (pH 7.5) composed of 25 mM HEPES, 4 mM $NaHCO_3$, 0.3% BSA, 0.1% collagenase, 0.25 mg/mL fungizone, and RPMI medium containing antibiotics (100 U/L penicillin and 100 mg/L streptomycin; penicillin–streptomycin, Gibco, Carlsbad, CA, USA). A scalpel was used to cut each brain into 1–3 mm^3 pieces. The pieces were then weighed, placed in a 24-well culture plate (SPL Life Science, Gyeonggi, Korea) containing 1 mL of medium. The cortisol (10 or 50 μg/mL) was dissolved in an equal volume of physiological saline (0.85% NaCl in distilled water, 10 μL/g BM) and added to the culture medium. The brain cells were cultured for 0, 6, 12, 24, and 48 h at 28°C, 100% humidity,

and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20°C, 10,000 ×*g*, 15 s), and the supernatant was removed and stored in individual microcentrifuge tubes at –80°C.

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. 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 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. 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 = 98.9%, GnIH = 97.8%, GnIH-R = 96.5%, sbGnRH = 95.8%. 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 (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated as ΔΔCt = 2^ − (ΔCt_{sample} − $\Delta Ct_{internal\ control}$). Based on qPCR assays, the intra- and inter-assay coefficients of variation (CV) for GnIH, GnIH-R, and sbGnRH mRNAs were less than 10% and 15%, respectively.

2.4. Plasma parameters analysis

Plasma FSH, LH, and cortisol levels were analyzed using the immunoassay technique with the ELISA kit (FSH (Catalog no. MBS035576; Mybiosource, USA), LH (Catalog no. MBS283097; Mybiosource, USA), and cortisol (Catalog no. E08487f; Cusabio Biotech, Hubei, China). An anti-antibody specific to the antibody of the hormones tested (FSH, LH, and cortisol) 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.

To assess assay parallelism, I compared the standard curve with a duplicate, and the standard curve was constructed with the following ranges: GTHs (FSH and LH; 0.625–20 and 3.56–100 mIU/mL, respectively) and cortisol (0.0023–10 pg/mL). The results of the ELISA kits indicated that there was no significant cross-reactivity or interference between these analysts and their analogues, and the intra-assay and inter-assay precisions were less than 10% and 15%, respectively.

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 1 μm thick sections, and prepared on individual microscope slides. For the double immunofluorescence staining of selected sections, both primary antibodies (rabbit GnIH [1:1,000] and mouse 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, 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°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. Photographs were taken using a fluorescence microscope (excitation filter 465–495 nm; Eclipse Ci, Nikon).

2.6. 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 ± SE.

3. Results

3.1. Time-course effects of cortisol on GnIH and GnIH-R (*in vivo* and *in vitro*)

Treatment with cortisol increased GnIH mRNA levels in a time and dose-related manner, reaching levels that were approximately 1.8-fold and 1.6-fold higher *in vivo* (Fig. 13A) and *in vitro* (Fig. 13C). For GnIH mRNA level, the effect of cortisol became significant after 6 h of treatment both *in vivo* and *in vitro*. Similar trend was observed for GnIH-R mRNA level, although the differences *in vivo* became significant after 12 h of treatment. Both concentrations of cortisol tested at 10 and 50 μg/g, *in vivo*, and 10 and 50 μg/mL, *in vitro* exerted significant effects, although the higher dose elicited the greatest response. (Figs. 13B and 13D). The *P* values of all of the experimental groups were less than 0.05.

In the long-term experiment, treatments with cortisol at both doses tested, *in vivo* (10 and 50 μg/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 beween 10 and 50 μg/g were not large, but statistically significant (Figs. 14A and 14B). The *P* values of all of the experimental groups were less than 0.05.

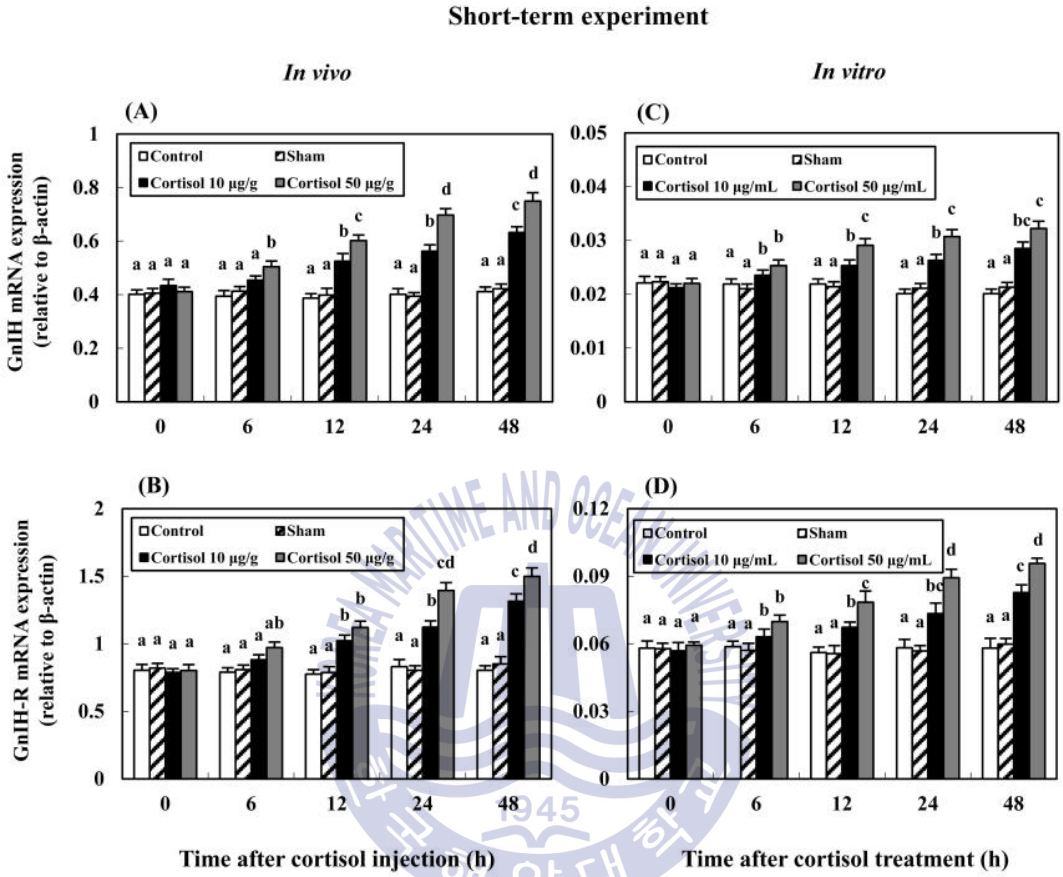

Fig. 13. mRNA expression levels of GnIH (A, C) and GnIH-R (B, D) in brains (*in vivo*; A, B) and cultured brain cells (*in vitro*; C, D) of cinnamon clownfish during 48 h after cortisol treatment (*in vivo*, 10 and 50 μg/g injection; *in vitro*, 10 and 50 μg/mL treatment). mRNA levels are relative to the β-actin mRNA levels in the cinnamon clownfish. Values with different letters indicate significant differences ($P < 0.05$). All values are means ± SE ($n = 5$).

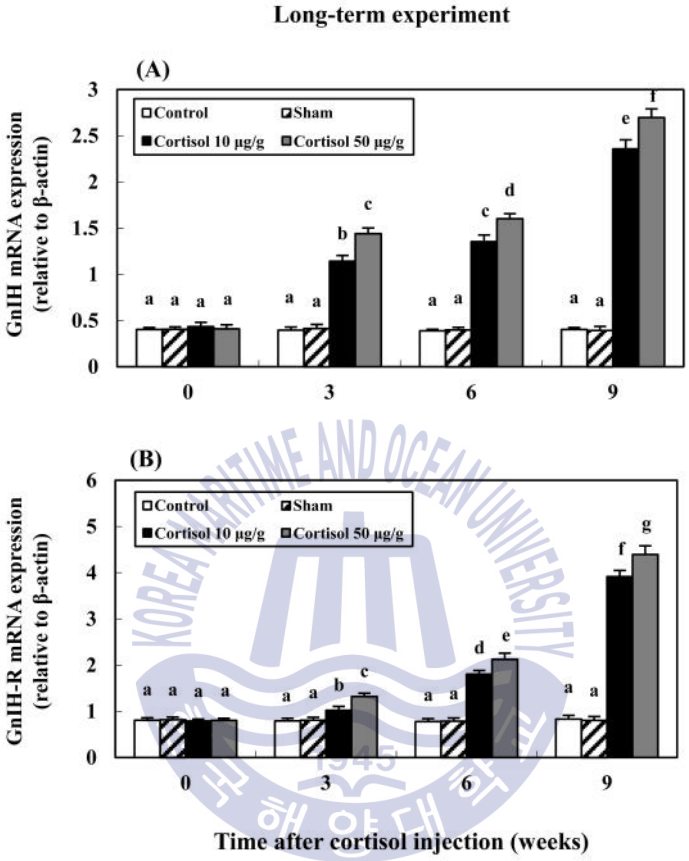

Fig. 14. mRNA expression of GnIH (A) and GnIH-R (B) in brains of cinnamon clownfish during 9 weeks after cortisol injection (10 and 50 μg/g) at weekly intervals. mRNA levels are relative to the β-actin mRNA levels in the cinnamon clownfish. Values with different letters indicate significant differences ($P < 0.05$). All values are means ± SE ($n = 5$).

3.2. Time-course effects of cortisol on sbGnRH

In the same experiments, I also measured sbGnRH mRNA levels. Treatment with cortisol had the opposite effect compared to GnIH. In this case, this study only measured sbGnRH following treatment with cortisol, *in vivo*, following short-term treatment (up to 48 h) and long-term treatment (up to 9 weeks). Here, this study also see a dose and time related reduction in sbGnRH mRNA level following treatment with cortisol, *in vivo* (Figs. 15A and 15B). The inhibitory action of cortisol was significant after 6 h of treatment (Fig. 3A). Long-term treatment also resulted in a dose-, and time-related reduction in sbGnRH mRNA level (Fig. 15B). The *P* values of all of the experimental groups were less than 0.05.

3.3. Double immunofluorescence staining during cortisol treatment

Double immunofluorescence staining demonstrated that the GnIH and GnRH proteins were expressed in the same reagion of diencephalon in the cinnamon clownfish brain (Fig. 16). The results are very consistent with the mRNA levels measured for both GnIH and GnRH. Similar to the expression of GnIH mRNA (Fig. 14A), GnIH protein level was more abundant after 9 weeks of treatment with cortisol (Fig. 16G). Also the GnRH protein levels decreased after 9 weeks of treatment with cortisol (Fig. 16H).

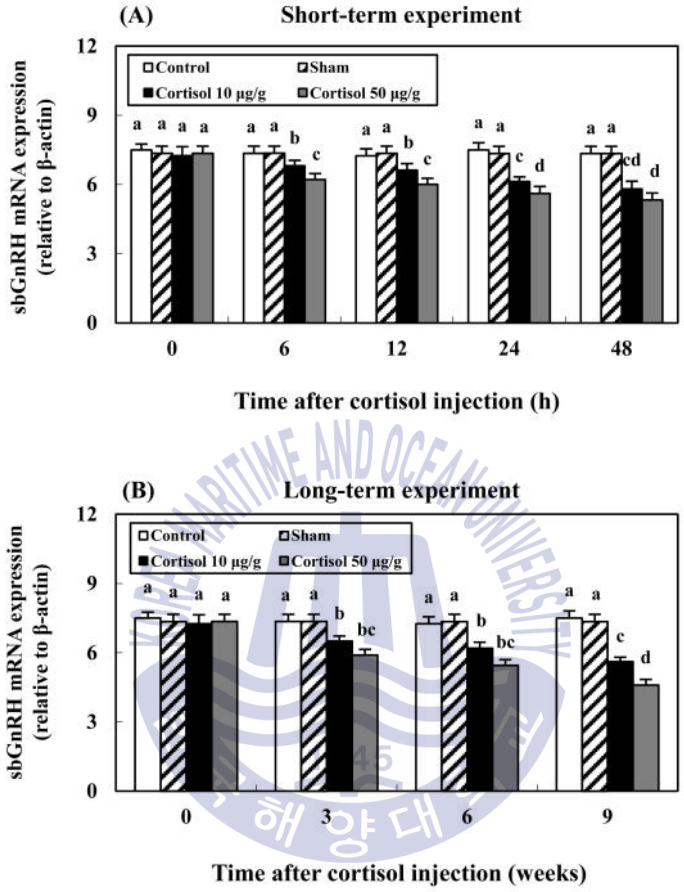

Fig. 15. Expression levels of sbGnRH mRNA in brains of cinnamon clownfish during 48 h (A) and 9 weeks (B) after cortisol injection (10 and 50 μg/g). mRNA levels are relative to the β-actin mRNA levels in the cinnamon clownfish. Values with different letters indicate significant differences ($P < 0.05$). All values are means ± SE ($n = 5$).

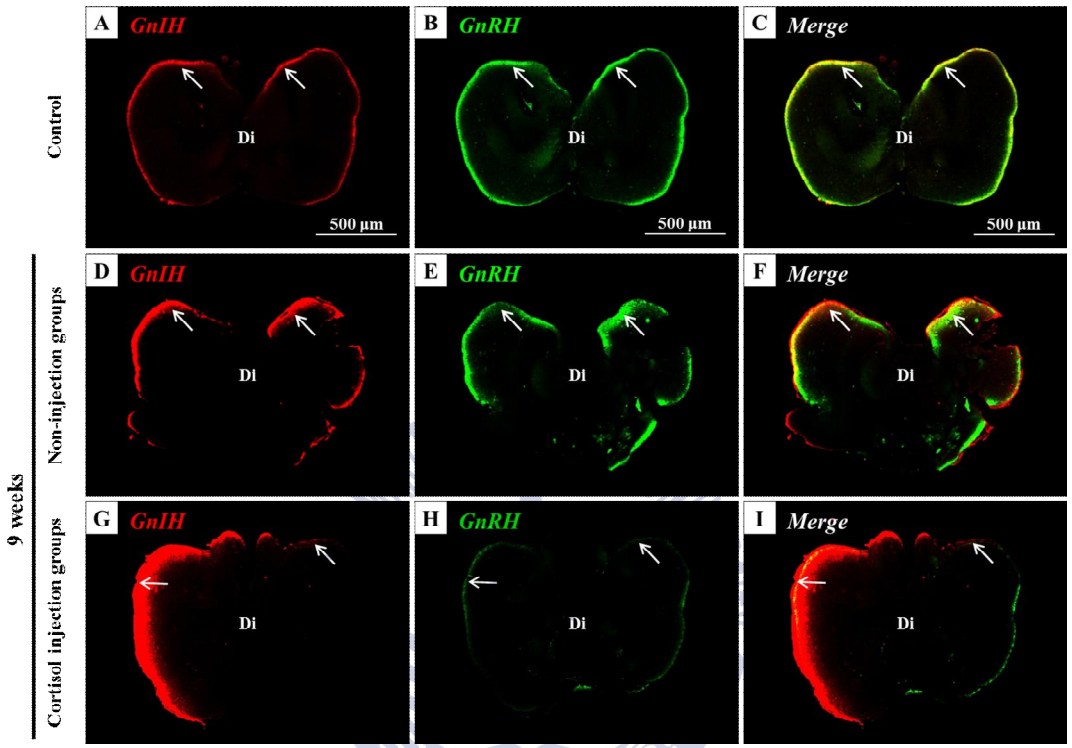

Fig. 16. Co-localization of GnIH and GnRH proteins in the diencephalon of cinnamon clownfish during 9 weeks after cortisol injection (50 μg/g), revealed by double immunofluorescence staining. (A–C) controls (non-injection groups), (D–F) 9 weeks after non-injection groups, (G–I) 9 weeks after cortisol injection. Diencephalons (Di) were stained with Texas Red (red; A, D, and G) or FITC (green; B, E, and 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, and I) and also indicated by arrows. Scale bars = 500 μm.

3.4. Time-course effects of hormone on GTHs

In the present study, I 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. 17). The results are shown are relative and are not absolute. The *P* values of all of the experimental groups were less than 0.05.

3.5. Time-course effects of GnIH and sbGnRH on cortisol

In a reverse experiment, present study investigated the effects of treatment with GnIH and sbGnRH analog on circulating cortisol level in the short-term experiment. Treatment with GnIH at 0.1 μg/g significantly increased circulating cortisol level in a time related manner. Following GnIH injection, plasma cortisol levels gradually increased by approximately 3.0-fold after 48 h (Fig. 18). Injection with sbGnRH, however, resulted in small but statistically significant reduction in plasma cortisol levels by approximately 1.5-fold (Fig. 18). The results for the sbGnRH analog serves two purposes; 1) as control to demonstrated that the increase in cortisol was not related to fish treatment and handling, and 2) the reduced level would be of significance for practical use of sbGnRH for induction of spawning. The *P* values of all of the experimental groups were less than 0.05.

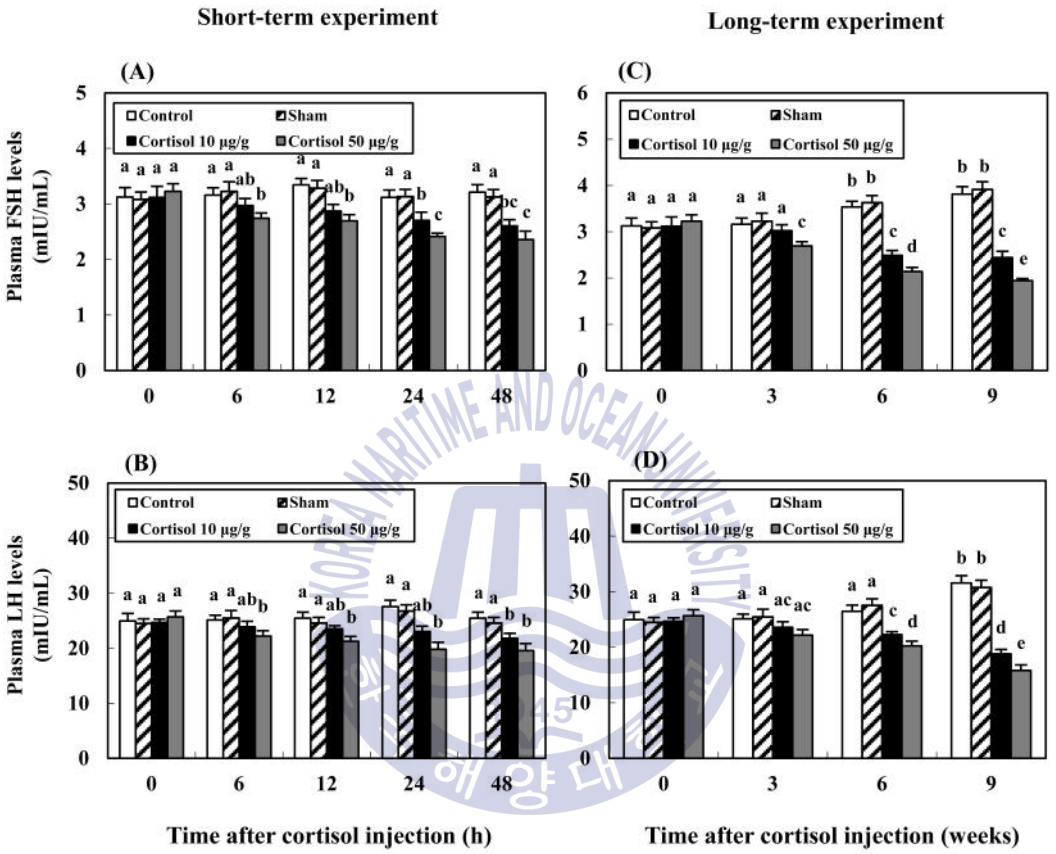

Fig. 17. The activity of plasma FSH (A, C) and LH (B, D) of cinnamon clownfish during 48 h (A, B) and 9 weeks (C, D) after cortisol injection (10 and 50 μg/g) was analyzed with a plate reader. Values with different letters indicate significant differences ($P < 0.05$). All values are means ± SE ($n = 5$).

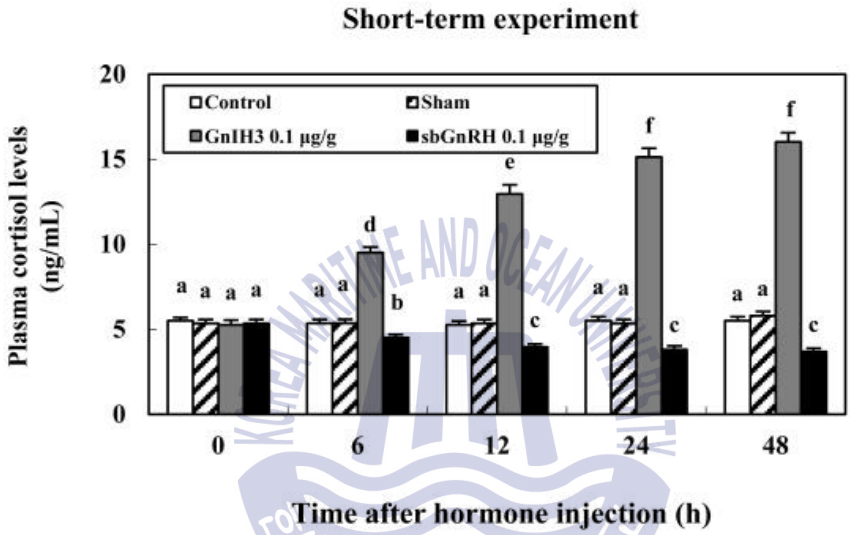

Fig. 18. The levels of plasma cortisol of cinnamon clownfish during 48 h after GnIH (0.1 μg/g) and sbGnRH injection (0.1 μg/g) assessed with a plate reader. Values with different letters indicate significant differences ($P < 0.05$). All values are means ± SE ($n = 5$).

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 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 reversal. The stress response in gonochoristic species may be different and dependent on the season based on the results observed in goldfish and salmonid (Moussavi et al., 2012, 2013). 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, I cannot rule out cross reactivity of the antibodies used for LH and FSH. However, the results are consistent with the observed effects on GnRH. This study used the heterologous assay since homologous immunoassay since homologous assay for cinnamon clownfish LH and FSH are not currently available.

The results obtained *in vitro* following treatment with cortisol and *in vivo* 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. (2010) demonstrating the presence of GnIH-R in the GnRH neurons, located in the hypothalamus of the brain. Also, Kirby et al. (2009) 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 hypothalamic–pituitary–adrenal axis (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 (Kirby et al., 2009). The results of Kirby et al. (2009) indicated that GRs are located in the 53% of GnIH (RFPR) cells of the hypothalamus, suggesting that glucocorticoids act directly on the RFRPs cells to increase GnIH, so inhibit the HPG axis. The present study 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, as well as cortisol, which increased during GnIH treatment, indicating that interactions of GnIH and cortisol have a positive feedback.

In this study, mRNA expression levels of sbGnRH and plasma levels of

gonadotropins significantly decreased after cortisol treatment as the concentration of cortisol increased (short-term experiment). However, mRNA expression of GnIH and GnIH-R significantly increased after cortisol treatment (short-term experiment). Also, sbGnRHa treatment significantly decreased the plasma levels of cortisol. 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. The observed effects of sbGnRH analog are importrnt, indication use of GnRH analogues for induction of spawning does not lead to stress response. It also seve as a positive control demonstration that the handling of fish in the same study did not significantly increase circulating cortisol level in the cinnamon clownfish.

Qi et al. (2013) reported that mRNA expression of GnRH, FSH, and LH significantly decreased in goldfish after GnIH treatment. Additionally, Kirby et al. (2009) 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 impare 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.

Chapter 5.

General Discussion

The identification of novel neurohormones that regulate the reproductive axis is essential for progress in neuroendocrinology. The decapeptide GnRH is the primary factor responsible for the hypothalamic control of gonadotropin secretion. However, GnIH has also been discovered in the brain of quail (Tsutsui et al., 2000), and a subsequent study reported that GnRH was not the only hormone that controlled vertebrate reproduction, as it interacted with GnIH. Furthermore, GnIH was shown to act on the pituitary gland and on GnRH neurons in the hypothalamus via a novel GnIH-R. GnIH decreased the synthesis and release of GTHs, inhibiting gonadal development.

The aim of this study was to examine the effects of GnIH on the GnRH axis, melatonin, and cortisol during different stages of gonadal maturation in male, female, and immature cinnamon clownfish *A. melanopus*.

I. Hypothalamic peptide neurohormones such as GnRHs and GnIH play pivotal roles in the control of reproduction and gonadal maturation in teleost fish (Tsutsui et al., 2012; Tsutsui and Ubuka, 2014). To study the effects of GnIH on fish reproduction, I investigated the influence of sbGnRH and GnIH (both alone and in combination) on levels of reproductive genes (GnIH, GnIH-R, MT-R, sbGnRH, and GTHs) during different stages of gonadal maturation in male, female, and immature cinnamon clownfish. For produced the family groups, 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 reversal 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 results showed that the expression

levels of GnIH, GnIH-R, and MT-R genes increased after the GnIH injection, but decreased after the sbGnRH injection. 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 reversal in cinnamon clownfish. In addition, these gene expression levels gradually lowered after GnIH3 and sbGnRH combination treatment, as compared to the MT-R mRNA levels of GnIH treatment alone. The same study suggested that when melatonin was combined with GnRH, other hormones in the HPG axis were suppressed because MT-R was presented on GnRH neurons (Sébert et al., 2008). However, the expression levels of the HPG axis genes (sbGnRH and GTHs) decreased after the GnIH injection, but increased after the sbGnRH injection. These results support previous evidence indicating that the GnIH-R located on GnRH nurone 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 *D. rerio* directly decreased following treatment with goldfish GnIH. 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. 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.

II. The annual changes in pineal melatonin secretion drive the reproductive responses of photoperiodic mammals (Sébert et al., 2008; McGuire et al., 2011). Recently, several studies indicated that melatonin stimulates the expression and release of GnIH via melatonin receptors expressed by GnIH neurons (Ubuka et al., 2005; McGuire et al., 2011). So, the present study aimed to determine the relationship between melatonin and GnIH and their effect on reproduction in cinnamon clownfish. Accordingly, this study investigated the expression pattern of GnIH, GnIH-R, and MT-R mRNA and protein, as well as the plasma levels of

melatonin, during sex reversal (mature male, male at 90 days after removing female, and female) in cinnamon clownfish. This present study found that GnIH and MT-R mRNA and melatonin activity were higher in fish with mature gonads than in fish with developing gonads. In fact, Tsutsui et al. (2010) 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, which suggests that melatonin directly controls GnIH expression and hormone levels (Chowdhury et al., 2010). So, this results 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). In addition, in the results of GnIH and MT-R expression on diencephalon by IF double staining, I observed the pattern that expression of GnIH and MT-R protein in the sex reversal groups reduced significantly in contrast to the mature male and female. These findings support the hypothesis that melatonin plays an important role in the negative regulation of maturation and GnIH regulation during reproduction. In particular, no studies have addressed the effects of GnIH during sexual maturation and sex reversal in hermaphroditic fish. Therefore, this 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.

III. Cortisol, a type of glucocorticoid hormone, is also associated with the stress response and is secreted by the HPI axis (Wendelaar Bonga, 1997; Flik et al., 2006). In vertebrates, glucocorticoid hormones, secreted from the center of the HPI axis include cortisol, and have a negative effect on the physiological aspects of vertebrate reproduction by inhibiting the activity of the HPG axis. Also, hypothalamus peptides, including the GnIH, play pivotal roles in the control of reproduction and gonadal maturation in fish and might lead to stress-mediated

reproductive dysfunction in teleosts (Rivier and Rivest, 1991; Moore and Jessop, 2003). In this study, I tested differences among the GnIH, GnIH-R, and HPG axis hormones (sbGnRH and GTH subunits (FSHβ and LHβ) on sex maturation of the cinnamon clownfish following treatment with cortisol (10 and 50 μg/g), which was used as an indicator for stress. The levels of GnIH mRNA expression increased after injection of cortisol (*in vivo* and *in vitro*), whereas the levels of sbGnRH mRNA expression and GTHs gradually decreased. These results support previous evidence indicating 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 (Kirby et al., 2009). This also leads to an increase in GnIH mRNA expression of, which was suggested to occur due to the activity of the HPI axis that increases the of levels of glucocorticoids, and decreases the activity of HPG. Using double immunofluorescence staining, I found that both GnIH and GnRH proteins were co-expressed in the diencephalon of cinnamon clownfish and the GnIH protein expression observed increased after 9 weeks of cortisol treatment, while GnRH decreased significantly. These results are consistent with the results of Kirby et al. (2009) that GRs are located in the 53% of GnIH (RFPR) cells of the hypothalamus, suggesting that glucocorticoids act directly on the RFRPs cells to increase GnIH, so inhibit the HPG axis. Also, cortisol levels gradually increased after GnIH treatment, although they decreased after sbGnRH treatment. These findings support the hypothesis that cortisol, an indicator of stress, effects reproduction and is mediated by GnRH-secreting neurons. Additionally, results of this study hypothesize that cortisol causes an increase in GnIH that contributes to hypothalamic suppression of reproductive function in the cinnamon clownfish. The findings provide a framework for better understanding of adverse stress response in teleosts and other vertebrates, in general.

In conclusion, the present study revealed changes in the expression of sex maturation-related genes (GnH, GnIH-R, GnRH, GTHs, and MT-R), as well as changes in the levels of plasma GnRH, FSH, LH, melatonin, and cortisol, during

the sex reversal from male to female and during hormone treatment (GnIH, sbGnRH, and cortisol). This study provides 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 and positive regulation of melatonin and cortisol in cinnamon clownfish. The cinnamon clownfish is a protandrous hermaphroditic teleost, and studies of its reproductive biology could enhance understanding of the evolution and diversity of reproductive endocrinology among teleosts, making the cinnamon clownfish a useful model species. The results are also significant because they enhance understanding of the possible role of GnIH in the control of sex reversal in cinnamon clownfish. Furthermore, the present study is the first to investigate the relationships among GnIH, melatonin, and cortisol in a target species, in this case, a hermaphroditic fish. Therefore, the present findings could provide useful basic data to identify the delay mechanism of sex conversion and maturation in teleosts.

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