Thesis for the Degree of Doctor of Philosophy

Molecular and Endocrine Regulation of Sex Change Mechanism in the Protandrous Cinnamon Clownfish *Amphiprion melanopus*

Na Na Kim

Department of Marine BioScience and Environment

The Graduate School

Korea Maritime and Ocean University

August 2014

Thesis for the Degree of Doctor of Philosophy

Molecular and Endocrine Regulation of Sex Change Mechanism in the Protandrous Cinnamon Clownfish *Amphiprion melanopus*

Na Na Kim

Department of Marine BioScience and Environment

The Graduate School

Korea Maritime and Ocean University

August 2014

Molecular and Endocrine Regulation of Sex Change Mechanism in the Protandrous Cinnamon Clownfish *Amphiprion melanopus*

Advisor: Prof. Cheol Young Choi

by

Na Na Kim

A dissertation submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

In the Department of Marine BioScience and Environment,

the Graduate School of Korea Maritime and Ocean University

August 2014

Molecular and Endocrine Regulation of Sex Change Mechanism in the Protandrous Cinnamon Clownfish *Amphiprion melanopus*

A dissertation

by

Na Na Kim

Approved as to style and content by:

Bong-Seok Kim, Ph.D.
Chairman

In-Seok Park, Ph.D.
Member

Sung Hwoan Cho, Ph.D.
Member

Young-Ung Choi, Ph.D.
Member

Cheol Young Choi, Ph.D.
Member

June 2014

CONTENTS

List of Tables

List of Figures

List of Abbreviations

AVPV	anteroventral periventricular nucleus
BM	body mass
BSA	bovine serum albumin
CCORA	Center of Ornamental Reef and Aquarium
cGnRH-II	chicken GnRH-II
cDNAs	complementary DNAs
Ct	cycle threshold
DBD	DNA-binding domain
ELISA	enzyme-linked immunosorbent assay
E. Coli	*Escherichia coli*
E_2	17β-estradiol
ER	estrogen receptor
ERE	estrogen response element
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
FSH	follicle-stimulating hormone
FSHR	FSH-receptor
GPR54	G protein coupled receptor 54
GVBD	germinal vesicle breakdown
GSI	gonadosomatic index
GTH	gonadotropin
GnRH	gonadotropin-releasing hormone
GnRHa	GnRH analogue
H&E	haematoxylin-eosin
HPG	hypothalamus-pituitary-gonad
RGD	integrin-recognition motif
11-KT	11-ketotestosterone

Kiss	kisspeptin
LBD	ligand-binding domain
LH	luteinizing hormone
LHR	LH-receptor
LRH13	luteinizing-releasing hormone 13
MBP	maltose binding protein
MEM	minimum essential medium
NCS	N-linked glycosylation sequence
ORF	open reading frames
PMSF	phenylmethylsulfonyl fluoride
PCR	polymerase chain reaction
PVDF	polyvinylidene diflouride
POA	pre-optic area
QPCR	quantitative real-time PCR
RIA	radioimmunoassay
RACE	rapid amplification of cDNA ends
rGTH	recombinant GTHs
RT	reverse transcription
sGnRH	salmon GnRH
sbGnRH	seabream GnRH
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
T	testosterone
MS-222	tricaine methane sulfonate
TBS	tris-buffered saline
Vtg	vitellogenenin

흰동가리 *Amphiprion melanopus*의 성 성숙 및 성 전환 메커니즘에 관한 분자내분비학적 조절

김 나 나

한국해양대학교 대학원 해양생명환경학과

요 약

어류의 성 성숙 및 발달은 시상하부-뇌하수체-생식소(HPG) 축을 중심으로 생식소자극호르몬방출호르몬(GnRH), 생식소자극호르몬(GTH)과 스테로이드호르몬 이외에 신경내분비물질, 생식소호르몬과 같은 다양한 성 호르몬에 의해 조절된다. 본 연구에 사용된 흰동가리 *Amphiprion melanopus*는 농어목(Perciformes) 자리돔과(Pomacentridae)에 속하는 종으로 암·수 한 쌍 또는 암·수 한 쌍과 미성숙 개체로 그룹을 형성하며 생활한다. 그룹 내에서 이들 개체의 성은 사회적 지위에 의해서 결정되는데, 암컷이 그룹 내에서 가장 크고 제일 높은 서열을 가지게 되며, 암컷이 죽거나 사라지게 되면 수컷이 암컷으로 성 전환을 하고, 미성숙 개체가 수컷으로 성 전환을 하는 대표적인 웅성선숙(protandry)형 어류이다. 따라서 본 연구는 흰동가리의 성 성숙 및 성 전환 과정 동안 HPG 축 활성에 영향을 미치는 유전자의 발현 및 성 스테로이드호르몬의 내분비학적 조절 메커니즘을 규명하기 위하여 수행되었다. 본 연구에서는 흰동가리의 성 성숙 및 성 전환 과정을 확인하기 위해, 암·수 한 쌍과 미성숙 개체로 이루어진 그룹에서 암컷을 제거한 후 60, 90 및 120 일째 수컷 개체를 관찰하였다. 본 연구를 진행하는 동안 수온은 28°C를 유지하였고, 일반 광주기(명기 12 시간: 암기 12 시간)를 사용하였다.

1. 흰동가리의 성 전환 단계별 3가지 타입 GnRH의 발현 변화 및 외인성 GnRH의 영향

GnRH는 어류의 번식과 성숙을 조절하는데 중요한 역할을 수행한다. 본 연구에서는 흰동가리에서 3가지 타입의 GnRH (sGnRH, sbGnRH 및 cGnRH-II) cDNA를 각각 클로닝 하였으며, 흰동가리가 수컷에서 암컷으로 성 전환 과정 동안 3가지 타입 GnRH의 mRNA 발현과 활성을 비교하여 성 전환에 따른 GnRH의 역할을 확인하였다. 그 결과, sGnRH, sbGnRH 및 cGnRH-II mRNA는 성숙한 개체의 정소와 난소에서 유의적으로 높은 발현량을 보였다. 성 전환 과정 동안은 GnRH mRNAs의 발현량이 감소하였다가 암컷으로 성 전환이 이루어지면서 다시 발현량이 증가하기 시작하여, 완전히 성숙한 암컷 생식소에서 가장 높은 발현량을 보였다. 또한, 3가지 타입의 GnRH를 각각 미성숙 개체에 각각 주입한 결과, 3가지 타입의 GnRH mRNA의 발현량(*in vivo* 및 *in vitro*) 뿐만 아니라 혈장 내 E_2 농도 또한 유의적으로 증가하였음을 확인하였다. 따라서 GnRH는 흰동가리의 HPG 축을 조절하는 데 중요한 역할을 수행하고 있으며, 생식소 발달 및 성 전환과 밀접한 관련이 있는 것으로 사료된다. 또한, 본 연구에서는 3가지 타입의 GnRH 중, sbGnRH가 가장 높은 발현량이 관찰되었는데, 이는 sbGnRH가 다른 2가지 타입의 GnRH에 비하여 시상하부에 많이 분포하고 있기 때문인 것으로 보이며, sbGnRH가 HPG 축을 직접적으로 활성화시켜 흰동가리의 성 전환 및 생식소의 최종 성숙 조절에 중요한 역할을 하고 있는 것으로 판단된다.

2. 재조합 GTH를 처리한 흰동가리에서 GTH, GTHR 및 Vtg의 발현 변화

GTH는 척추동물의 생식 조절에 중요한 역할을 하고 있는 호르몬으로, 여포자극호르몬(FSH)과 황체형성호르몬(LH)으로 구분된다. 이들 호르몬은 뇌하수체 전엽에서 합성되며, 생식소로 분비되어 각각의 수용체인 FSHR 및 LHR과 결합한 후, 에스트로겐과 테스토스테론과 같은 성 스테로이드를 조절함으로써 생식소의 발달과 성숙을 제어하는 것으로 알려져 있다. 본 연구에서는 흰동가리의 뇌하수체에서 GTH를 분리하였으며 이를 이용하여 2가지 타입의 재조합

GTH (rFSH, rLH)를 제작하였다. GTH의 발현 변화에 따른 각각의 호르몬 및 수용체와의 상관관계를 조사하기 위해, 미성숙 개체에 rFSH와 rLH를 각각 주입한 후, 경과 시간대별로 3가지의 GTH (GTHα, FSHβ 및 LHβ)와 각각의 수용체(FSHR, LHR) 그리고 Vtg mRNA의 발현(*in vivo* 및 *in vitro*) 및 혈장 E_2 농도 변화를 관찰하였다. 그 결과, 3가지의 GTH와 각각의 수용체 및 Vtg mRNA의 발현량 모두 증가하였으며, 혈장 E_2 농도 또한 증가하였다. 따라서 본 연구에서는 재조합 GTH가 흰동가리의 생식소 발달 및 성 성숙에 직접적으로 영향을 미치고 있음을 확인할 수 있었다.

3. 흰동가리의 성 전환 단계별 ER와 Vtg의 발현량 변화 및 E_2의 영향

에스트로겐은 어류를 포함한 척추동물에서 난자 형성, 난황 형성, GTH의 분비, 정소 발달 등과 같은 성 발달 및 성숙 과정 동안 세포핵 내에 존재하는 특이적 수용체인 에스트로겐 수용체(ER)와 결합하여 다양한 기능을 하는 스테로이드호르몬이다. 또한, 간에서 유도되는 비텔로제닌(Vtg)은 ER 관련 경로에 의하여 에스트로겐과 반응하는 난황전구단백질로 ER과 상호작용을 통하여 성 성숙 및 번식을 조절한다. 본 연구에서는 흰동가리가 수컷에서 암컷으로 성 전환이 이루어지는 동안 3가지 타입의 ER (ERα, β1 및 β2)와 Vtg mRNA의 발현량 변화를 비교하였으며, 미성숙 개체에 E_2를 복강 주사한 후 ER와 Vtg mRNA의 발현량 및 혈장 E_2 농도의 변화를 통해 E_2와 이들 유전자간의 상호작용에 대하여 확인하였다. 본 연구 결과, 흰동가리의 성 전환 과정 동안 생식소와 간에서 3가지 타입의 ER과 Vtg mRNA의 발현이 증가되었으며, E_2를 복강 주사한 미성숙 개체에서도 3가지 타입의 ER와 Vtg mRNA의 발현량이 증가되었다. 또한, E_2가 ER와 Vtg을 직접적으로 조절하여 흰동가리의 성 전환 과정에 있어서 중요한 역할을 하고 있음을 시사하고 있다.

4. 흰동가리의 성 전환 단계별 caspase-3 mRNA의 발현 변화

Apoptosis는 세포사멸의 유형으로, 생식소에서 apoptosis는 GTH와 생식소 스테로이드 인자를 통해 조절된다. 본 연구에서는 흰동가리에서 caspase-3 cDNA를 클로닝 하였으며, 성 전환 과정 동안 caspase-3 mRNA의 발현량과 활성을 비교하여 흰동가리가 수컷에서 암컷으로 성 전환 과정 동안 caspase-3의

역할에 대해 조사하였다. 그 결과, 미성숙에서 수컷, 수컷에서 암컷으로 성 전환이 이루어지는 동안 apoptosis를 유도하는 caspase-3 mRNA의 발현량이 증가하였으며, 이와 동시에 난모세포의 발달과 더불어 성 성숙 또한 촉진되었음이 확인되었다. 또한, 미성숙 개체에 GnRH analogue를 주입함에 따라 caspase-3 mRNA의 발현량이 증가된 점으로 볼 때, GnRH가 HPG 축을 활성화시켜 생식소 발달에 영향을 미치는 apoptosis를 조절하고 있는 것으로 판단되었다.

5. Kisspeptin이 흰동가리의 성 성숙 및 HPG 축 유전자의 발현에 미치는 영향

Kisspeptin (Kiss)은 RF-amide peptide 그룹에 속하는 신경단백질로, 시상하부의 시삭전영역(POA)에 분포하고 있으며, 척추동물에서는 번식을 조절하는 신경내분비 인자로 알려져 있다. 본 연구에서는 Kiss에 의한 HPG 축의 조절과 성 성숙과의 관련성을 파악하고자 인위적으로 흰동가리 암·수 한 쌍을 만든 후, 각각의 암·수 개체에 Kiss를 주사하였다. Kiss를 주사한 각각의 암·수 개체를 6주간 사육하면서, 2주 간격으로 암·수 개체를 각각 샘플링하여 성 성숙과 직접적으로 관련되어 있는 유전자인 GnRH, GTH, FSHR, LHR, ER 및 Vtg mRNA의 발현량 변화를 조사하였다. 또한, 생식소스테로이드 호르몬의 전구체인 17α-hydroxypregnenolone와 E_2, 11-keto- testosterone (11-KT)의 농도를 측정하여 Kiss 처리에 의한 흰동가리의 성 성숙 조절 메커니즘을 조사하였다. 그 결과, Kiss의 처리 시간이 경과함에 따라 3가지 타입 GnRH의 mRNA 발현량 및 혈장 GnRH 농도가 모두 증가하는 경향을 보였으며, GTH mRNA 발현량과 혈장 FSH와 LH 농도뿐만 아니라 FSHR와 LHR mRNA의 발현량 또한 모두 증가된 것으로 관찰되었다. 또한, Kiss 처리에 따라 ER과 Vtg mRNA의 발현 및 혈장 내 17α-hydroxypregnenolone, E_2 및 11-KT의 농도가 모두 증가하였으며, 조직학적 관찰 결과에서도 Kiss를 처리한 흰동가리의 암컷과 수컷 생식소 조직에서 성 성숙이 활발히 진행되고 있음을 확인할 수 있었다. 본 연구 결과, 시상하부 POA 세포에 존재하는 Kiss는 흰동가리의 암·수 개체 모두에 작용하여 GnRH의 분비를 촉진시키고, HPG 축을 활성화시켜 생식소의 최종 성숙이 이루어지는 동안 중요한 역할을 하고 있는 것으로 판단되었다.

Chapter 1.

General Introduction

Sexual development and maturation in teleost fish are regulated by various sex hormones in the hypothalamus-pituitary-gonad (HPG) axis, including gonadotropin-releasing hormone (GnRH), gonadotropin (GTH), steroid hormones, and other neurohormones (Lee et al., 2001a,b; Habibi and Andreu-Vieyra, 2007). A key regulator of this system is GnRH, which stimulates the synthesis and the release of pituitary hormones, most notably follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In turn, FSH and LH stimulate gonadal gametogenesis and steroidogenesis (Andrews et al., 1988). This process involves a complex interplay between neuroendocrine and endocrine inputs from multiple receptors, local paracrine and autocrine regulation, and feedback controls (Weltzien et al., 2004; Habibi and Andreu-Vieyra, 2007; Taranger et al., 2010).

To date, 15 GnRH isoforms have been isolated from vertebrates, and they comprise a family of highly conserved, decapeptide neurohormones that are responsible for the control and co-ordination of reproduction in all vertebrates (Fernald and White, 1999; Adams et al., 2002). Many studies suggest that GnRH-expressing neurons 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). sGnRH is produced in neuronal groups localized in the ventral forebrain along the terminal nerve, controls GTH secretion, and has been implicated in the regulation of spawning behavior (Senthilkumaran et al., 1999). The cGnRH-II neurons are localized in the mid-brain tegmentum, project their axons widely throughout the central nervous system, and modulate sexual and feeding behaviors (Millar, 2003). sbGnRH neuronal cell bodies are localized in the pre-optic area (POA), project their axons throughout various brain loci, have neuromodulatory activities (Oka, 2009), and exert effects on pheromone production

(Steven et al., 2003).

FSH regulates both 17β-estradiol (E_2) for vitellogenesis and spermatogenesis, and LH promotes follicular maturation, ovulation, and the synthesis of steroid hormones in teleosts (Nagahama et al., 1995; Ando and Urano, 2005; Kobayashi et al., 2006). Specific FSH-receptor (FSHR) and LH-receptor (LHR) on target cells mediate FSH and LH-induced stimulation of gametogenesis and gonadal steroidogenesis (Nagahama et al., 1995). There is evidence that FSHR gene is expressed in the granulosa cells of the ovary and in the Sertoli cells of the testis, whereas the LHR gene is expressed primarily in the theca and granulosa cells of preovulatory ovarian follicles and in the Leydig cells of the testis (Rocha et al., 2007). It is established that LH and FSH are key regulators of gonadal development and differentiation and control the synthesis of gonadal hormones that regulate reproduction in vertebrates, including fish (Amano et al., 1997; Colombo and Chicca, 2003; An et al., 2008a, 2009). Furthermore, gonadal steroid hormones such as estrogen and testosterone (T) play an important role in sex-related gonadal development and sexual characteristics (Dickey and Swanson, 2000).

Estrogen is an essential steroid hormone in reproduction and plays important roles in sexual maturation and differentiation, which include oogenesis, vitellogenesis, and testicular development. Estrogen action is primarily mediated by nuclear estrogen receptors (ERs; ERα and ERβ). In addition, vitellogenin (Vtg), a precursor yolk protein, in response to estrogens by an ER-mediated pathway is well documented in several oviparous (egg-laying) fish species (Ryffel, 1978; Pakdel et al., 1991)

Teleosts rely heavily on various environmental cues, such as temperature and light, during spawning (Billard, 1986), and leads to gonadal regression, a process that may occur via the induction of apoptosis. Apoptosis due to hormones and other chemicals that occurs during embryonic development and metamorphosis is involved in cell death (Corriero et al., 2009). Furthermore, testicular apoptosis is also known to occur during normal spermatogenesis in both mammals (Hong et al., 2008) and fish (Prisco et al., 2003), and it is thought to be essential for

maintaining the correct ratio between sertoli cells and gametes during this process (Lee et al., 1997). In particular, caspase-3 appears to be a key protease in the apoptotic pathway (Porter and Janicke, 1999) and the most characterized effector caspase. In addition, caspase-3 levels were reported to increase in follicles undergoing atresia (Boone and Tsang, 1998). Thus, caspase-3 seems to be involved in several cell death processes in the ovary.

Recently, kisspeptin (Kiss), a neuropeptide that regulates sexual differentiation and spawn time in vertebrates, and its receptor, GPR54 (G protein coupled receptor 54), have been shown to play major roles in the central regulation of the HPG axis (Roa et al., 2011; Chang et al., 2012). Kiss, a member of the Arg-Phe (RF)-amide peptide family, is located in the POA of the hypothalamus and regulates sexual maturation and regulation factors (Roa et al., 2011). Mammalian Kiss1 is suggested to be a key regulator of the HPG axis, and in teleosts, its ortholog is named Kiss1 (Kanda et al., 2008). In addition, the Kiss-GPR54 signal systemis one of the circuits regulating GnRH secretion in the hypothalamus (Colledge, 2009). According to a recent study performed in goldfish, both signal peptides could stimulate or inhibit the HPG axis dependent on the stage of gonadal development (Zmora et al., 2012). Many findings have led a number of authors to suggest an important role played by the Kiss system in the activation of the HPG axis in fish (Parhar et al., 2004; Filby et al., 2008; Kitahashi et al., 2009). The hypophysiotropic actions of Kiss1 was also confirmed *in vitro* on primary pituitary cell culture from goldfish (Yang et al., 2010), and results also showed that Kiss1 could stimulate prolactin and growth hormone secretion and gene expression in goldfish pituitaries (Yang et al., 2010). The synthesis and secretion of FSH and LH in the pituitary are stimulated by Kiss protein synthesis and secretion in the hypothalamus; in particular, Kiss plays an important role in regulating reproduction and stimulates LH secretion before reproduction and ovulation (Chang et al., 2012).

Cinnamon clownfish *Amphiprion melanopus* typically consist of a mated adult pair and an immature individual, and social ranking in the group controls the sexes of the fish. In general, the female is the largest in size and is dominant in the

group. If a dominant female dies or is absent, the male partner undergoes a sex change to become a female (Godwin and Thomas, 1993). Thus, cinnamon clownfish are an interesting model for studying the mechanism of sex change in protandrous hermaphroditic fish. So far, only studies investigating changes in steroid hormones (Godwin and Thomas, 1993) and gonad histological analyses (Godwin, 1994) have been reported with regard to sex change processes in cinnamon clownfish; neither expression of HPG axis and apoptosis involved in sex change progression nor expression of these genes by steroid hormone treatment has yet been examined.

To investigate the roles of HPG axis gene in cinnamon clownfish sex differentiation and gonadal development, the mRNA and protein expression patterns of these genes, such as GnRHs (sGnRH, sbGnRH, and cGnRH-II), GTHs (GTHα, FSHβ, LHβ), GTHR (FSHR, LHR), ERs, and Vtg was investigated during the sex change process. In addition, the effects of three GnRHs (sGnRH, sbGnRH, and cGnRH-II) on GnRH mRNA expression (*in vivo* and *in vitro*) and the E_2 levels was studied in immature cinnamon clownfish. Also, recombinant GTHs (rGTH; rFSH and rLH) was produced for investigate GTH-mediated control of reproduction in cinnamon clownfish. The results provide the information on autoregulatory mechanisms of reproduction and vitellogenic/spermatogenic synthesis in cinnamon clownfish. Here, the relationship between HPG axis regulation and sexual maturation have been studied using Kiss. This study aim was to produce a pair (female and male) of cinnamon clownfish with artificially induced sexes and to investigate the effects of weekly injections of Kiss on the regulation mechanism of sexual maturation in pairs of cinnamon clownfish, as assessed by the effects of Kiss on GnRHs, GTHs, GTH receptors, ERs, Vtg mRNA expression, and plasma concentrations of 17α-hydroxypregnenolone, E_2, and 11-ketotestosterone (11-KT).

Chapter 2.

Expression profiles of three types of GnRH during sex-change in the protandrous cinnamon clownfish *Amphiprion melanopus*: Effects of exogenous GnRHs

1. Introduction

The regulation of reproduction in teleost fish is a complex process that involves interactions between several factors, including GnRH, GTHs, gonadal hormones, and other neurohormones (Lee et al., 2001a,b; Habibi and Andreu-Vieyra, 2007). A key regulator of this system is GnRH, which stimulates the synthesis and release of pituitary hormones, most notably, FSH and LH. In turn, FSH and LH stimulate gonadal gametogenesis and steroidogenesis (Andrews et al., 1988). This process involves a complex interplay of neuroendocrine and endocrine inputs with multiple receptors, local paracrine and autocrine regulation, and feedback controls (Weltzien et al., 2004; Habibi and Andreu-Vieyra, 2007; Taranger et al., 2010).

To date, 15 GnRH isoforms have been isolated from vertebrates, and they comprise a family of highly conserved, decapeptide neurohormones that are responsible for the control and co-ordination of reproduction in all vertebrates (Fernald and White, 1999; Adams et al., 2002). GnRH-producing neurons within the brain play distinct roles in the regulation of reproduction (Zohar et al., 2010). The majority of studies conducted in fish suggest that GnRH-expressing neurons are distributed among three distinct populations within the brain, probably reflecting distinct phylogenetic relationships and functions (Andersson et al., 2001). The cGnRH-II, is expressed ubiquitously in vertebrates, including fish and mammals.

cGnRH-II neurons are localized in the midbrain tegmentum, project their axons widely throughout the central nervous system, and modulate sexual and feeding behaviors (Millar, 2003). In certain teleosts, such as striped bass *Morone saxatilis*, gilthead seabream *Sparus aurata*, and Nile tilapia *Oreochromis niloticus*, sGnRH is produced as the third form in neuronal groups localized in the ventral forebrain along the terminal nerve. sGnRH controls GTH secretion and has also been implicated in the regulation of spawning behavior (Gothilf et al., 1995; Senthilkumaran et al., 1999). The sbGnRH neuronal cell bodies are localized in the pre-optic area, project their axons throughout various brain loci, have neuro modulatory activities (Oka, 2009), and exert effects on pheromone production (Steven et al., 2003).

Interestingly, extra-hypothalamic GnRH has been reported in the ovaries and testes of various species, including the gilthead seabream (Nabissi et al., 2000; Andreu-Vieyra et al., 2005; Soverchia et al., 2007). It has been suggested that GnRH is involved in the regulation of gonadal function as an autocrine or paracrine regulator (Leung and Steele, 1992; Andreu-Vieyra et al., 2005; Habibi and Andreu-Vieyra, 2007). Gonadal GnRH peptides directly stimulate the resumption of oocyte meiosis and steroidogenesis in goldfish *Carassius auratus* (Habibi et al., 1989; Pati and Habibi, 1998, 2000, 2002). In addition, gonadal GnRH appears to regulate testicular and ovarian apoptosis in goldfish and gilthead seabream, which may be an important factor in follicular atresia, control of spermatogenesis, and early sex differentiation in fish (Andreu-Vieyra and Habibi, 2000; Andreu-Vieyra et al., 2005; Soverchia et al., 2007). Despite these recent insights, the molecular mechanism underlying gonadal sex change in protandrous hermaphroditic fish remains poorly understood.

In particular, a few studies have suggested that GnRH affects the gonads of fish that undergo sex change. However, given the phylogenetic and physiologic differences among the three GnRHs, the expression of the three GnRHs was anlayzed during sex change. Cinnamon clownfish typically consist of a mated adult pair and an immature individual, and social ranking in the group controls the sexes

of the fish. In general, the female is the largest in size and is dominant in the group. If a dominant female dies or is absent, the male partner undergoes a sex change to become a female (Godwin and Thomas, 1993). Thus, cinnamon clownfish are an interesting model for studying the mechanism of sex change in protandrous hermaphroditic fish.

To investigate the roles of GnRH in cinnamon clownfish sex differentiation and gonadal development, the complementary DNAs (cDNA) species was isolated for three types of prepro-GnRH from cinnamon clownfish and investigated the mRNA and protein expression patterns during the sex change process. In addition, the effects of three GnRHs (sGnRH, sbGnRH, and cGnRH-II) on GnRH mRNA expression (*in vivo* and *in vitro*) and the E_2 levels was studied in immature cinnamon clownfish.

2. Materials and methods

2.1. Experimental fish

The study was conducted with immature males (7.1 ± 0.8 g), mature males (10.5 ± 1.2 g), males at 60 days after removal of the female (12.5 ± 1.4 g), males at 90 days after removal of the female (15.2 ± 0.9 g), males at 120 days after removal of the female (20.1 ± 0.5 g), and female (22.2 ± 2.1 g) clownfish. Fish were purchased from CCORA (Center of Ornamental Reef and Aquarium, Jeju, Korea). Sexual maturity was determined by the presence of mature ova and sperm. All fish were anesthetized in tricaine methanesulfonate (MS-222; Sigma-Aldrich Chemical Co., MO, USA) and decapitated prior to tissue collection.

The experimental design for sex reversal was a modified version of the methods described by Godwin and Thomas (1993). After the groups consisting of a mated pair (dominant female and male) were made, sex change was induced in male fish by removing the female from each group and adding the immature clownfish. After 60, 90, and 120 days after removal of the female, the male fish underwent sex change from male to female. The process of sex reversal into five developmental stages was divided as follows: I, mature male; II, male at 60 days after female removal; III, male at 90 days after female removal; IV, male at 120 days after female removal (mostly ovary); V, mature female. Gonads obtained from fish representing the five stages of the sex change process were removed, immediately frozen in liquid nitrogen, and stored at −80°C until total RNA was extracted for analysis.

2.2. Treatment with GnRHs

To investigate the roles of GnRHs in the reproductive physiology of cinnamon clownfish, immature males were treated with GnRH, and GnRH gonadal expression was measured thereafter. The fish were reared in 220-L filtered water tanks in the laboratory and were anesthetized with MS-222 (200 mg/L; Sigma-Aldrich) prior to

injection. sGnRH [Gly^{10}-($D\text{-}Trp^{6}$) LHRH (salmon), H6845; Bachem, Torrance, CA, USA], sbGnRH [des Gly^{10}-($D\text{-}Leu^{6}$) LHRH (seabream), H4284; Bachem], and cGnRH-II [des Gly^{10}-($D\text{-}Ala^{6}$) LHRH II, H4288; Bachem] were dissolved in 0.9% physiological saline. Each fish was given an injection of GnRH [0.1 and 0.5 μg/g body mass (BM)] in a volume corresponding to 10 μL/g BM and sham group of fish was injected with a equal volume of 0.9% NaCl (10 μL/g BM). After injection, gonad samples were removed from five fish at 0, 6, 12, 24, and 48 h. During the experimental period, the water temperature and photoperiod were maintained at 26 ± 1°C and 12-h light:12-h dark (lights on from 07:00 to 19:00 h), respectively.

2.3. *In vitro* culturing of tissues

After the gonadal tissues were removed from ten immature cinnamon clownfish, they were placed in a 24-well culture plate (SPL Life Sciences, Seoul, South Korea). The gonadal tissues were treated with the GnRHs (sGnRH, sbGnRH, and cGnRH-II) at 10^{-9} and 10^{-7} M in medium 199 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and cultured for 0, 6, 12, 24, and 48 h. Testes were cultured under these conditions for 48 h in an incubator at 18°C, 100% humidity, and 5% CO_2 in air. Following incubation, 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.

2.4. Identification of different prepro-GnRHs

The primers used for amplification of the three prepro-GnRHs were designed using highly conserved sequences from other teleost fish; the primers used for reverse transcription polymerase chain reaction (RT-PCR) are shown in Table 1. Total RNA was extracted from the gonads using the TRIzol kit (Gibco/BRL, Grand Island, NY, USA). PCR amplification was performed using 2×Taq Premix I (Solgent, Daeseon, Korea), according to the manufacturer's instructions. The PCR was performed as follows: initial denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 40 s, and extension at 72°C for

Table 1. Primers of GnRHs used for PCR amplification

PCR	Genes	DNA sequences
RT-PCR	sGnRH-F	5′-AGC AGC AGA GTG ACG GTG C-3′
	sGnRH-R	5′-TCT TGG GTT TGG GCA CTC G-3′
	sbGnRH-F	5′-ACT TCA AAC CTC TGG ATC C-3′
	sbGnRH-R	5′-ACG CTG CAG GGA GAG TC-3′
	cGnRH-II-F	5′-TGC TGCT GGG GCT GCT TC-3′
	cGnRH-II-R	5′-TCT CCG GCC TCG CAC AGC TT-3′
RACE-PCR	5′-sGnRH	5′-ACC TGG TAG CCA TCC GTA GGA CCA GTG-3′
	3′-sGnRH	5′-TGG GAG AGC TGG AGG CAA CCA TCA GAG-3′
	5′-sbGnRH	5′-ACT CAG TCC ATA GGA CCA GTG CTG AC-3′
	3′-sbGnRH	5′-AAG AGA GAC CTG GAC AGC CTG TCG GAC-3′
	5′-cGnRH-II	5′-CTC CGG GGT ACC AAC CAT GAG ACC AG-3′
	3′-cGnRH-II	5′-TTC AGC ACA TCA GAG ATT TCA GAG GAG-3′
QPCR	sGnRH-F	5′-GAA GAG AAG TGT GGG AGA G-3′
	sGnRH-R	5′-CGA AAG GAC TGG AAT CAT CT-3′
	sbGnRH-F	5′-CTG CTG GTG GTG ATG ATG-3′
	sbGnRH-R	5′-GGT CTC TCT TCC CTC CTG-3′
	cGnRH-II-F	5′-AGC ACA TCA GAG ATT TCA GAG-3′
	cGnRH-II-R	5′-CCA AGG CAT CCA AAA GAA TG-3′
	β-actin-F	5′-GGA CCT GTA TGC CAA CAC TG-3′
	β-actin-R	5′-TGA TCT CCT TCT GCA TCC TG-3′

60 s; followed by 7 min at 72°C for the final extension. Amplified PCR products were electrophoresed in a 1% agarose gel that contained ethidium bromide (Biosesang, Sungnam, Korea). The PCR product was purified and cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The colony formed by transformation was cultivated into competent DH5α cells (RBC Life Sciences, Seoul, Korea) and that plasmid DNA was subsequently isolated from the transformants that grew on the selective medium. Then, plasmid DNA was extracted using the LaboPass Plasmid DNA Purification kit (Cosmo Genetech, Seoul, Korea) and the plasmid DNA was subjected to digestion with EcoRI (Fermentas, Glen Burnie, MD, USA). The sequence of the plasmid cDNA inserts were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Rapid amplification of prepro-GnRHs cDNA 3′ and 5′ ends

For the PCR reaction, total RNA was extracted from the gonads using the TRIzol kit. Using 3 μg of total RNA as a template, 3′-rapid amplification of cDNA ends (RACE) cDNA and 5′-RACE cDNA were synthesized using the CapFishing Full-length cDNA Premix kit (SeeGene, Seoul, Korea). First-strand cDNA synthesis was synthesized using an oligo-$(dT)_{18}$ anchor primer and the CapFishing adaptor (SeeGene). Gene-specific primers were selected from the PCR products obtained by RT-PCR. For 3′-RACE, the 50-μL PCR mixture contained 5 μL of 3′-RACE cDNA, 1 μL of 10 μM 3′-target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 μM 3′-RACE-specific primer (Table 1), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was conducted as follows: one cycle of denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 60 s; followed by one cycle of 5 min at 72°C for the final extension. For 5′-RACE, the 50 μL PCR mixture contained 5 μL of 5′-RACE cDNA, 1 μL of 10 μM 5′-target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 μM 5′-RACE-specific primer (Table 1), and 25 μL of SeeAmp Taq Plus Master Mix. The PCR was conducted as follows: one cycle of denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at

72°C for 60 s; followed by one final extension cycle of 5 min at 72°C. The amplified PCR products were electrophoresed in a 1% agarose gel that contained ethidium bromide (Biosesang). Transformation was conducted using the methods described above.

2.6. Phylogenetic analysis of the different prepro-GnRHs

Phylogenetic analyses were conducted by alignment with the known vertebrate sGnRH, sbGnRH, and cGnRH-II amino acid sequences using the BioEdit software (Hall, 1999). A phylogenetic tree was constructed using the neighbor-joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

2.7. Western blot analysis

Total protein isolated from gonads during sex change was extracted using a protein extraction buffer [5.6 mM Tris, 0.55 mM ethylenediaminetetraacetic acid (EDTA), 0.55 mM ethylene glycol tetraacetic acid (EGTA), 0.1% sodium dodecyl sulfate (SDS), 0.15 mg/mL phenylmethylsulfonyl fluoride (PMSF), and 0.15 mg/mL leupeptin], sonicated, and quantified using the Bradford method (Bio-Rad, Hercules, CA, USA). Total protein (30 μg/lane) was loaded onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. A protein ladder (Fermentas) was used for reference. Samples were electrophoresed at 80 V until they ran through the stacking gel, and then at 150 V until they ran through the resolving gel or until the bromophenol blue dye front had run off the gel. The gels were then immediately transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 1.5 h at 4°C. Subsequently, the membranes were blocked with 5% milk in tris-buffered saline (TBS, pH 7.4) for 45 min, followed by washing in TBS. Membranes were incubated with a monoclonal mouse antiserum that recognizes most vertebrate GnRH forms [luteinizing-releasing hormone 13 (LRH 13), dilution 1:5,000; courtesy of M.K. Park; Park and Wakabayashi, 1986],

followed by horseradish peroxidase conjugated anti-mouse IgG secondary antibody (1:5,000; Bio-Rad) for 60 min. The internal control was β-tubulin (dilution 1:5,000, ab6046, Abcam, Cambridge, MA, USA), followed by horseradish peroxidase- conjugated anti-rabbit IgG secondary antibody (1:5,000; Bio-Rad) for 60 min. GnRH immunoreactive bands were detected using the standard ECL, as well as the more sensitive ECL system (ECL Advance; GE Life Sciences, Uppsala, Sweden) and using exposure to autoradiographic film for 5 min.

2.8. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression levels of the mRNA species for the three GnRH subunits (sGnRH, sbGnRH, and cGnRH-II) using cDNA samples extracted from the gonads and cultured gonads of cinnamon clownfish. The primers used for QPCR are listed in Table 1. QPCR amplification was conducted using the BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System and a mixture that contained 0.5 μL of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, Sybr Green and Taq polymerase in buffer [10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein] in a total volume of 25 μL. The QPCR was conducted as follows: one 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. After the QPCR, the data from four replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. 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 $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$. Also, to ensure that the primers amplified a specific product, performed a melt curve, a single melting point was observed for the products of each primer pairs.

2.9. Plasma analysis

Plasma E_2 levels were analyzed by radioimmunoassay (RIA) using an E_2 RIA kit (DIASORIN, Antony, France).

2.10. Statistical analysis

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

3. Results

3.1. Identification of the three prepro-GnRHs

The sGnRH cDNA included an open reading frame (ORF) that was predicted to encode a protein of 90 amino acids (GenBank accession no. **HQ883477**). The sbGnRH cDNA consisted of an ORF that was predicted to encode a protein of 96 amino acids (**HQ883476**), and the full-length cGnRH-II cDNA contained an ORF that was predicted to encode a protein of 85 amino acids (**EU908057**).

Using the BLAST algorithm (Blastp; National Center for Biotechnology Information), the prepro-GnRH amino acids shared high-level identity with the prepro-GnRHs of other species. The amino acid sequences of the three prepro-GnRHs were compared with those deduced from the cDNA species of other teleost species (Fig. 1). The following amino acid similarities were deduced: for sGnRH, 97% with black porgy *Acanthopagrus schlegelii* sGnRH, 97% with red seabream *Pagrus major* sGnRH, 94% with bluefin tuna *Thunnus thynnus* GnRH3, and 89% with spotted weakfish *Cynoscion nebulosus* sGnRH; for sbGnRH, 86% with black porgy sbGnRH, 87% with red seabream sbGnRH, 74% with bluefin tuna GnRH-I, and 63% with European sea bass *Dicentrarchus labrax* sbGnRH; for cGnRH-II; 97% with black porgy cGnRH-II, 95% with bluefin tuna GnRH2, 94% with Nile tilapia cGnRH-II, and 94% with spotted weakfish cGnRH-II.

The three prepro-GnRH cDNAs found in cinnamon clownfish all contain the characteristic signal peptides [sGnRH, amino acids (aa) 1–23; sbGnRH, aa 1–26; and cGnRH-II, aa 1–26], specific GnRH amino acids (sGnRH, aa 24–33; sbGnRH, aa 27–36; and cGnRH-II, aa 24–33), enzymatic processing site [Gly-Lys-Arg (GKR)] (sGnRH, aa 34–36; sbGnRH, aa 37–39; and cGnRH-II, aa 34–36), and GnRH-associated peptides (GAPs) (sGnRH, aa 37–90; sbGnRH, aa 40–96; and cGnRH-II, aa 37–85).

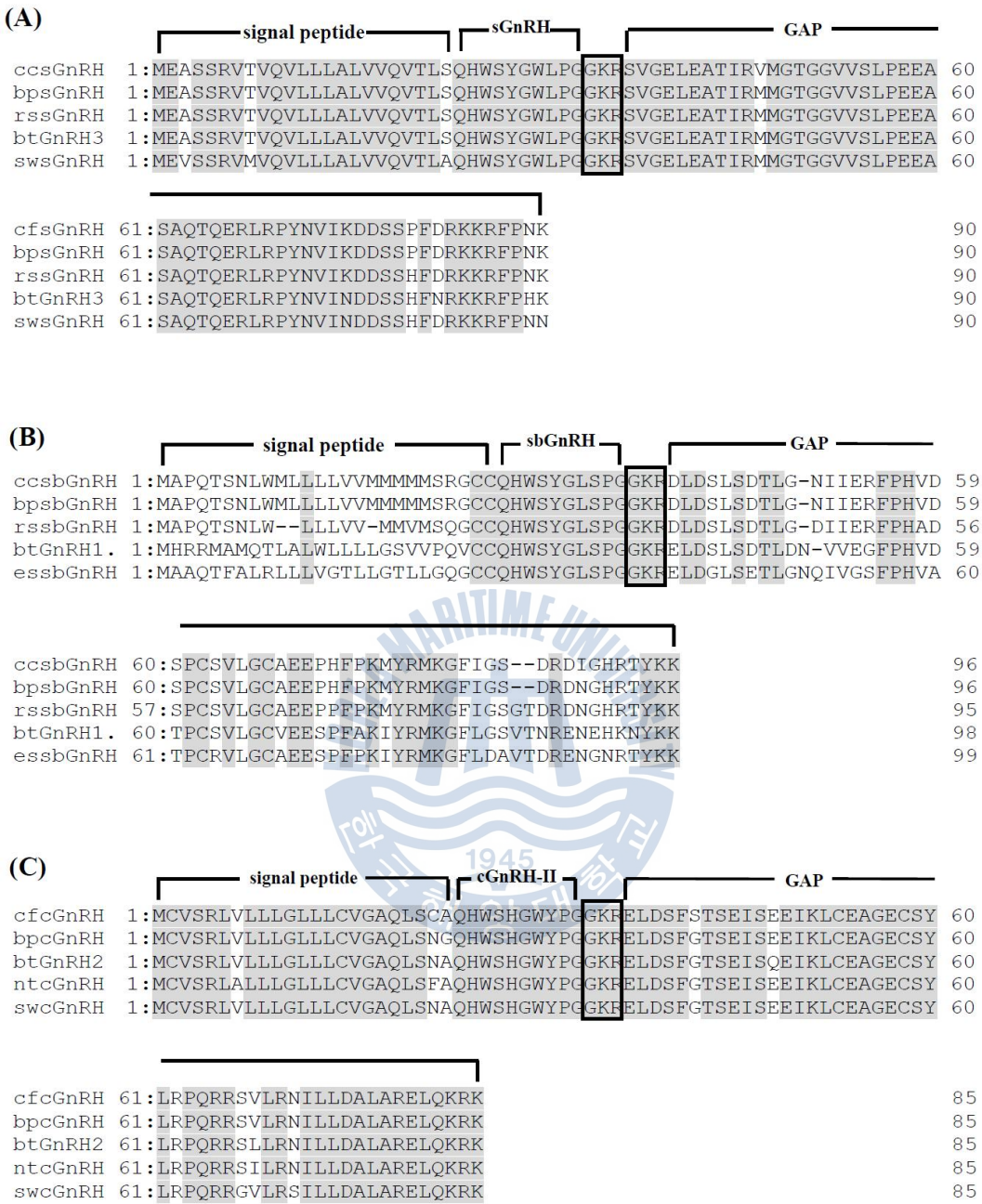

Fig. 1. Comparison of the amino acid sequences of sGnRH (A), sbGnRH (B), and cGnRH-II (C). The sequences were taken from the GenBank/EMBL/DDBJ databases. The amino acid sequences of cinnamon clownfish sGnRH (ccsGnRH, GenBank accession no. **HQ883477**), black porgy sGnRH (bpsGnRH, **EU117212**),

red seabream sGnRH (rssGnRH, **D26108**), bluefin tuna GnRH3 (btGnRH3, **ABX10868**), spotted weakfish sGnRH (swsGnRH, **AAV74403**), cinnamon clownfish sGnRH (ccsbGnRH, **HQ883476**), black porgy sbGnRH (bpsbGnRH, this study, **EU099997**), red seabream sbGnRH (rssbGnRH, **D86582**), bluefin tuna GnRH1 (btGnRH1, **EU239500**), European sea bass sbGnRH (essbGnRH, **AF224279**), cinnamon clownfish cGnRH-II (cccGnRH, **EU908057**), black porgy cGnRH-II (bpcGnRH, this study, **EU099996**), bluefin tuna GnRH-II (btGnRH2, **EU239502**), Nile tilapia cGnRH-II (ntcGnRH, **AB104862**), and spotted weakfish cGnRH2 (swcGnRH, **AY796309**) are optimally aligned to match identical residues, indicated by the shaded box. The enzymatic processing site (GKR) is boxed.

3.2. Phylogenetic analysis

The phylogenetic tree using the amino acid sequences of the prepro-GnRHs [signal peptide, specific GnRH amino acids (decapeptide), enzymatic processing site, and GAPs] obtained by cluster analysis described below is shown in Fig. 2. The phylogenetic analysis indicate a strong relationship among the same prepro-GnRH forms, designated as the three cinnamon clownfish prepro-GnRH isoforms. It is important to note that the cinnamon clownfish GnRHs are the most closely related to the prepro-GnRHs of closely related perciform members, such as the black porgy and gilthead seabream (sGnRH, sbGnRH, and cGnRH-II).

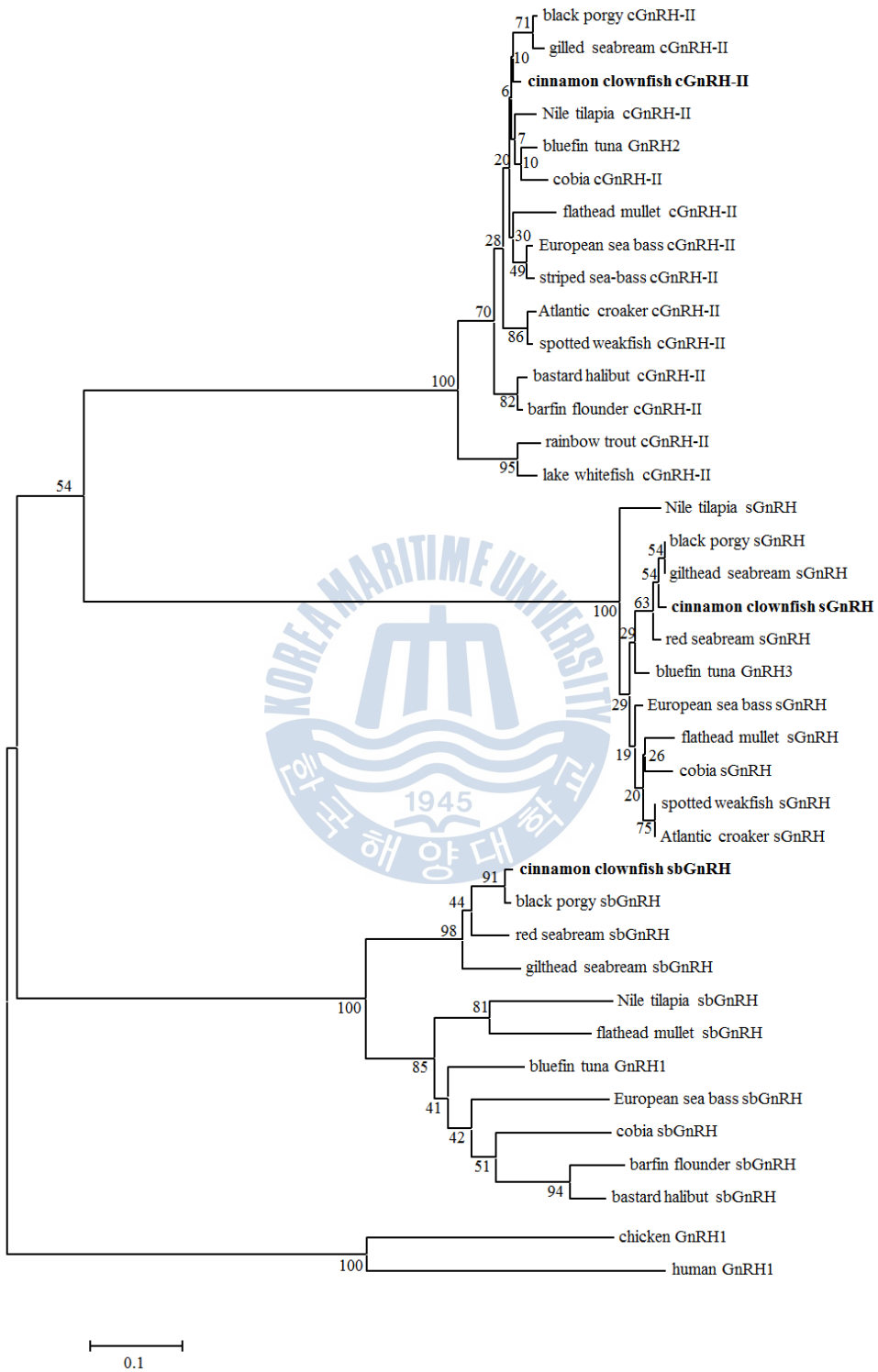
black porgy cGnRH-II
gilled seabream cGnRH-II
cinnamon clownfish cGnRH-II
Nile tilapia cGnRH-II
bluefin tuna GnRH2
cobia cGnRH-II
flathead mullet cGnRH-II
European sea bass cGnRH-II
striped sea-bass cGnRH-II
Atlantic croaker cGnRH-II
spotted weakfish cGnRH-II
bastard halibut cGnRH-II
barfin flounder cGnRH-II
rainbow trout cGnRH-II
lake whitefish cGnRH-II
Nile tilapia sGnRH
black porgy sGnRH
gilthead seabream sGnRH
cinnamon clownfish sGnRH
red seabream sGnRH
bluefin tuna GnRH3
European sea bass sGnRH
flathead mullet sGnRH
cobia sGnRH
spotted weakfish sGnRH
Atlantic croaker sGnRH
cinnamon clownfish sbGnRH
black porgy sbGnRH
red seabream sbGnRH
gilthead seabream sbGnRH
Nile tilapia sbGnRH
flathead mullet sbGnRH
bluefin tuna GnRH1
European sea bass sbGnRH
cobia sbGnRH
barfin flounder sbGnRH
bastard halibut sbGnRH
chicken GnRH1
human GnRH1
0.1

Fig. 2. Phylogenetic tree based on amino acid alignment of the sequences of sGnRH, sbGnRH, and cGnRH-II in teleost fish. Bootstrap values (%) are indicated by 1,000 replicates. The number associated with each internal branch is the local bootstrap probability. The GenBank accession numbers of the sequences are: for cinnamon clownfish sGnRH, **HQ883477**; black porgy sGnRH, **EU117212**; gilthead seabream sGnRH, **D26108**; flathead mullet sGnRH, **AY373449**; Nile tilapia sGnRH, **AB104863**; spotted weakfish sGnRH, **AAV74403**; bluefin tuna GnRH3, **ABX10868**; Atlantic croaker sGnRH, **AAQ16503**; European sea bass sGnRH, **AF224280**; cobia sGnRH, **AY677173**; cinnamon clownfish sbGnRH, **HQ883476**; black porgy sbGnRH, **EU099997**; red seabream sbGnRH, **D86582**; gilthead seabream sbGnRH, **AF046801**; Nile tilapia sbGnRH, **AB104861**; flathead mullet sbGnRH, **AY373450**; cobia sbGnRH, **AY677175**; bluefin tuna GnRH1, **EU239500**; barfin flounder sbGnRH, **AB066360**; bastard halibut sbGnRH, **DQ074693**; European sea bass sbGnRH, **AF224279**; cinnamon clownfish cGnRH-II, **EU908057**; black porgy cGnRH-II, **EU099996**; gilthead seabream cGnRH-II, **U30325**; rainbow trout cGnRH-II, **AF125973**; lake whitefish cGnRH-II, **AY245102**; Nile tilapia cGnRH-II, **AB104862**; Atlantic croaker cGnRH-II, **AY324669**; European sea bass cGnRH-II, **AF056313**; bluefin tuna GnRH2, **EU239502**; cobia cGnRH-II, **AY677174**; spotted weakfish cGnRH-II, **AY796309**; bastard halibut cGnRH-II, **DQ008580**; barfin flounder cGnRH-II, **AB066359**; and flathead mullet cGnRH-II, **AY373451**.

3.3. Expression profiles of sGnRH, sbGnRH, and cGnRH-II mRNA during sex change

All three identified GnRHs were found to be present at higher levels in mature testes and mature ovaries than in either of the sex-switching gonads (Fig. 3) ($P < 0.05$). The levels of sbGnRH and cGnRH-II transcripts were significantly higher in mature ovaries than in the testes, whereas the levels of sGnRH transcripts in the mature testes and mature ovaries were similar. Western blot analysis revealed a protein with GnRH-specific immunoreactivity and with a mass that corresponded to the predicted mass for cinnamon clownfish GnRH (52 kDa). The expression pattern of the protein resembled the pattern of the GnRH mRNA expressed in cinnamon clownfish gonads.

3.4. Plasma E_2 levels during sex change

The plasma E_2 level was 182.1 ± 30.2 pg/mL in males when a female was present, increased to 893.1 ± 55.3 pg/mL in males after removal of the female, and was increased again when the male changed to a female (1,192.4 ± 40.4 pg/mL) (Fig. 4) ($P < 0.05$). Overall, the plasma E_2 levels were higher in fish with mature gonads, than in fish in the gonadal development stage.

3.5. Time-related levels of the three GnRHs mRNA levels following GnRH injection

The expression of GnRH mRNA in the gonad following GnRH injection is shown in Fig. 5. Treatment with 0.1 μg/g BM and 0.5 μg/g BM of the GnRHs (sGnRH, sbGnRH, and cGnRH-II) significantly increased the GnRH mRNA levels in the gonads of the cinnamon clownfish ($P < 0.05$). In particular, the level of sbGnRH mRNA was higher (as compared to the untreated control and the levels of the other GnRH forms) at 6 h (at 0.5 μg/g BM) and 48 h (at 0.1 μg/g BM) post-injection, respectively (Fig. 5B, E, H).

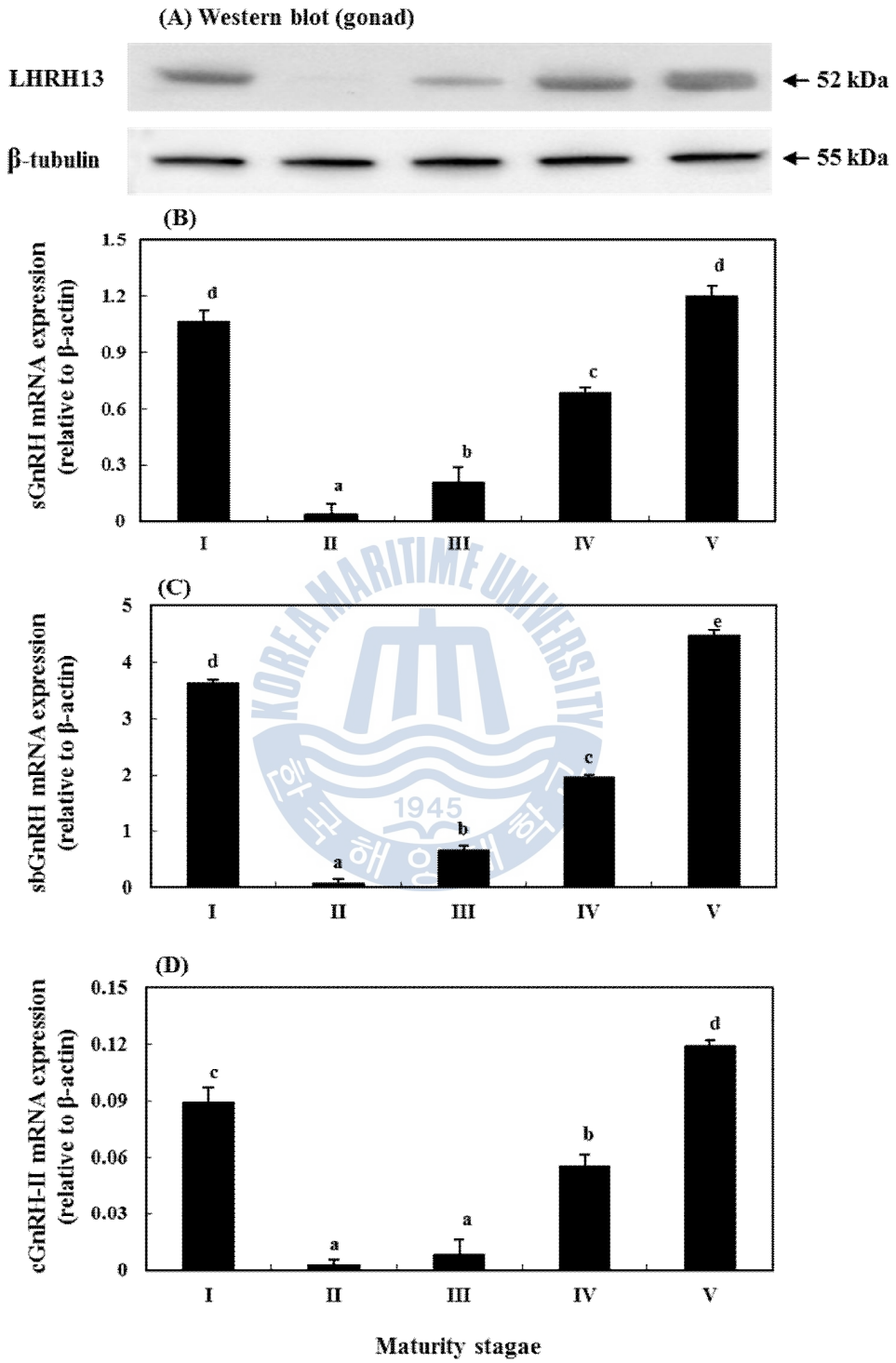

Fig. 3. sGnRH, sbGnRH, and cGnRH-II mRNA expression levels in the gonads of cinnamon clownfish during sex change (I, mature males; II, males at 60 days after removal of the female; III, males at 90 days after removal of the female; IV, males at 120 days after removal of the female; and V, mature females). (A) Western blot using LRH13 (dilution 1:5,000; 52 kDa) to examine protein expression in the gonads of cinnamon clownfish during sex change. The β-tubulin (dilution 1:2,000; 55 kDa) was used as the internal control. (B) sGnRH, sbGnRH, and cGnRH-II mRNA levels relative to the β-actin mRNA levels in the gonads of cinnamon clownfish during sex change were analyzed by QPCR. Values with dissimilar letters are significantly different ($P < 0.05$). Values are expressed as mean ± SE ($n = 5$).

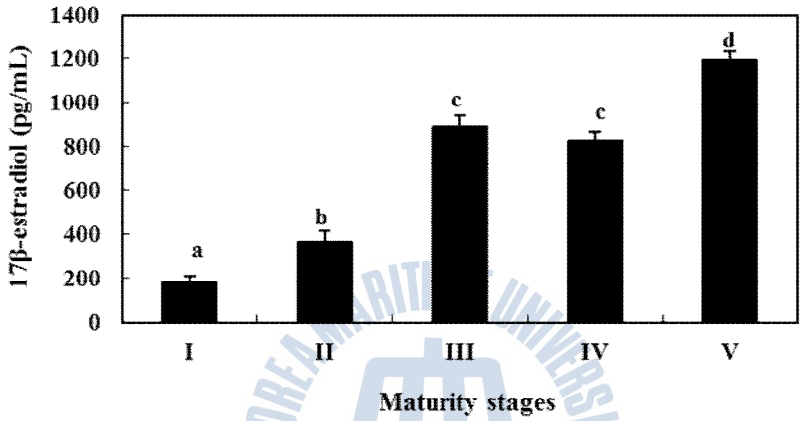

Fig. 4. Plasma E_2 levels during sex change in cinnamon clownfish (I, mature males; II, males at 60 days after removal of the female; III, males at 90 days after removal of the female; IV, males at 120 days after removal of the female; and V, mature females). Values with dissimilar letters are significantly different ($P < 0.05$). Values are expressed as mean ± SE ($n = 5$).

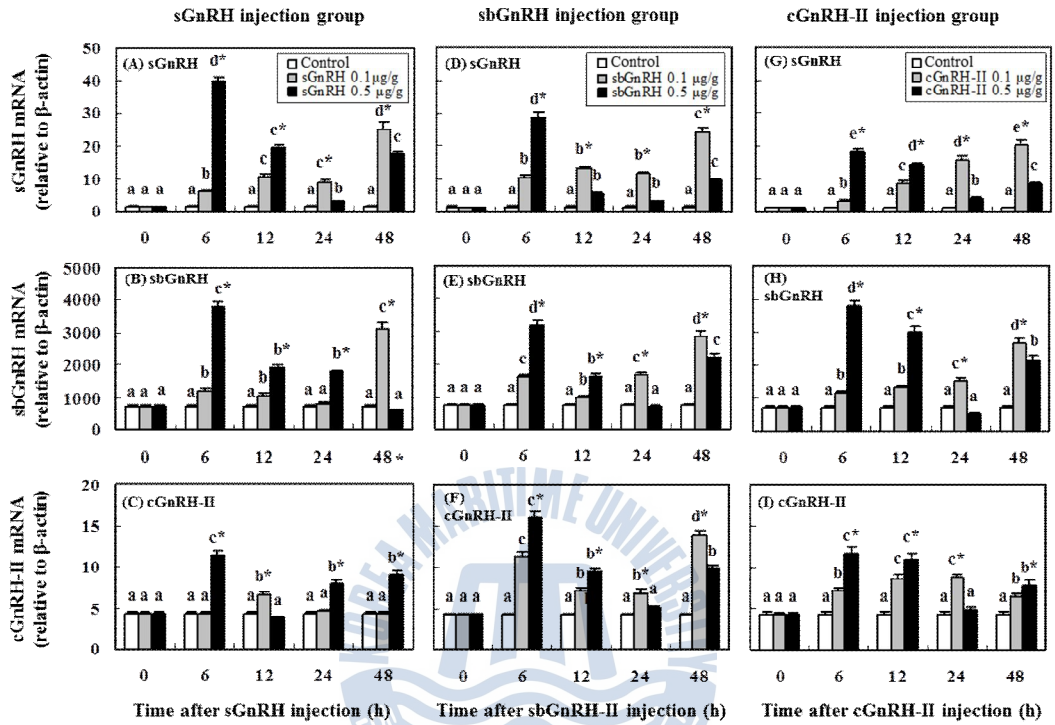

Fig. 5. Time-course effects of the three GnRHs [sGnRH (A, B, C), sbGnRH (D, E, F), cGnRH-II (G, H, I); 0.1 μg/g and 0.5 μg/g BM] on the cGnRH-II (A, D, G), sGnRH (B, E, H), and sbGnRH (C, F, I) mRNA levels in the cinnamon clownfish gonads. Total RNA was extracted 0, 6, 12, 24, and 48 h after treatment, and 3 μg was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is presented as relative expression. Values with dissimilar letters are significantly different ($P < 0.05$). The asterisks were indicated significant differences between each of the injected GnRH concentration on same time after GnRH injection ($P < 0.05$). Values are expressed as mean ± SE ($n = 5$).

3.6. Time-related expression of the three GnRHs in cultured gonads treated with GnRH

The GnRH mRNA mRNA levels were estimated in cultured gonads following treatment with GnRH (Fig. 6). Treatment with 10^{-9} M and 10^{-7} M sGnRH, sbGnRH, and cGnRH-II significantly increased the GnRH mRNA levels in cinnamon clownfish gonads ($P < 0.05$). In particular, the level of sbGnRH mRNA was higher than the levels of the untreated control and the other GnRHs at 24 h (at 10^{-7} M GnRH) and 48 h (at 10^{-9} M GnRH) post-injection, respectively (Fig. 6B, E, H) ($P < 0.05$).

3.7. Plasma E_2 concentrations following GnRH injection

The circulating E_2 concentration was measured in cinnamon clownfish following injection with the three molecular forms of GnRH (sGnRH, sbGnRH, and cGnRH-II) (Fig. 7). The plasma E_2 level was 35.9 ± 5.2 pg/mL at the start of the experiment. Most notably, the levels of E_2 increased to 167.4 ± 40.1 pg/mL at 6 h (0.5 μg/g BM sGnRH; Fig. 7A), to 487.4 ± 40.1 pg/mL at 12 h (0.5 μg/g BM sbGnRH; Fig. 7B), to 89.5 ± 15.3 pg/mL at 12 h (0.5 μg/g BM cGnRH-II; Fig. 7C), and decreased at 24 h all GnRH treatment grout, subsequently ($P < 0.05$).

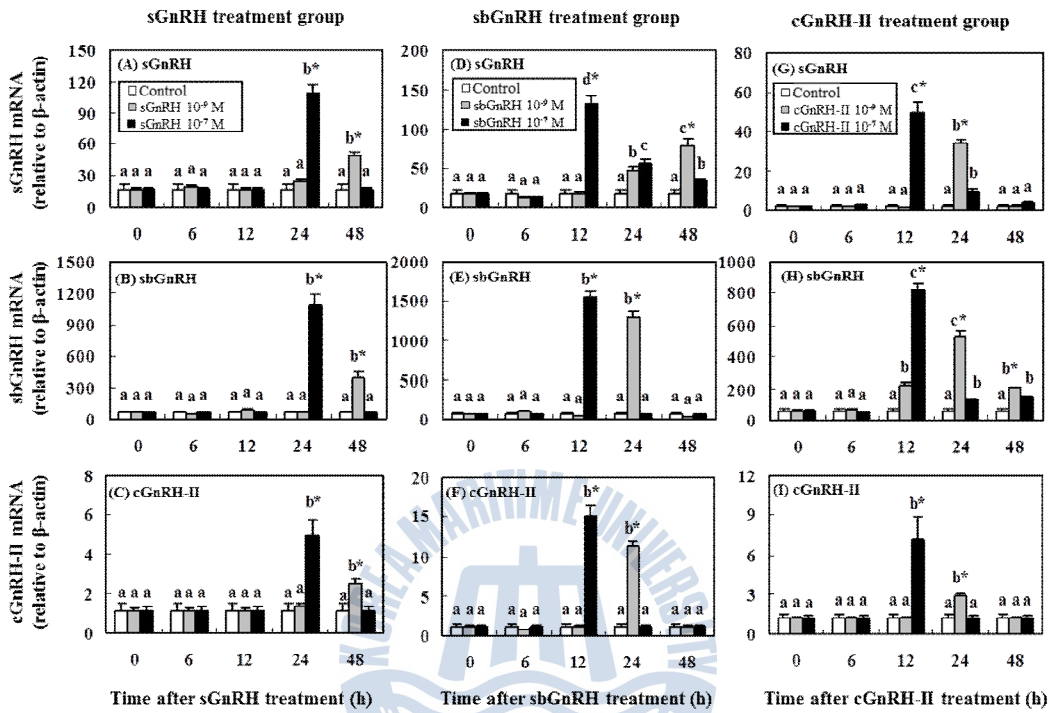

Fig. 6. Time-course effects of the three GnRHs [sGnRH (A, B, C), sbGnRH (D, E, F), cGnRH-II (G, H, I); 10^{-9} and 10^{-7} M] on the cGnRH-II (A, D, G), sGnRH (B, E, H), and sbGnRH (C, F, I) mRNA levels in the cinnamon clownfish cultured gonads. Total RNA was extracted 0, 6, 12, 24, and 48 h after treatment, and 3 μg was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is presented as relative expression. Values with dissimilar letters are significantly different ($P < 0.05$). The asterisks were indicated significant differences between each of the injected GnRH concentration on same time after GnRH injection ($P < 0.05$). Values are expressed as mean ± SE ($n = 5$).

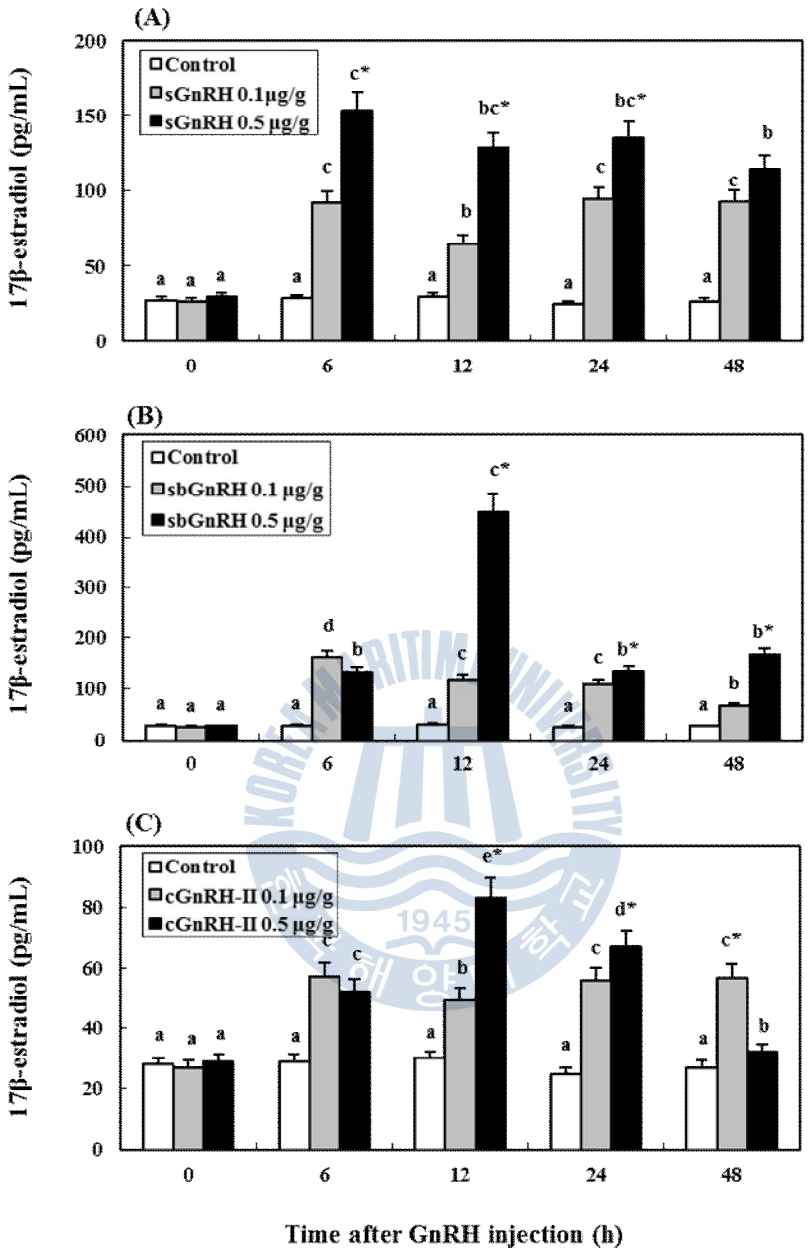

Fig. 7. Plasma E_2 levels during treatment with the three GnRHs [sGnRH (A), sbGnRH (B), cGnRH-II (C); 0.1 and 0.5 μg/g]. Values with dissimilar letters are significantly different ($P < 0.05$). The asterisks were indicated significant differences between each of the injected GnRH concentration on same time after GnRH injection ($P < 0.05$). Values are expressed as mean ± SE ($n = 5$).

4. Discussion

The full-length cDNAs of three molecular forms of prepro-GnRH (sGnRH, sbGnRH, and cGnRH-II) was isolated in the gonads of cinnamon clownfish. This study subsequently was investigated gonadal GnRH variant mRNA levels during sex change and following treatment with GnRH peptides. In this study, the circulating levels of E_2 was also measured during sex change from male to female in cinnamon clownfish and following injection with GnRH. Since the changes in GnRH variant expression was observed at different stages of gonadal development, the effects of GnRH peptides were investigated on sGnRH, sbGnRH, and cGnRH-II mRNA levels, *in vivo* and *in vitro* in immature cinnamon clownfish gonads.

The deduced amino acid sequences of the three prepro-GnRHs were found to be highly similar to those of other species (Fig. 1). All the prepro-GnRHs identified have a primary gene structure that is highly conserved. Prepro-GnRH is encoded as a prepro-hormone, consisting of a signal peptide, directly followed by the decapepdide, and by a GAP as described previously (Guilgur et al., 2006). The cinnamon clownfish GnRHs have the general organization similar to other prepro-GnRH molecular forms, including signal peptide, specific GnRH amino acids [sGnRH (^{24}QHWSYGWLPG^{33}), sbGnRH (^{27}QHWSYGLSPG^{36}), and cGnRH-II, (^{24}QH WSHGWPG^{33})], an enzymatic processing site (GKR), and the GAP (Guilgur et al., 2006). Comparison of the amino acid sequences of the three cinnamon clownfish GnRHs with other fish species revealed that the cinnamon clownfish specific GnRH amino acids and enzymatic processing site are similar to those in other fish species (Amano et al., 1997; Guilgur et al., 2007). Based on the specific GnRH amino acid sequences determined, the presence of three GnRH forms, sGnRH, sbGnRH, and cGnRH-II was demonstrated in cinnamon clownfish. This phylogenetic analyses suggest that the three prepro-GnRHs are derived from the same gene and that they are most closely related to the corresponding prepro-GnRHs of the gilthead seabream (Powell et al., 1994), African cichlid *Haplochromis burtoni* (White et al.,

1995), and red seabream (Senthilkumaran et al., 1999) of Sparidae.

Although several studies have shown GnRH mRNA expression in the gonads of vertebrates, including teleost fish (Nabissi et al., 2000; Uzbekova et al., 2001; Andreu-Vieyra et al., 2005), few studies have examined the roles of GnRHs during sexual differentiation and sex change in fish. The present study provides information on gonadal GnRH mRNA expression during the sex-change process in cinnamon clownfish. The transcript levels of all three GnRHs and protein levels of LRH13 were high in the mature testes and ovaries. However, the gonads involved in the switching process had very low levels of GnRH mRNA (Fig. 3). The anti-LRH13 directed at the common amino acid sequence of mammalian, avian, and fish GnRHs (Park and Wakabayashi, 1986). These results support previous evidence indicating that the level of GnRH mRNA decreases in switching testes compared to mature testes, and that the level of GnRH mRNA is generally highest in the mature ovaries of the gilthead seabream (Soverchia et al., 2007) and black porgy (An et al., 2008a). Soverchia et al. (2007) provided information on GnRH transcription in different areas of the switching gonads, including the nascent ovary, parts of regressing testes, and switching areas of the gonads. In gilthead seabream, the GnRH expression was found to be low in the regressing testis and in the testicular portion of the switching gonad; higher sGnRH and cGnRH-II transcript levels were observed in the emerging ovary (Soverchia et al., 2007). In the present study, low level of GnRH transcript was also observed in the gonads switching to ovary. The present study was observed a gradual increase in GnRH transcript level during the stages of ovarian development. The present results suggests that the three GnRH forms might be important players in paracrine control of ovarian development and sex-change process in cinnamon clownfish and other group of sequential hermaphrodites. So, it is demonstrate that the high levels of GnRH in mature testes and ovaries may help to maintain the appropriate ratio of Sertoli cells to gametes by stimulating apoptosis in the testes and ovaries.

In the present study, the circulating level of E_2 was found to increase slowly in the developing ovary reaching statistically significant in mature ovary (Fig. 4).

These observations are consistent with previous studies on sea bass (Guiguen et al., 1993) and anemone fish (Godwin and Thomas, 1993), in which plasma E_2 levels were found to be the highest in mature females.

In view of the evidence that GnRH plays a role in regulation of ovarian development, the expression levels of the three GnRH mRNA species was investigated directly *in vitro*, and indirectly *in vivo*, presumably via increase in gonadal GTH level in immature cinnamon clownfish. The developmental changes in frequency of GnRH secretion *in vitro* were associated with an increase in three GnRH activity and expression in neurons and to stimulate its secretion via a transcription-dependent mechanism. The present results demonstrate increase in sGnRH, sbGnRH, and cGnRH-II mRNA following treatment with GnRH variants, *in vivo* and *in vitro* with differences in time course and concentration. The expression of three GnRH forms in the gonad (*in vivo*) increased significantly at 6 h at an earlier time than in cultured gonad (*in vitro*) at 24 h. The results shown that cultures from dispersed fish gonads have been used successful to analyze the regulation of GnRH release. This is also provides that the support that sex steroids stimulate the release of sbGnRH in dispersed gonad through a direct pathway (Lee et al., 2004). Therefore, gonadal GnRH is hypothesized to be release through GnRHs mRNA expression and protein levels. In cultured gonads, changes in the expression of the three GnRHs were similar to the changes seen after injection of the three GnRHs *in vivo*. Nabissi et al. (2000) isolated seabream follicle-enclosed oocytes *in vitro* to investigate the direct actions of sGnRH and cGnRH-II on the reinitiation of oocyte meiosis, as indicated by germinal vesicle breakdown (GVBD). As a result, a significantly higher GVBD response was observed after treatment with GnRH. The present findings strongly support the hypothesis that sGnRH, and possibly other forms of GnRHs, are involved in paracrine/autocrine regulation of seabream ovarian function. The magnitude of the increase in sbGnRH gene expression was greater than those of the other GnRH forms (sGnRH, cGnRH-II) following injection of the three GnRH. This suggests that sbGnRH plays a central role in final sexual maturation, although the functional significance of sbGnRH in

the regulation of spawning and migratory behaviors remains to be determined (Lee et al., 2001a). Shahjahan et al. (2010) showed that levels of sbGnRH gene expression increased significantly in May in the spawning grass puffer *Takifugu niphobles*. sbGnRH plays a central role in final sexual maturation by stimulating GTH secretion in the grass puffer. The physiologic significance of sbGnRH as a regulator of GTH secretion and gametogenesis has been established in various teleosts. In gilthead seabream, the levels of sbGnRH in the pituitary of sexually mature fish are higher than those in recrudescent fish, and pituitary sbGnRH levels in sexually mature fish are higher than those in recrudescent fish, whereas pituitary cGnRH-II content remains unchanged during these two stages and sGnRH was not detected in the pituitary irrespective of reproductive state (Holland et al., 1998). A temporal increase in sbGnRH pituitary content during the spawning season has also been reported in striped bass (Senthilkumaran et al., 1999) and turbot *Scophthalmus maximus* (Andersson et al., 2001). Therefore, it was hypothesized that sbGnRH increasing in the brain of spawning fish, suggesting that it may play a role in stimulating spawning and/or migratory behavior in teleosts.

The E_2 levels increased significantly following injection with sbGnRH (Fig. 7). GnRH treatment has been shown to regulate the gonadal ER (Choi et al., 2007), implicating E_2 as a mediator of sex change. Therefore, while exo-genous E_2 can induce sex change, endogenous E_2 is more likely to be the result of increased ovarian tissue rather than the initial signal for sex change (Lee et al., 2001b; An et al., 2008b). In this context, estrogen treatment was shown to increase ERs expression in goldfish liver, testis and ovary (Nelson et al., 2007; Nelson and Habibi, 2010). Therefore, it is likely that GnRH-induced increase in E_2 level also increase estrogen responsiveness by increasing expression of ERs. Especially Lee et al. (2004) investigated the regulation of sbGnRH release using *in vivo* and *in vitro* approaches in the black porgy. This study showed that steroids (E_2, T, and 11-KT) and cortisol have direct effect on brain neuronal cells stimulating the release of sbGnRH. Therefore, sbGnRH is suggested as the physiological and main form of GnRH in the relation to plasma LH concentrations in the black porgy (Lee et al., 2004).

In summary, it is demonstrate that cinnamon clownfish ovary and testis express three conserved types of GnRH (sGnRH, sbGnRH, cGnRH-II) and the levels changes as testis switches to ovary. The sGnRH, sbGnRH, and cGnRH-II mRNA expression levels were found to be higher in mature testes and ovaries, as compared to gonads at different stages of maturity. The levels of sbGnRH gene expression increased significantly following injection of the three GnRHs. These findings support the hypothesis that especially sbGnRH plays important roles in the regulation of gonadal development and sex change in cinnamon clownfish.

Chapter 3.

Effects of recombinant gonadotropin hormones on the expression of vitellogenin, gonadotropin subunits and gonadotropin receptors in cinnamon clownfish *Amphiprion melanopus*

1. Introduction

The regulation of reproduction is a complex process involving the interaction of a number of factors including GnRHs, GTHs, gonadal steroid hormones, and other neurohormones in fish and other vertebrates (Habibi and Matsoukas, 1999; Lee et al., 2001ab; Habibi and Andreu-Vieyra, 2007; Chang et al., 2009; Zohar et al., 2010). The pituitary GTHs, FSH and LH are members of the glycoprotein hormone family consisting of a common α subunit and a specific β subunit (Pierce and Parsons, 1981). The α/β subunits are highly complex in structure and are identified by the presence of different N-linked oligosaccharide chains and the formation of cross-linked disulfide bonds between cysteine residues. The non-covalent association of the two subunits determines the formation and release of the bioactive dimeric hormone. These hormones carry highly conserved N-linked glycosylation sites, and the attached oligosaccharides are important in hormone bioactivity in teleosts (Kamei et al., 2003). FSH regulates both E_2 for vitellogenesis and spermatogenesis, and LH promotes follicular maturation, ovulation, and the synthesis of steroid hormones in teleosts (Nagahama et al., 1995; Ando and Urano, 2005; Kobayashi et al., 2006). Specific FSHR and LHR on target cells mediate FSH and LH-induced stimulation of gametogenesis and gonadal steroidogenesis (Nagahama et al., 1995). There is evidence that FSHR gene is expressed in the granulosa cells of the ovary and in the Sertoli cells of the testis, whereas the LHR gene is expressed primarily in the theca

and granulosa cells of preovulatory ovarian follicles and in the Leydig cells of the testis (Rocha et al., 2007). It is established that LH and FSH are key regulators of gonadal development and differentiation and control the synthesis of gonadal hormones that regulate reproduction in vertebrates, including fish (Amano et al., 1997; Colombo and Chicca, 2003; An et al., 2008a, 2009). Furthermore, gonadal steroid hormones such as estrogen and testosterone play an important role in sex-related gonadal development and sexual characteristics (Dickey and Swanson, 2000).

A key step in control of reproduction in fish and other oviparous vertebrates is the production of Vtg, a precursor yolk protein, under the regulation of E_2. The GTH-induced ovarian E_2 production in turn stimulate synthesize and release of Vtg from hepatocytes into the bloodstream, where it is taken up and incorporated into the growing oocytes via receptor mediated endocytosis (Sawaguchi et al., 2006). The E_2-mediated expression of Vtg and ER-mediated pathways have been investigated in fish (Pakdel et al., 2000; Nelson and Habibi, 2010). The Vtg gene is also present in male fish and can be stimulated by E_2. However, under normal condition Vtg level in male is very low, and can be used as an effective biomarker for environmental contaminants with estrogen-like activity (Filby et al., 2006; Jeffries et al., 2008, 2010; Kim et al., 2010).

Cinnamon clownfish is a protandrous hermaphroditic fish, typically found as a mated adult pair and an immature individual. Social ranking in the group normally controls fish gender. In general, the female is the largest in size and is dominant in the group. If a dominant female dies or is absent, the male partner undergoes a sex change to become a female (Godwin and Thomas, 1993). Currently, little studies are known about hormonal control of reproduction and gonadal sex change in cinnamon clownfish, which is used in this study as a suitable experimental model. Therefore, recombinant GTHs (rFSH and rLH) was produced to investigate GTH-mediated control of reproduction in cinnamon clownfish. However, it is unclear whether the maturational GTH is important for regulation of gonadal steroidogenesis. The results provide the information on autoregulatory mechanisms of GTH-mediated control of reproduction and vitellogenic/spermatogenic synthesis in immature cinnamon clownfish.

2. Materials and methods

2.1. Experimental fish

The study was conducted with immature clownfish (7.1 ± 0.8 g). Fish were purchased from CCORA, and reared in the six 50-L circulation filter tanks in the laboratory. All fish were anesthetized in tricaine methane sulfonate (MS-222; Sigma-Aldrich) and decapitated prior to tissue collection. Gonads and pituitaries were removed from the fish, immediately frozen in liquid nitrogen, and stored at −80°C until total RNA was extracted for analysis.

2.2. Production of rFSH and rLH

ORF of the cinnamon clownfish FSHβ and LHβ were amplified by polymerase chain reaction (PCR) using previously cloned cinnamon clownfish cDNAs for the FSH and LH subunits (An et al., 2010). The GenBank accession numbers for GTH α, FSHβ, LHβ are **EU908056**, **FJ868867**, and **FJ868868**, respectively. The strategy to construct the tethered single-chain FSHα/β and LHα/β are shown in Fig. 8. At first, the ORF regions of mature β subunits encoding the FSHβ (26 amino acids of signal sequence and 118 amino acids of the mature protein without stop codon) or the LHβ (25 amino acids of signal sequence and 138 amino acids of the mature protein without stop codon), and mature GTHα were generated by overlapping PCR method. Recombinant protein included the maltose binding protein (MBP) in the N-terminals and a synthetic N-linked glycosylation sequence (NCS) (Fig. 8). In each cDNA construct, a synthetic DNA encoding Ser-Gly-Ser-Asn-Ala-Thr-Gly-Ser-Gly-Ser-Asn-Ala-Thr-Ser-Gly-Ser (NCS) was inserted between the β and α chain by overlapping PCR strategy. In addition, an EcoRI site (FSHβ and LHβ) was placed at the 5′-end of the DNA constructs and a HindIII (FSHβ), and SalI (LHβ) site was placed at the 3′-end of the DNA constructs immediately following the terminator codon of the common GTHα subunit. PCR was performed in 50 μL final volume containing the subunit cDNA templates (An et al., 2010), 50 μL 10×reaction buffer,

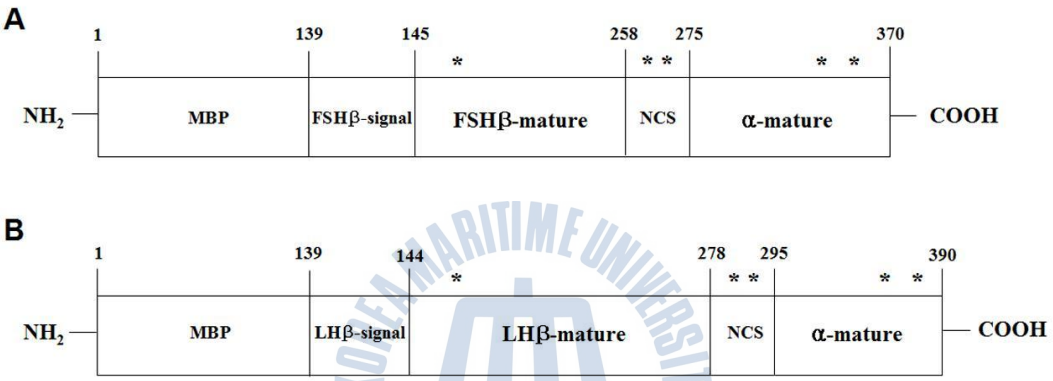

Fig. 8. Schematic diagrams of recombinant single-chain cinnamon clownfish rFSH (A) and rLH (B). The numbers above each box refer to the amino acid position in each fragment or GTH subunit. Asterisks above each box indicate the putative N-linked glycosylation sites (Asn-X-Ser/Thr).

2 mM $MgCl_2$, 200 mM dNTP, 2 mM each primer, and 2.5 U Pfu-Taq DNA polymerase (Fermentas). After an initial 5 min denaturing step at 94°C, 30 cycles of amplification were performed using a cycle profile of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. After the last cycle, elongation was extended to 5 min at 72°C. Next, the PCR products were cut with EcoRI and HindIII (FSHβ-GTHα), EcoRI and SalI (LHβ-GTHα), purified by gelextraction and inserted into EcoRI-HindIII sites and EcoRI-SalI of a transfer vector (pYNG: Katakura Industry, Sayama, Saitama, Japan). Finally, the plasmid DNA was sequenced in both strands by chaintermination method using a Big Dye Terminator Ready Reaction Mix (Applied Biosystems) and an Applied Biosystems' Prism 377 DNA Sequencer.

The recombinant proteins pre-test for activation assay was overexpressed in *Escherichia coli* (*E. coli*) BL21 (DE3) pLysS cells. Briefly, 10 mL of *E. coli* DE3 pLysS cells starter culture was inoculated into 1,000 mL Luria broth with 1 mL ampicillin (50 mg/mL). The culture was incubated at 37°C with shaking at 180 ×*g* until the cell count reached 0.7 at an optical density of 600 nm. Then, the culture was shifted to 20°C for 15 min and induced with 0.5 mM isopropyl-β-thiogalactopyranoside for 6 h at FSHβ-GTHα 28°C and LHβ-GTHα 37°C. After 6 h of induction, the cells were cooled on ice for 30 min and harvested by centrifugation at 6,000 ×*g* for 15 min at 4°C. The cells were re-suspended in 30 mL 1×phosphate buffered saline (PBS) buffer and frozen at −70°C. After thawing, the bacterial cells were placed in an ice-water bath and sonicated 10 times using short 10 s pulses. After centrifugation at 10,000 ×*g* for 20 min and 4°C, the recombinant proteins were purified in the form of fusion protein as MBP using a pMAL protein fusion and purification system (vector: amylose resin E8021S, Cosmo Genetech). Briefly, amylose resin was poured into a poly-chromatography column and washed three times with PBS, and the fusion protein was eluted with an elution buffer (2^{-10} mM maltose). The purified proteins were run on 10–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel with a protein marker (Fermentas). Gels were stained using 0.05% Coomassie Blue R-250, followed by a standard de-staining procedure.

2.3. Electrophoresis and Western blot analysis

SES-PAGE was performed using 12% polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane (Pall Corp., Ann Arbor, MI, USA). After blocking, the membrane was sequentially incubated with a 1:2,000 dilution of polyclonal mouse anti-MBP (Abcam; rFSHβ-GTHα and rLHβ-GTHα) overnight at 4°C and a peroxidase-conjugated polyclonal antibody to mouse IgG (1:2,000; goat anti-mouse IgG-HRP; Santa Cruz Biotechnology, Santa, Cruz, CA, USA) for 2 h at room temperature. After washing, the membrane was incubated with an enhanced chemiluminescent detection reagent (Amersham Biosciences, Fairlawn, CT, USA) according to the manufacturer's directions. Bands were visualized by exposure to X-ray film (Amersham Biosciences).

2.4. Treatment procedure

To investigate the effects of rLH and rFSH, *in vivo*, fish were reared in 220-L circulating filter tanks in the laboratory and were anesthetized with MS-222 prior to injection. rLH and rFSH were dissolved in 0.9% physiological saline, and each fish was given an injection of the either rLH or rFSH (0.1 and 1 μg/g, BM) at a volume of 10 μL/g BM and sham group of fish was injected with a equal volume of 0.9% NaCl (10 μL/g BM). After the injection, gonad samples were removed from five fish at 0, 6, 12, 24, and 48 h. During the experimental period, the water temperature and photoperiod were maintained at 26 ± 1°C and 12L/12D, respectively.

2.5. Gonadal culture, *in vitro*

For *in vitro* experiments, the gonads were dissected in to small pieces in cold culture phenol red free Medium 199 (Invitrogen) from 10 immature cinnamon clownfish. Approximately 5 mg of testes slices (20-μm thick) were added per well for 1 h prior to hormone treatment in 24-well culture plates (SPL Life Sciences). Cultured gonadal tissues were treated with 0.1 and 1 μg/mL of either rLH or rFSH in M199 for 0, 6, 12, 24, and 48 h at 18°C, 100% humidity, and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20°C, 10,000 ×*g*, 15 s),

the supernatant and gonadal tissues were removed and stored separately in micro-centrifuge tubes at −80°C.

2.6. QPCR

QPCR was conducted to determine the relative expression of GTH subunits (GTH α, FSHβ, and LHβ), GTH receptors (FSHR, LHR), and Vtg, using total RNA extracted from the pituitaries (GTH subunits), gonad (GTH receptors), and liver (Vtg) of cinnamon clownfish, respectively. Primers for QPCR are shown in Table 2. QPCR amplification was conducted as described previously (Kim et al., 2010) using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad) under the following conditions: 0.5 μL of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, Sybr Green and Taq polymerase in buffer [10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein] to a total volume of 25 μL. QPCR was conducted as follows: one cycle of denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 20 s and annealing at 55°C for 20 s. After the PCR program, QPCR data from three replicate samples were analyzed with system analysis software (Bio-Rad) to estimate transcript copy numbers for each sample. Each experimental group was run in triplicate to ensure consistency. Experiments were duplicated with β-actin (**JF273495**) as an internal control, and all data are expressed as the change with respect to the corresponding β-actin calculated ΔCt levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$. Also, to ensure that the primers amplified a specific product, performed a melt curve, and a single melting point was observed for the products of each primer pairs.

Table 2. Primers of GTHs used for QPCR amplification

Genes (accession no.)	Primer	DNA sequences
GTHα	Forward	5′-AAG TCC ATG AAG ACG ATG ACA ATT CC-3′
(**EU908056**)	Reverse	5′-GTG GCA CTG TGT ATG GTT TCT CAC-3′
FSHβ	Forward	5′-AGC GGC GAC TGG TCC TAC G-3′
(**FJ868867**)	Reverse	5′-CGT CTC CAT CAA ACC TCC CAC AG-3′
LHβ	Forward	5′-GGT GTC TCT GGA GAA GGA GGG ATG-3′
(**FJ868868**)	Reverse	5′-TGA ACA GCG TCT TGA TGA CTG GAT C-3′
FSHR	Forward	5′-CCT CTC ATT ACC GTG TCC GAC TC-3′
(**GU722648**)	Reverse	5′-GGG TGA AGA AGG CAT ACA GGA AGG-3′
LHR	Forward	5′-GGA AAC AGA AAT AGA GCC CAC TAC AG-3′
(**GU722649**)	Reverse	5′-CAC TTG ACG AAG GGG TTG TTA AGA C-3′
Vtg	Forward	5′-GAG ATT CTG AAA CAC CTG-3′
(**HM185181**)	Reverse	5′-ACA GCG TCT TGA TGA CTG GAT C-3′
β-actin	Forward	5′-GGA CCT GTA TGC CAA CAC TG-3′
(**JF273495**)	Reverse	5′-GCT GAA ATA ATT CCA CAA ACT T-3′

2.7. Plasma parameters analysis

Plasma E_2 levels were analyzed by RIA usingan E_2 RIA kit (DIASORIN). Duplicate 200 μL aliquots of plasma samples were extracted in 3 mL of ethyl-acetate/cyclohexane (v/v) mixed for 5 min. After 2 h, the tubes were centrifuged for 15 min and frozen in liquid nitrogen. The solvent layer was decanted, transferred into glass tubes, and evaporated under nitrogen. Standards and samples were reconstituted in 150 μL of 0.1 M PBS (pH 7.4) that contained 1 g/mL bovine serum albumin (BSA) and 1 mM EDTA. Samples were incubated in primary antibody (150 μL) for 24 h at 4°C, and ^{125}I estradiol (100 μL) was added to each tube for 24 h at 4°C. The secondary antibody (500 μL of precipitating solution) was added, mixed, and incubated at 4°C for 1 h. Subsequently, 2 mL of 0.025 M Tris buffer (pH 7.4) was added, the tubes were centrifuged at 1,000 ×*g* for 30 min, the supernatant was discarded, and the radioactivity was measured.

2.8. Statistical analysis

All data were analyzed using the SPSS statistical package version 10.0 (SPSS Inc.). A one-way analysis of variance followed by Tukey's post hoc test was used to compare differences in the data ($P < 0.05$). Values are expressed as mean ± SE ($n = 5$).

3. Results

3.1. Production of rGTHs

To study the biological activities and physiological significance of the two cinnamon clownfish GTHs, rLH and rFSH were produced using the pMAL protein fusion and purification system with *E. coli*. The result showed that the concentration of the purified MBP-tagged FSHβ-GTHα and LHβ-GTHα protein was 0.2 mg/mL and 0.3 mg/mL by Bradford assay, respectively. Specific bands corresponding to molecular sizes of 68 kDa for rFSHβ-GTHα and 69 kDa for rLH β-GTHα were obtained, using MBP tagging of *E. coli* BL21 (DE3) pLysS cells resolved by SDS-PAGE (Fig. 9A). Also, anti-MBP antibodies (FSHβ-GTHα and LH β-GTHα) was used to detect the same bands in a western blot analysis (Fig. 9B). In the present study, rLH and FSH were used, *in vivo* and *in vitro* experiments. For *in vitro* experiments, rLH and rFSH were used at 0.1 and 1 μg/mL. These doses correspond to 1.5 and 15 nM, respectively. For *in vivo* experiments, fish were injected with rLH and rFSH at 0.1 and 1 μg/g BM which corresponds to 1.5 and 15 nM/g of BM.

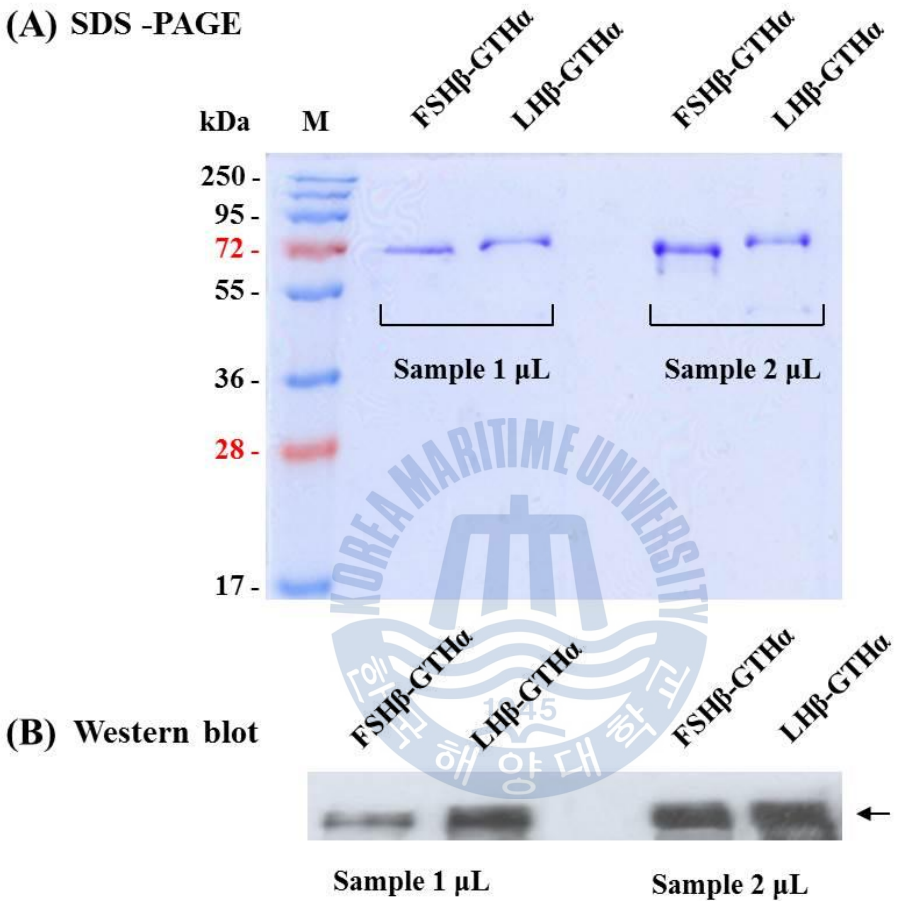

Fig. 9. Production of rFSH and rLH. (A) SDS-PAGE analysis of purified rFSH and rLH. (B) Western blot analysis of purified rFSH and rLH using a MBP-Tag probe. M, protein markers.

3.2. Time and dose-related effects of rLH and rFSH on pituitary GTH subunits mRNA levels, *in vivo*

Pituitary expression levels of GTH subunits (GTHα, FSHβ, and LHβ) were measured following treatment with rLH and rFSH (Fig. 10). The results provide information on time and dose related effects of FSHβ and LHβ and their autoregulatory properties on GTH subunit mRNA levels *in vivo*. Treatments with both rLH and rFSH significantly increased GTHα, mRNA level, although rFSH exerted a greater effect, especially at higher dose tested (Fig. 10A) ($P < 0.05$). Treatments with both rLH and rFSH significantly increased FSHβ, and LHβ mRNA levels. The results demonstrate that rFSH exerted greater stimulation of FSHβ than LHβ mRNA level, and rLH exerted greater stimulation of LHβ than FSHβ mRNA levels (Fig. 10B, C).

3.3. Time and dose-related effects of rLH and rFSH on gonadal LH and FSH receptor mRNA levels, *in vivo*

To further elucidate the autoregulatory effects of LH and FSH, expression of LH and FSH receptor (LHR and FSHR) mRNA levels was measured in the gonadal tissues following treatments of rLH and rFSH, *in vivo* in cinnamon clownfish. Injection with rFSH, significantly increased FSHR after 6 h and the rFSH-induced response at the 15 nM/g BM remained significant even after 48 h of treatment ($P < 0.05$). At the lower dose of rFSH (1.5 nM/g BM) tested, significant increase in FSHR was observed after 12 and 24 h of injection (Fig. 12). Injection with rLH resulted in small but significant increase in FSHR mRNA level at the higher dose tested after 12 and 24 h of administration (Fig. 12A). For LHR, only injection with rLH significantly increased LHR mRNA level, with the exception of very small increase in LHR following 12 h of higher dose of rFSH treatment. In terms of magnitude of response, rLH-induced increase in LHR mRNA level was detected earlier, following 6 h of the higher dose of rLH injection, compared to rFSH-induced increase in FSHR observed after 24 h of injection (Fig. 12).

3.4. Time and dose-related effects of rLH and rFSH on gonadal GTH subunits and receptors mRNA levels, *in vitro*

In this experiment, direct actions of rLH and rFSH on GTH subunits (GTHα, FSHβ, and LHβ) and receptors (FSHR and LHR) mRNA were tested in cultured gonads (Figs. 11 and 13). Overall, treatments with both rLH and rFSH significantly increased all three GTH subunits mRNA levels ($P < 0.05$). Treatments with both rLH and rFSH significantly increased GTHα mRNA level in the cultured gonadal tissue. However, rFSH exerted a greater effect on GTHα after 12 and 48 h of treatment (Fig. 11A). Similar to pituitary, treatment with rFSH exerted greater stimulation of FSHβ than LHβ mRNA level, and rLH treatment exerted greater stimulation of LHβ than FSHβ mRNA levels, although not consistently at all time points (Fig. 11B, C). In all cases, no significant change was observed following treatments with rLH and rFSH after 6 h in cultured cinnamon clownfish gonads (Fig. 11).

Both rLH and rFSH significantly increased FSHR mRNA levels after 12 h of incubation, *in vitro*. However, the magnitude of rFSH-mediated response on FSHR mRNA level was greater for rFSH than rLH at all time points (Fig. 13A). The response for LHR was somewhat different. Treatment with rLH significantly increased LHR after 12 h with maximum effect observed at higher level of rLH after 24 h of incubation. Treatment with rFSH also caused a significant increase in LHR after 24 h, although to a much lesser extent compared to rLH.

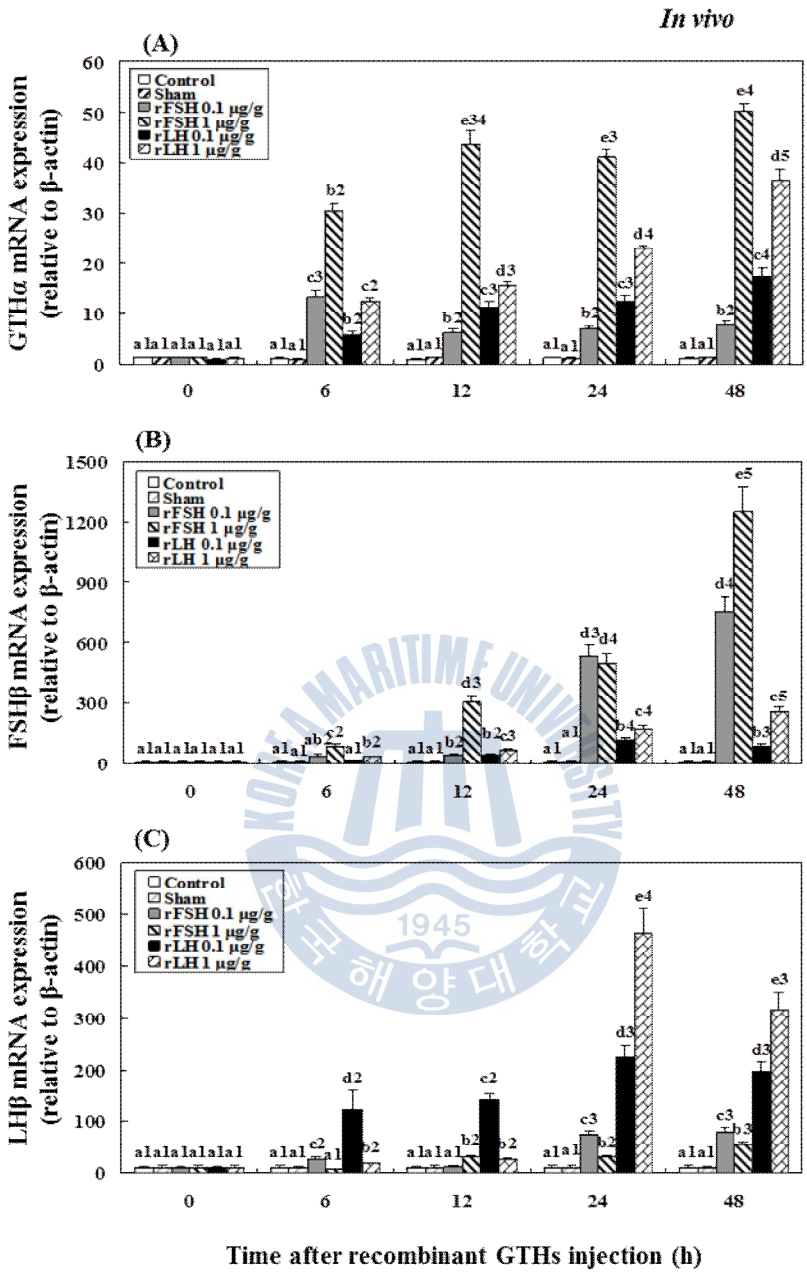

Fig. 10. Time-related effect of rFSH and rLH (0.1 and 1 μg/g) on GTHα (A), FSH β (B), and LHβ (C) mRNA levels in cinnamon clownfish pituitary. Total RNA was extracted at 0, 6, 12, 24, and 48 h after treatment, and 3 μg of RNA was used for PCR. The expression level of each sample was normalized with respect to the β-actin

signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P < 0.05$). All values are mean ± SE ($n = 5$).

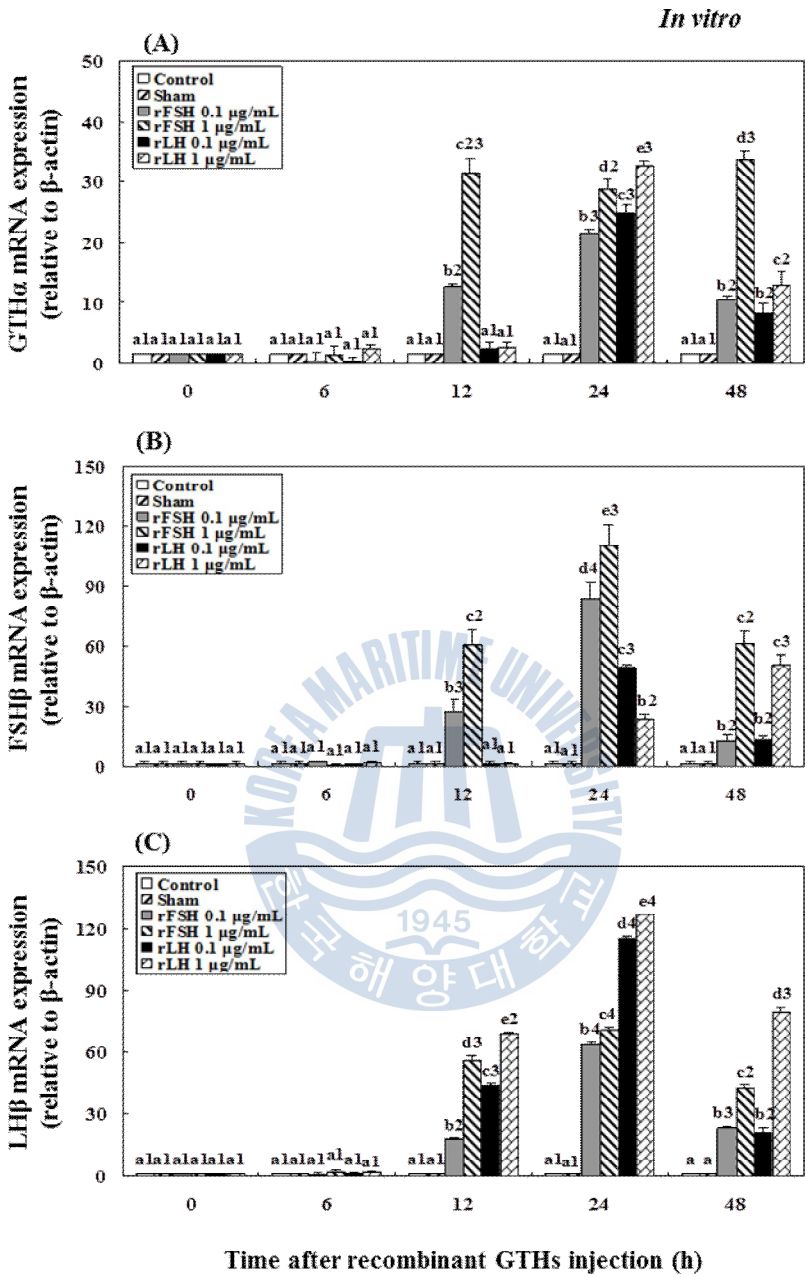

Fig. 11. Time-related effect of rFSH and rLH (0.1 and 1 μg/mL) on GTHα (A), FSHβ (B), and LHβ (C) mRNA levels in cinnamon clownfish cultured gonads. Total RNA was extracted at 0, 6, 12, 24, and 48 h after treatment, and 3 μg RNA was used for the PCR. The expression level of each sample was normalized with respect

to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P < 0.05$). All values are mean ± SE ($n = 5$).

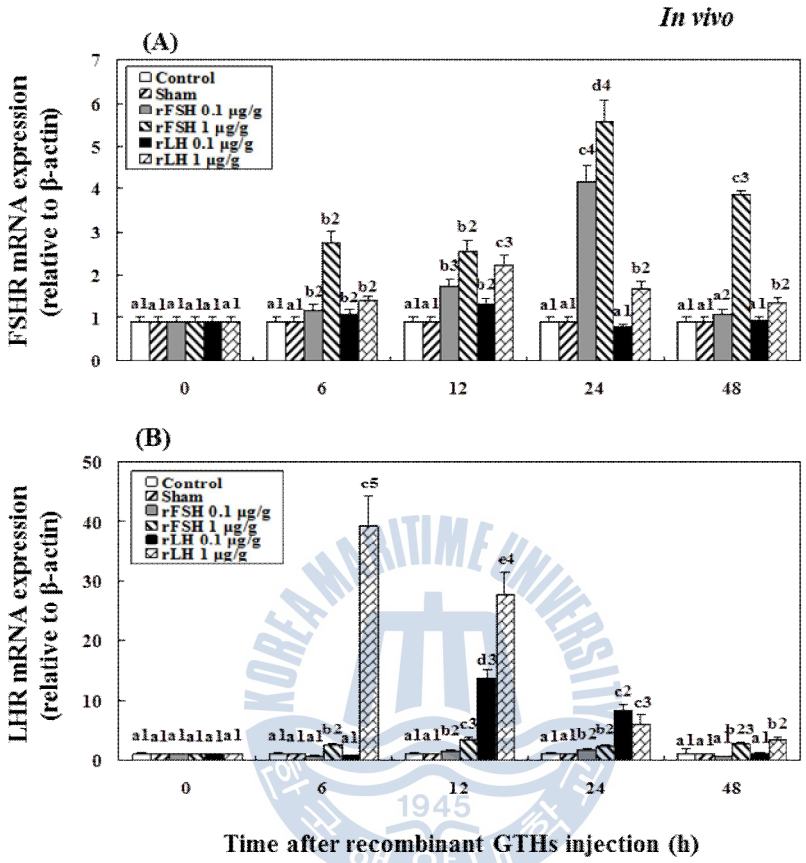

Fig. 12. Time-related effect of rFSH and rLH (0.1 and 1 μg/g) on FSHR (A) and LHR (B) mRNA levels in the cinnamon clownfish gonad. Total RNA was extracted 0, 6, 12, 24, and 48 h after treatment, and 3 μg RNA was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P < 0.05$). All values are mean ± SE ($n = 5$).

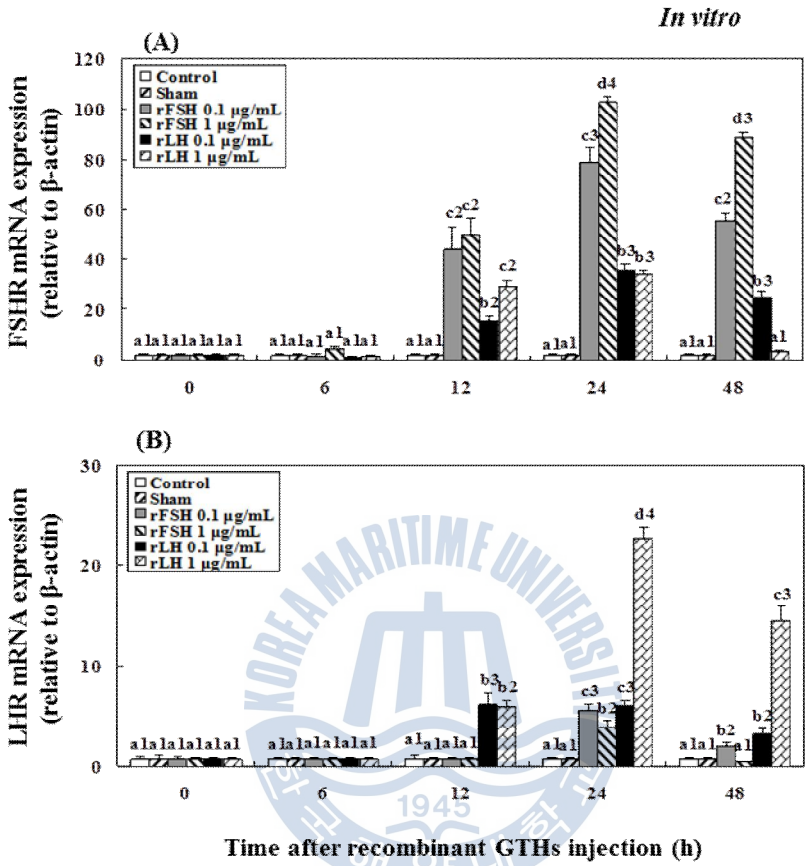

Fig. 13. Time-related effect of rFSH and rLH (0.1 and 1 μg/mL) on FSHR (A) and LHR (B) mRNA levels in cinnamon clownfish cultured gonad. Total RNA was extracted at 0, 6, 12, 24, and 48 h after treatment, and 3 μg RNA was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P < 0.05$). All values are mean ± SE ($n = 5$).

3.5. Time and dose-related effects of rLH and rFSH on liver Vtg mRNA level, *in vivo*

Production of Vtg is estrogen dependent and provides a suitable parameter to estimate efficacy of rLH and rFSH, *in vivo*. Injection with both rLH and rFSH significantly increased Vtg mRHA level after 6 h of treatment (Fig. 14) ($P < 0.05$). rFSH-induced increase in Vtg mRNA level appeared earlier than rLH induced response. After 24 h of injection, however, rLH stimulated Vtg expression to a greater extent than rFSH. After 48 h, only rLH (1.0 μg/g) significantly increased Vtg mRNA level (Fig. 14).

3.6. Time and dose-related effects of rLH and rFSH circulating E_2 concentration, *in vivo*

As a follow up to measuring Vtg production, circulating levels of E_2 was also measured in cinnamon clownfish following injection with rFSH and rLH. Injection with both rLH and rFSH significantly increased circulating E_2 concentration after 6 h ($P < 0.05$). As in Vtg level, the effect of rFSH peaked earlier and the effect of rLH was greater at later time course by 24 h. After 48 h, the rLH and rFSH induced response were lower relative to earlier time points, but remained statistically significant (Fig. 15).

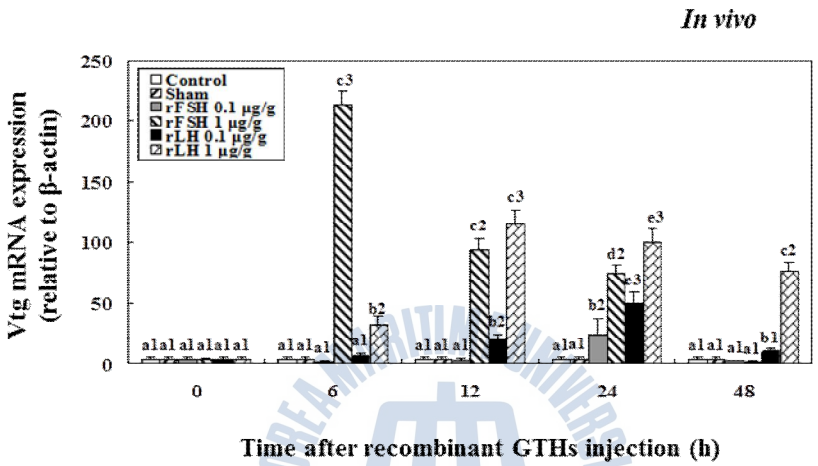

Fig. 14. Time-related effects of rFSH and rLH (0.1 and 1 μg/g) on Vtg mRNA levels in the cinnamon clownfish liver *in vivo*. Total RNA was extracted at 0, 6, 12, 24 and 48 h after treatment, and 3 μg RNA was used for the PCR. The expression level of each sample was normalized with respect to the β-actin signal and is expressed as relative expression level. The numbers were indicated for time after same rGTH injection concentration, and the lower-case letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P < 0.05$). All values are mean ± SE ($n = 5$).

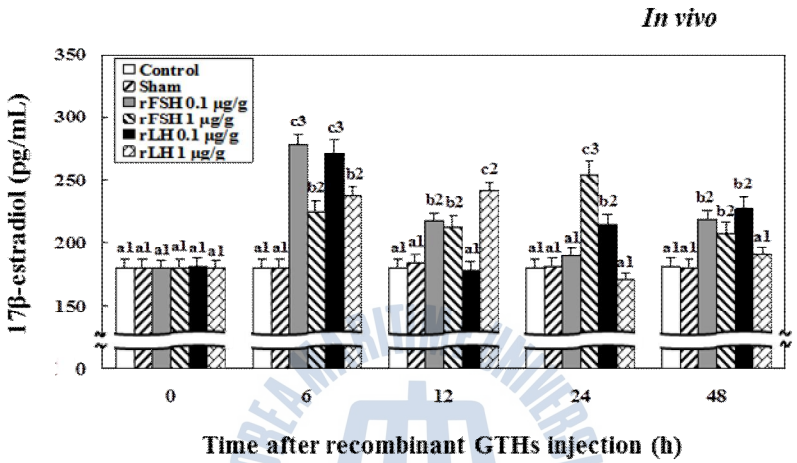

Fig. 15. Plasma E_2 levels during treatment with rFSH and rLH. The numbers were indicated for time after same rGTH injection concentration, and the lowercase letters indicate significant differences between the treated rGTH concentrations at the same time after the rGTH treatment ($P < 0.05$). All values are mean ± SE ($n = 5$).

4. Discussion

Teleosts are among the most diverse species and display a wide range of plasticity in reproduction and gonadal development. There are both hermaphroditic and gonochoristic species that reproduce once a year, once in a lifetime or several times per year. While reproduction of gonochoristic teleosts has been studied extensively over many decades, much less information is available on reproductive biology of hermaphroditic species. Among hermaphroditic teleosts more information is available on reproductive endocrinology of protogynous hermaphrodite teleosts than protandrous species that begins life as a male and then changes into a female at a later stage. Cinnamon clownfish is a protandrous hermaphroditic teleosts, and study of its reproductive biology as a model species would be important to understand evolution and diversity of reproductive endocrinology among teleosts. To better investigate control of reproduction of cinnamon clownfish, molecular characterization of GTH and GTH receptors (An et al., 2010), and GnRH forms (Kim et al., 2012) have been provided at pervious study, respectively. Here, this study was describe a method to produce biologically active rGTHs, rLH and rFSH, which was used to provide novel information on autoregulation of LH and FSH-induced responses. Given the size of these fish, it would be very difficult to extract and purify pituitary LH and FSH, and production of recombinant LH and FSH are the best approach. The present study is also important as it validate previously described approach to produce single chain recombinant LH and FSH (Kobayashi et al., 2010).

Previous studies provided molecular characterization and cDNA sequence for FSHβ and LHβ of cinnamon clownfish, encoding predicted proteins of 119 and 139 amino acids, respectively (An et al., 2010). The three GTH subunits contain cysteine residues and a highly conserved N-linked glycosylation site, which are reportedly sites for receptor binding in mammals and fish (Gen et al., 2000). Phylogenetic analyses suggest that the clownfish GTH subunits are closely related

to those of other teleost species such as Atlantic salmon *Salmo salar* (Ando and Urano, 2005), rainbow trout *Oncorhynchus mykiss* (Sambroni et al., 2007), black porgy (An et al., 2009), and red seabream (Gen et al., 2000).

In the present study, single chain recombinant FSH and LH (rFSH and rLH) were produced to consisted FSHβ-GTHα and LHβ-GTHα, fusion proteins, respectively. Each fusion protein contains N-glycosylated protein, which is important for extending its half-life (Klein et al., 2003). The present results demonstrate that the single-chain rFSH and rLH as heterodimeric fusion proteins are biologically active and exert specific LH and FSH-like activity at least up to 48 h post treatment. This is consistent with pervious report on production of recombinant Manchurian trout FSH and LH (Ko et al., 2007). Overall, there is evidence that pituitary glycoprotein hormones carry highly conserved N-linked glycosylation sites, and the attached oligosaccharides are important for hormone bio-activity in teleosts (Klein et al., 2003).

Production of rFSH and rLH enabled us to investigate autoregulatory control of LH and FSH by investigating expression of pituitary and gonadal GTHα, FSHβ, and LHβ, gonadal LHR and FSHR, liver Vtg mRNA levels as well as circulating E_2 level in cinnamon clownfish. The results indicate that single chain rLH and rFSH are biologically active and exert specific autoregulatory actions by upregulation of GTH subunits in the pituitary and gonads. The present results demonstrate that rFSH has greater potency in up regulating FSHβ transcript level as well as FSHR in cinnamon clownfish. Likewise, rLH was found to have greater potency in up regulating LHβ transcript level as well as LHR. These observations provide novel information on homologous upregulatory mechanisms underlying GTH-mediated control of reproduction in cinnamon clownfish. The results hypothesized to have provided indirect evidence for LH and FSH receptor specificity. The observed autoregulatory mechanism may be particularly important during ovulation to facilitate maximum gonadotropic response to LH and FSH by upregulating LHR and FSHR in gonadal tissue as well as increasing production of GTHs in cinnamon clownfish. The present results are also consistent with previous

observations on specificity of LH and FSH in Coho salmon *Oncorhynchus kisutch* (Miwa et al., 1994), channel catfish *Ictalurus punctatus* (Zmora et al., 2003), and zebrafish *Danio rerio* (So et al., 2005), which suggested that FSH and LH exert their effects on ovarian functions through membrane receptors (GTHR) on the granulosa and theca cells.

The observed action of rFSH in immature clownfish is consistent with the view that FSH is generally involved in the formation of yolk and gametes, and LH is involved in final oocyte maturation and spermiation (Nagahama, 1994; Swanson et al., 2003). The present results are in agreement with previous report that FSH increases significantly at the early maturation stage in red seabream, striped bass, and black porgy (Gen et al., 2000; Hassin et al., 2000; An et al., 2009). Specially, Ko et al. (2007) reported that a single injection with rFSH and rLH significantly increased mean GSI and follicle diameters as compared with those of immature Manchurian trout (Ko et al., 2007).

As expected, plasma E_2 level increased following injection with rLH and rFSH which is consistent with previous studies on goldfish (Kobayashi et al., 2006), and African catfish (Vischer et al., 2003). Also, Ko et al. (2007) suggested that rmtFSH elevated plasma E_2 levels and in turn E_2 stimulated Vtg synthesis and to some extent its uptake into immature ovarian follicles of immature Manchurian trout. The observed increases in GTH-induced E_2 level as well as LHR and FSHR suggest that gonad development is controlled through the combination of GTH and GTH-receptor activity in the gonads. Furthermore, increasing plasma E_2 levels results in oocyte development and maturation in immature teleosts, and plays an important role in sex change from male to female (Lee et al., 2000, 2001; An et al., 2008b, 2009). Thus, rGTH activates the brain–pituitary–gonad axis pathway in cinnamon clownfish.

A likely consequence of increase in rLH, and rFSH-induced circulating E_2 concentration was increased level of Vtg mRNA. The present results provide a better understanding on the mechanisms of LH and FSH stimulated production of E_2 and vitellogenesis which are critical steps in hormonal control of reproduction.

The multistep autoregulatory process may in part explain the observed temporal increase in FSHR mRNA content during early vitellogenesis in other species, including channel catfish (Kumar and Trant, 2004), zebrafish (Kwok et al., 2005), and Manchurian trout (Ko et al., 2007). These previous studies suggest that FSH/FSHR increases during early vitellogenesis and promotes oocyte development, whereas LH/LHR is associated with gonadal maturation and sex change during late vitellogenesis (Rocha et al., 2007).

In summary, the present study provides information on the production of biologically active single chain recombinant cinnamon clownfish LH and FSH. The rLH and rFSH were subsequently used in experiments that provide valuable insight into autoregulatory mechanisms of GTH control of reproduction in cinnamon clownfish. The further investigation will enhance understanding of hormonal control of reproduction in cinnamon clownfish which is used here as a suitable model to investigate reproductive endocrinology in a protandrous hermaphroditic teleost.

Chapter 4.

Upregulation of estrogen receptor subtypes and vitellogenin mRNA in cinnamon clownfish *Amphiprion melanopus* during the sex change process: Profiles on effects of 17β-estradiol

1. Introduction

Estrogen is an essential steroid hormone in reproduction and plays important roles in sexual maturation and differentiation, which include oogenesis, vitellogenesis, and testicular development. In addition, estrogen is a sex steroid hormone that influences growth, gonad sex differentiation, the reproductive cycle and lipid and bone metabolisms (Auchus and Fuqua, 1994; Pakdel et al., 2000). Estrogen action is primarily mediated by nuclear ERs (ERα and ERβ), which function as ligand-dependent transcription factors that regulate transcription of target genes containing the consensus estrogen response element (ERE) in their promoter regions. Also, estrogen exerts its action through a membrane ER, and various signaling pathways (Ca^{2+}, cyclic AMP, protein kinase cascades) are rapidly activated and ultimately influence downstream transcription factors (Zhang and Trudeau, 2006).

Members of this superfamily share several common features that can be divided into 6 distinct domains (Krust et al., 1986; Kumar et al., 1987), including the highly conserved C (DNA-binding domain; DBD) and E (ligand-binding domain; LBD) domains, as well as many variable regions at the N and C termini between the DBD and LBD (the A/B, F and D domains) (Krust et al., 1986). In fish, 3 ER have been identified and characterized: ERα and ERβ1 (Chang et al., 1999; Tchoudakova et al., 1999; Xia et al., 1999; Hawkins et al., 2000; Ma et al., 2000; Pakdel et al., 2000; Patiño et al., 2000; Rogers et al., 2000; Socorro et al., 2000; Huang and Chang, 2002;

Choi and Habibi, 2003; Halm et al., 2004), ERβ2 (Tchoudakova et al., 1999; Ma et al., 2000; Menuet et al., 2002; An et al., 2008b), and ERγ (Hawkins et al., 2000; Halm et al., 2004), which has been cloned and is genetically distinct from the other two.

Induction of Vtg, a precursor yolk protein, in response to estrogens by an ER-mediated pathway is well documented in several oviparous (egg-laying) fish species (Ryffel, 1978; Pakdel et al., 1991), and Vtg in males is widely accepted as a biomarker of exposure to environmental estrogens (Hutchinson and Pickford, 2002; Filby et al., 2006). To date, more than two Vtg transcripts have been discovered in at least 17 teleost species (Hiramatsu et al., 2006).

In this study, cDNAs encoding one type of Vtg have been cloned and appear to belong to the VtgA group (Finn and Kristoffersen, 2007). In largemouth bass *Micropterus salmoides* and Atlantic salmon, the ERα subtype is highly correlated with Vtg mRNA levels in the liver (Sabo-Attwood et al., 2004; Meucci and Arukwe, 2006). However, in cinnamon clownfish, the role of ER subtypes in the regulation of genes such as Vtg *in vivo* and *in vitro* is unknown.

Cinnamon clownfish groups consist of a mated adult pair and an immature individual, and sex is controlled by social rank in the group. The female is largest and is dominant in the group. If the dominant female dies or is absent, the male undergoes a sex change to female (Godwin and Thomas, 1993; Godwin, 1994). Hence, cinnamon clownfish are a good model to study the mechanisms of male to female sex change in protandrous hermaphrodite fish. So far, only studies investigating changes in steroid hormones (Godwin and Thomas, 1993) and gonad histological analyses (Godwin, 1994) have been reported with regard to sex change processes in cinnamon clownfish; neither expression of ER subtypes and Vtg involved in sex change progression nor expression of these genes by steroid hormone treatment has yet been examined. Thus, the aims of this study were to determine the changes in the mRNA expression of the 3 ER subtypes and Vtg during sex change from male to female, to determine the role of E_2 in the changes in ER and Vtg gene expressions and in plasma E_2 levels, and to characterize the interaction between E_2 and these genes in cinnamon clownfish.

2. Materials and methods

2.1. Experimental fish

The cinnamon clownfish [immature (7.1 ± 0.8 g), mature male (10.5 ± 1.2 g), and mature female (22.2 ± 2.1 g)] were purchased from the CCORA (Jeju, Korea). Groups consisting of a mated pair (dominate female and male) were made in 12 of 50-L circulation filter tanks prior to the experiments. The temperature was maintained at 28 ± 0.5°C, and the photoperiod was a 12-h light:12-h dark. Fish were fed a commercial marine aquarium fish feed (Jeilfeed Company, Kyoungnam, Korea) twice a day.

In the present study, the experimental design for sex reversal was a modified version of methods described by Godwin and Thomas (1993). Sex change was induced in male fish by removing their female mate from each group and adding one immature clownfish in its place. Ninety days after the female was removed, the male fish was observed undergo the sex change process from male to female. The fish were anesthetized with MS-222 (200 mg/L, Sigma-Aldrich) prior to blood and tissues collection. Blood was taken from the caudal vasculature using a 1 mL heparinized syringe. After centrifugation (1500 ×*g*, 4°C, 5 min), the plasma was stored at −80°C before analysis, and fish were killed by spinal transection for collection of the pituitary glands and gonads. Pituitary glands and gonads from the fish of the sex change process (I, mature male; II, male at 90 days after removal of the female; and III, mature female) were removed, immediately frozen in liquid nitrogen, and stored at −80°C until total RNA was extracted for analysis.

2.2. E_2 treatment

E_2 (Sigma-Aldrich) was dissolved and diluted in 0.9% physiological saline. After anesthesia, immature fish were injected intraperitoneally of E_2 (0.1 and 1 μg/g BM) at a volume of 10 μL/g BM. After injection, the gonad, liver and bloodwere sampled from the 5 fish at each of the following time periods: 0, 6, 12, 24, and 48 h. Water temperature was maintained at 28 ± 0.5°C during the injection periods.

2.3. Primary hepatic culture

Livers were removed from 5 cinnamon clownfish, and cut by scalpel into 1–3mm^3 pieces were weighed, placed in a 24-well culture plate. Under a sterile hood, the pieces were washed several times with culture medium solution (M199, Invitrogen). The liver pieces were added in equal amounts (approximately 50 mg) to each well of a 24-well plate and a total of 2 mL of fresh culture media was added. The liver pieces were allowed to acclimatize at room temperature with access to ambient air for 2 h, after which the indicted concentrations of E_2 (10^{-6} and 10^{-5} M) were added, with equal volume of distilled water (dH_2O) being added to the control group. The liver pieces were cultured for 0, 6, 12, 24, and 48 h in an incubator 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.

2.4. Total RNA extraction and reverse transcription (RT)

Total RNA from the gonads and liver at each maturity stage during the sex change processwas extracted using the Trizol-method according to the manufacturer's instructions (Gibco/BRL). Concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Total RNA (3 μg) was reverse transcribed in a total volume of 20 μL using an oligo-$d(T)_{15}$ anchor and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at −20°C for use in PCR and QPCR.

2.5. QPCR

QPCR was conducted to determine the relative expression of the ER subtypes (ERα, ERβ1, and ERβ2) and Vtg mRNA using total RNA extracted from the gonads and livers of cinnamon clownfish. Primers for QPCR were designed with reference to the known sequences of cinnamon clownfish as follows (ERα, **HM185179**; ERβ1, **HM185180**; ERβ2, **HM185178**; Vtg, **HM185181**): ERα forward

primer (5′-CTG GGC TGG AGG TGC TGA TG-3′), ERα reverse primer (5′-TCA ACA CAG TCG CCT TCG TTC C-3′), ERβ1 forward primer (5′-GCT TCA GGC TAC CAC TAT-3′), ERβ1 reverse primer (5′-CGG CGG TTC TTG TCT ATA-3′), ERβ2 forward primer (5′-GTC TCG GTT CCG TGA GCT GAA G-3′), ERβ2 reverse primer (5′-GCA CAG ATT GGA GTT GAG GAG GAT C-3′), Vtg forward primer (5′-CGA GAT TCT GAA ACA CCT G-3′), Vtg reverse primer (5′-GCT GAA ATA ATT CCA CAA ACT T-3′), β-actin forward primer (5′-GCG ACC TCA CAG ACT ACC TCA TG-3′), and β-actin reverse primer (5′-AAG TCC AGG GCA ACA TAG CAG AG-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad) and the iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The QPCR conditions were 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, and extension at 72°C for 20 s. Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were normalized to the β-actin calculated Ct level. All analyses were based on the Ct values of the PCR products. Ct level was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve.

2.6. Western blot analysis

Western blots were performed based on the protocols described previously (An et al., 2010). Briefly, the total protein isolated from the gonads of cinnamon clownfish during salinity changes was extracted using protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL PMSF, and 0.15 mg/mL leupeptin, pH 7.4), sonicated, and quantified using the Bradford method (Bio-Rad). Total protein (30 μg) was loaded per lane onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. A protein ladder (PageRuler™ Prestained Protein Ladder, SM0671, Fermentas) was used for reference. Samples were electrophoresed at 80 V through the stacking gel and 150 V

through the resolving gel until the bromophenol blue dye front ran off of the gel. The gels were then immediately transferred to a 0.2-μm polyvinylidene diflouride (PVDF) membrane (Bio-Rad) at 85 V for 1.5 h at 4°C. Thereafter, the membranes were blocked with 5% milk in TBS (pH 7.4) for 45 min followed by washing in TBS. Membranes were incubated with polyclonal rabbit antibody to ERα (dilution 1:1,000, E1528, Sigma-Aldrich) followed by horseradish peroxidase conjugated antirabbit IgG secondary antibody (dilution 1:2,000, Bio-Rad) for 60min. Bands were detected using standard ECL as well as more sensitive ECL systems (ECL Advance; GE Life Sciences) and exposure to autoradiography-sensitive film for 3 min.

2.7. Plasma analysis

Plasma E_2 levels were analyzed by RIA using an E_2 RIA kit (ADALTIS).

2.8. Statistical analysis

The data from each experiment were tested for significant differences using the Statistical Package for the Social Sciences software program (version 10.0; SPSS Inc.). One-way analysis of variance followed by a post hoc multiple comparison test (Newman Keuls multiple range test) was used to compare differences in the data at a significance level of $P < 0.05$. Results are presented as mean ± SE. The inter- and intra-assay CV were calculated as the SE divided by the mean.

3. Results

3.1. Quantification of ERα, ERβ1, ERβ2, and Vtg mRNA expressions during sex change

The ERα, ERβ1, ERβ2, and Vtg mRNA expression changes in the gonads during sex change from male to female by QPCR are shown in Fig. 16. Expressions of ERα, ERβ1, ERβ2, and Vtg mRNA in the gonads were increased as the fish progressed toward the mature female state ($P < 0.05$). Expression of these genes in the livers was similar to that in the gonads (Fig. 17).

3.2. Western blot analysis

The ERα protein corresponding to the predicted size (approximately 66 kDa) for cinnamon clownfish was detected only in the female ovary, and not in males or transitioning fish (Fig. 18).

3.3. Plasma E_2 concentration during sex change

The plasma E_2 level was 182.1 ± 30.2 pg/mL in male with a female present, elevated to 893.1 ± 55.3 pg/mL in the male after removal of the female, and was higher once the male changed to a female (1,192.4 ± 40.4 pg/mL) (Fig. 19) ($P < 0.05$).

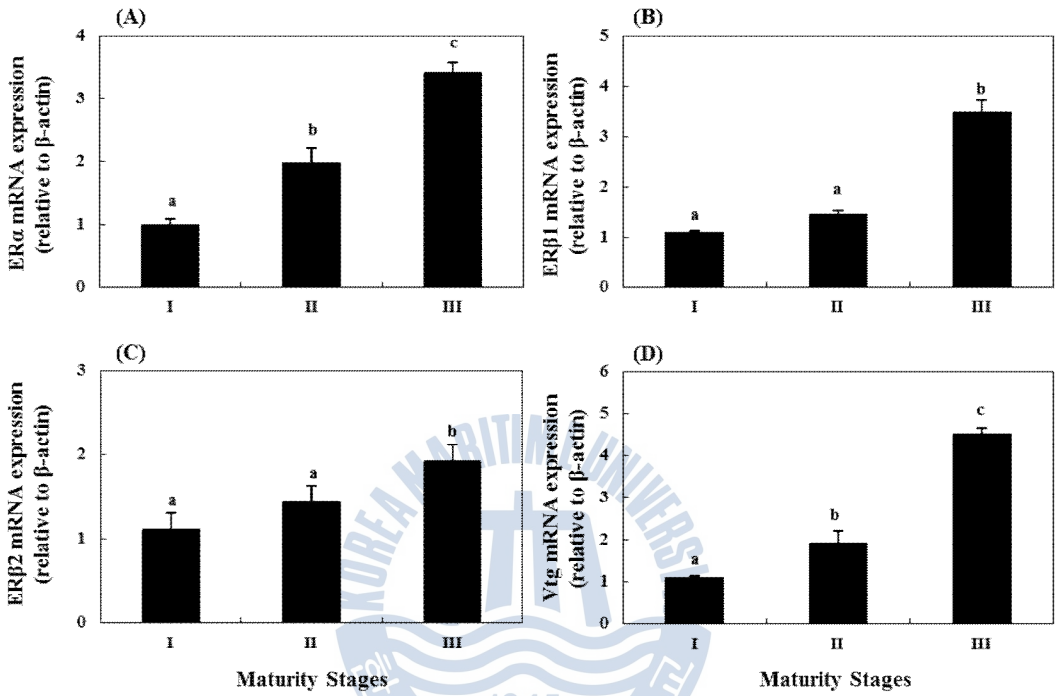

Fig. 16. Expressions of ERα (A), ERβ1 (B), ERβ2 (C), and Vtg (D) mRNA in the gonads of cinnamon clownfish using QPCR. I: mature male; II: male at 90 days after removal of the female; III: mature female. Total RNA (3 μg) prepared from the gonads was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P < 0.05$). All values are mean ± SE ($n = 5$).

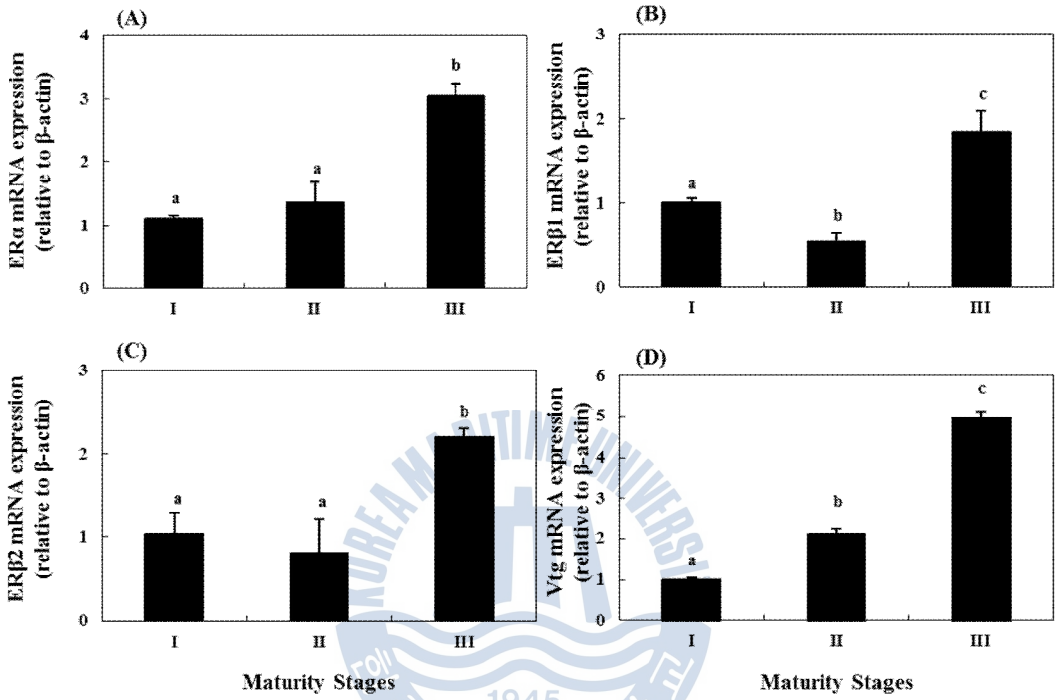

Fig. 17. Expressions of ERα (A), ERβ1 (B), ERβ2 (C), and Vtg (D) mRNA in the livers of cinnamon clownfish using QPCR. I: mature male; II: male at 90 days after removal of the female; III: mature female. Total RNA (3 μg) prepared from the livers was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P < 0.05$). All values are mean ± SE ($n = 5$).

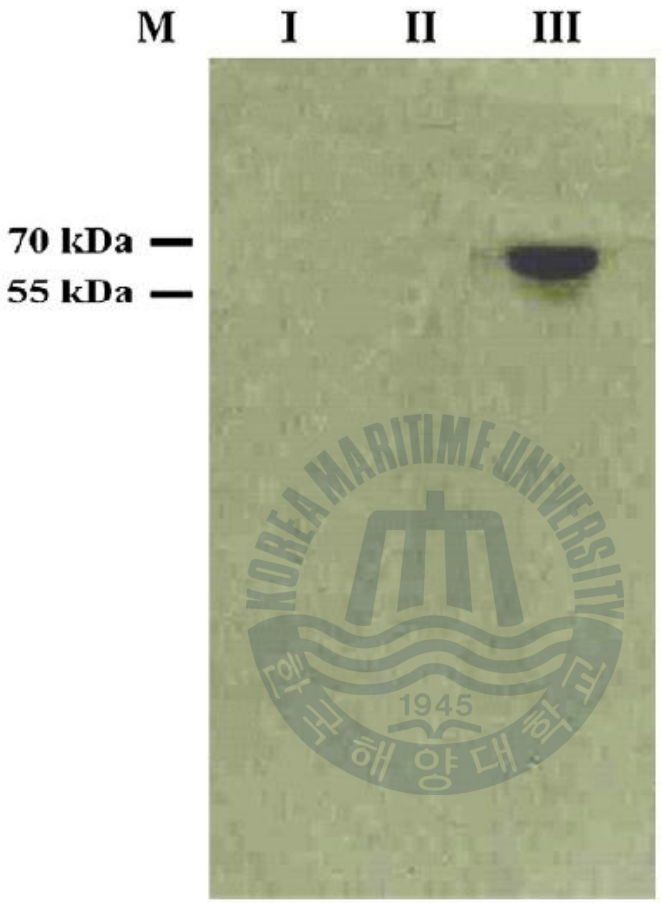

Fig. 18. Western blot of ERα protein (approximately, 66 kDa) expression in cinnamon clownfish gonads during sex change. M: protein marker; I: mature male; II: male at 90 days after removing the female; III: mature female.

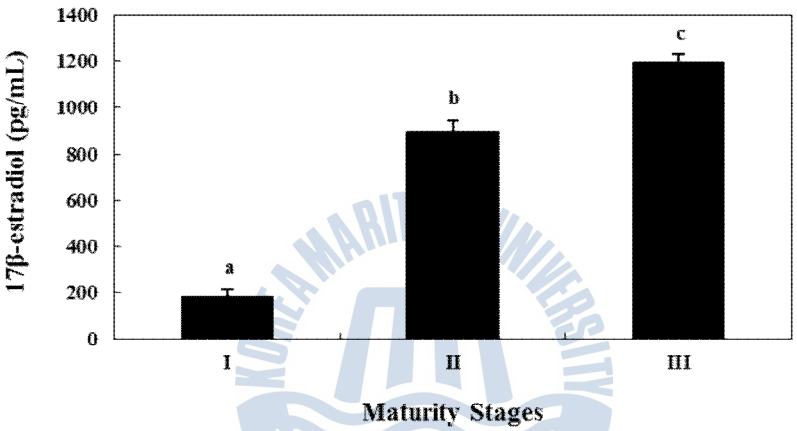

Fig. 19. Plasma E_2 levels during sex change in cinnamon clownfish. I: mature male; II: male at 90 days after removal of the female; III: mature female. Values with dissimilar letters are significantly different ($P < 0.05$). All values are mean ± SE ($n = 5$).

3.4. Quantification of ERα, ERβ1, ERβ2, and Vtg mRNA expressions by E_2 *in vivo*

The ERα, ERβ1, ERβ2, and Vtg mRNA expression changes in the gonads by E_2 injection are shown in Fig. 20. The ER mRNA expression was highest at 12 h after injection (ERα: approximately 20-fold; ERβ1: 12.1-fold, and ERβ2: 15.4-fold, respectively, at 1 μg/g E_2) and then decreased, and Vtg mRNA continuously increased during the experimental period. The ERα mRNA expression was highest in the liver at 6 h after injection (approximately 6-fold at 1 μg/g E_2) and then decreased, and the ERβ1 mRNA expression increased up to 24 h and then decreased at 48 h. The ERβ2 was highest at 12 h (approximately 2.5-fold, at 1 μg/g E_2), and Vtg mRNA increased at 12 h (7.5-fold, at 1 μg/g E_2) and then decreased (Fig. 21) ($P < 0.05$).

3.5. Plasma E_2 concentration by E_2 injection

The plasma E_2 level was 35.9 ± 5.2 pg/mL at the start of the experiment, increased to 375.5 ± 15.3 pg/mL at 24 h (0.1 μg/g E_2) and 560.4 ± 40.1 pg/mL at 24 h (1 μg/g E_2), and then decreased (Fig. 22) ($P < 0.05$).

3.6. Quantification of the ERα, ERβ1, ERβ2, and Vtg mRNA expressions by E_2 *in vitro*

The expression changes of ERα, ERβ1, ERβ2, and Vtg mRNA by QPCR are shown in Fig. 23. The expression of ER mRNA was highest at 12 h (ERα: approximately 58.4-fold, ERβ1: 13.7-fold, and ERβ2: 17.6-fold, respectively at 10^{-5} M E_2) and then decreased, and Vtg mRNA was highest at 12 h (approximately 25.2-fold, at 10^{-5} M E_2) and then decreased ($P < 0.05$).

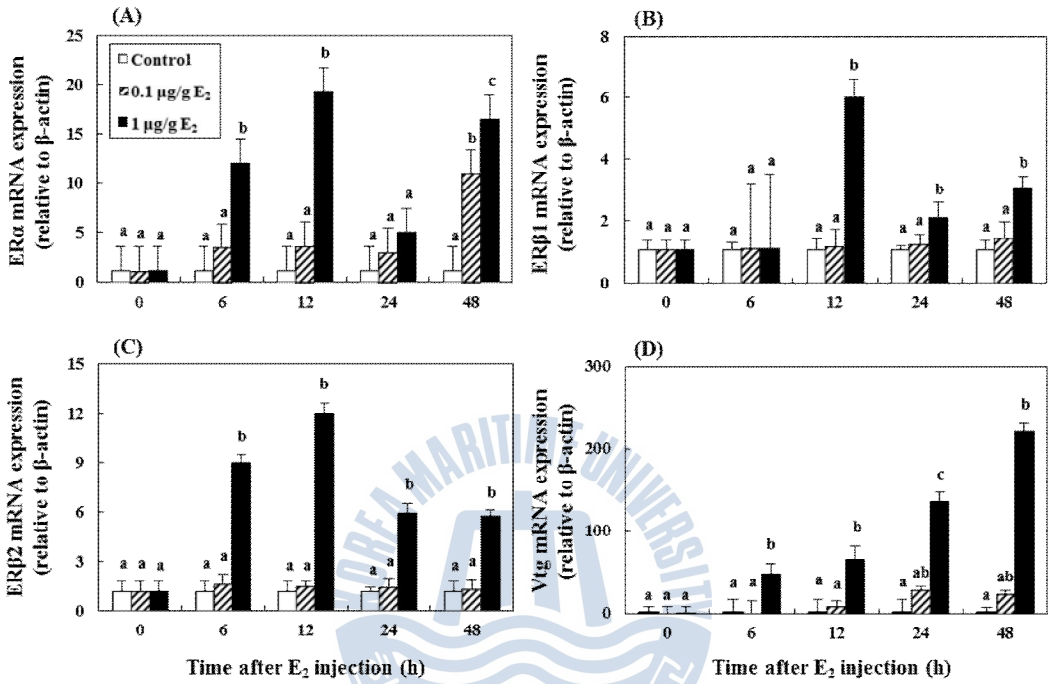

Fig. 20. Expressions of ERα (A), ERβ1 (B), ERβ2 (C), and Vtg (D) mRNA in the gonads of cinnamon clownfish using QPCR after E_2 injection. Total RNA (3 μg) prepared from the gonads was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P < 0.05$). All values are mean ± SE ($n = 5$).

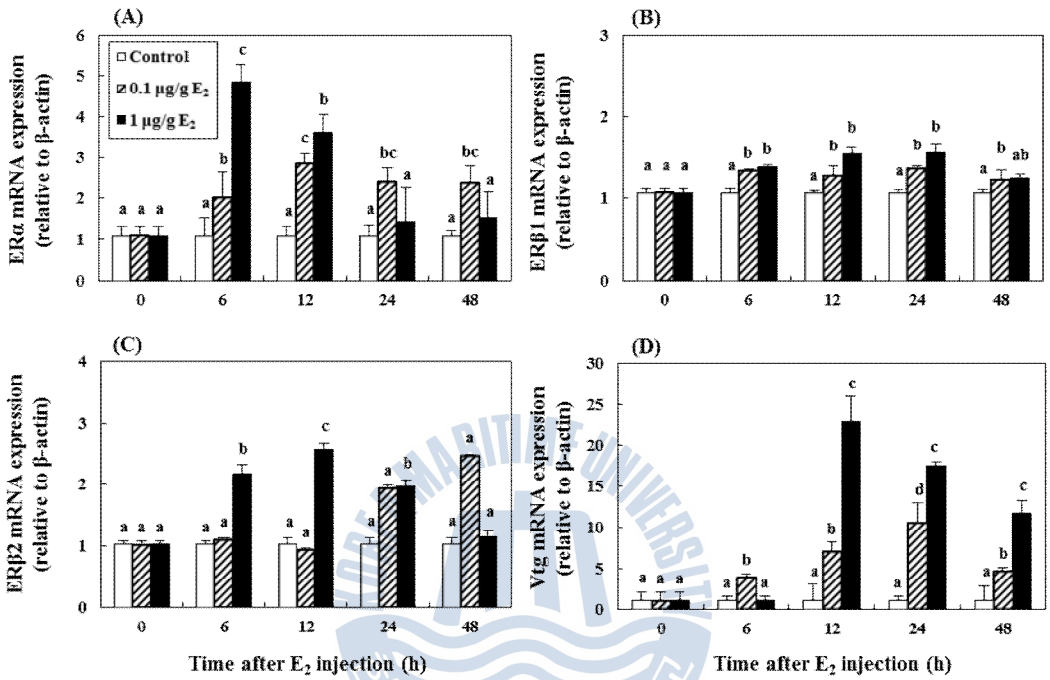

Fig. 21. Expressions of ERα (A), ERβ1 (B), ERβ2 (C), and Vtg (D) mRNA in the livers of cinnamon clownfish using QPCR after E_2 injection. Total RNA (3 μg) prepared from the livers was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P < 0.05$). All values are mean ± SE ($n = 5$).

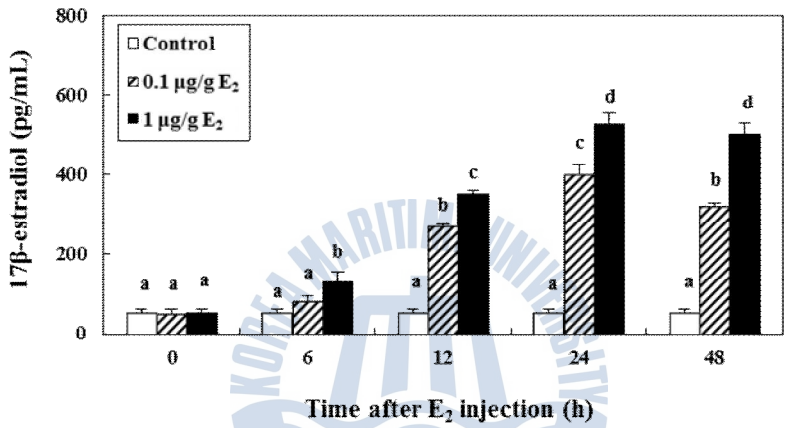

Fig. 22. Plasma E_2 levels after E_2 injection in cinnamon clownfish. Values with dissimilar letters are significantly different ($P < 0.05$) from each other. Values indicate mean ± SE ($n = 5$).

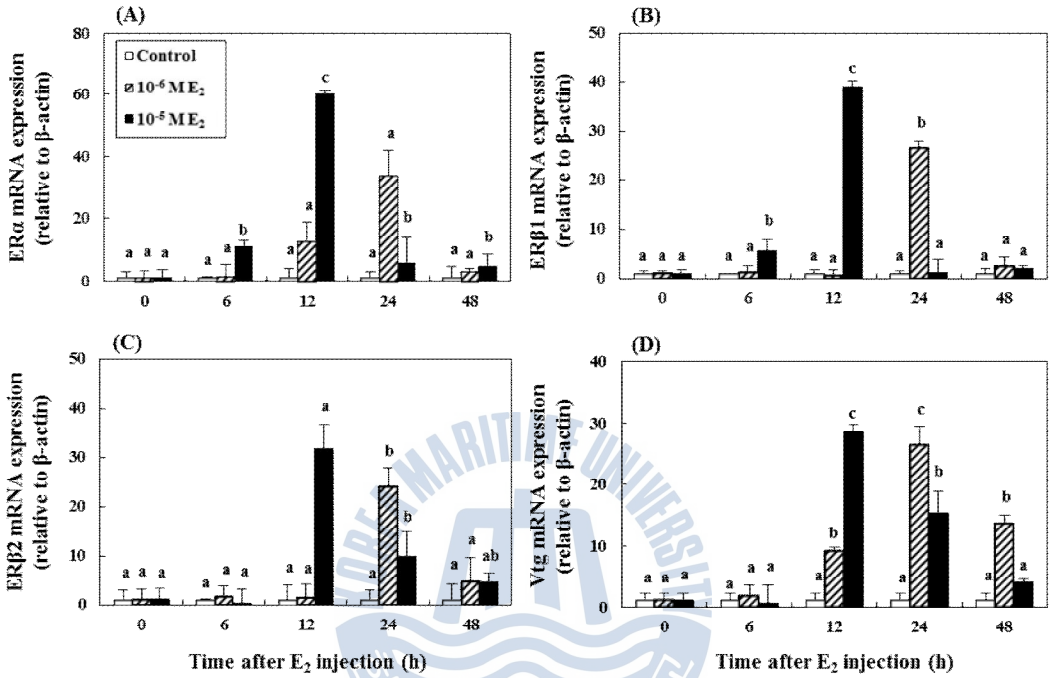

Fig. 23. Expressions of ERα (A), ERβ1 (B), ERβ2 (C), and Vtg (D) mRNA in hepatic cultures of cinnamon clownfish using QPCR after E_2 injection. Total RNA (3 μg) prepared from hepatic cultures was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels of the same sample. Values with dissimilar letters are significantly different ($P < 0.05$). All values are mean ± SE ($n = 5$).

4. Discussion

In the present study, ER subtype and Vtg mRNA expression and plasma E_2 levels was examined during a gonadal sex change from male to female in cinnamon clownfish. Also, to understand the role of ER and Vtg genes in the gonads and livers, the expression of ER subtypes and Vtg mRNA and plasma E_2 levels was examined after E_2 treatment (*in vivo* and *in vitro* experiments) in the gonads and livers of cinnamon clownfish.

During the sex change process of cinnamon clownfish, ER subtypes and Vtg mRNA expressions was increased in the gonads and livers of fish that changed to female (Figs. 16 and 17). In addition, ERα protein in western blot analysis was only detected in the female ovary (Fig. 18), and plasma E_2 was highest in females during the sex change (Fig. 19); these data supported the expression data. Generally, ER and Vtg genes are involved in development and maturity of oocytes in fish, as well as E_2 (Nagahama et al., 1995; Tyler and Sumpter, 1996). It has been reported that ER and Vtg are involved in maturity of oocytes after synthesis of Vtg by binding E_2 and ER in the liver (Flouriot et al., 1996; Bowman et al., 2002; Davis et al., 2009), so it can be concluded that oocytes developed as a result of increasing ER and Vtg mRNA levels during sex change to female in cinnamon clownfish. The ER were detected in the males, which agreed with the studies that ER were detected in the testes and sperm of rainbow trout and channel catfish, implying that E_2 and ER are important in the regulation of gametogenesis in both sexes (Bouma and Nagler, 2001; Wu et al., 2001).

To study the role of E_2, ER and Vtg mRNA expressions and the levels of plasma E_2 was investigated after intraperitoneal injection of E_2 in cinnamon clownfish. The ER and Vtg mRNA expressions in the gonads tended to increase (Fig. 20). This result concurred with studies finding that expression of ER mRNA was increased by E_2 injection in goldfish gonads (Nelson et al., 2007; Marlatt et al., 2008). Among ER subtypes, ERα had the highest levels after E_2 injection, and

previous studies demonstrated that ERα was important for the synthesis of Vtg after E_2 injection (Sabo-Attwood et al., 2004; Marlatt et al., 2008). In the present study, ERα mRNA was highest, and it was thought that ERα affected Vtg synthesis in cinnamon clownfish. Generally, although Vtg was mainly synthesized in the liver, Vtg was detected in the testes of various fish including tilapia *Oreochromis mossambicus*, seabream *Sparus auratus*, and zebrafish (Wang et al., 2005; Pinto et al., 2006; Davis et al., 2008). In previous studies, ERα, ER2, and Vtg mRNA increased in the testes of male tilapia by injection of E_2 (Davis et al., 2008), and the increases of Vtg mRNA seen in the testes of seabream after E_2 injection (Pinto et al., 2006) were similar to present results. Also, Chang et al. (1995) and An et al. (2009) mentioned that using protandrous black porgy, the plasma E_2 level was 9.4 ± 0.7 pg/mL at the start of the experiment, increased to a maximum of 260.1 ± 23.54 pg/mL after 3 days, and then decreased to 96.0 ± 13.2 pg/mL after 9 days by E_2 injection (1.5 μg/g) in black porgy. An et al. (2008b) found that the maximum plasma E_2 level (3 days; 260.1 ± 23.54 pg/mL) following E_2 injection was lower than that of mature female black porgy (705.6 ± 70 pg/mL) during sex change determined in this previous study. These data suggest that the plasma E_2 concentration attained following the exogenous administration of E_2 did not affect sex change but only temporarily increased plasma levels in immature black porgy. Therefore, these results combined with these previous reports suggest that E_2 is involved in ovarian maturation as well as the male to female sex change in cinnamon clownfish.

As in the gonads, expressions of the 3 ER and Vtg mRNA in the livers of immature cinnamon clownfish increased (Fig. 21). Marlatt et al. (2008) reported increased ERα mRNA expression in the livers of male goldfish after E_2 injection, and Soverchia et al. (2005) also found increased ERβ1 mRNA expression in the livers of immature goldfish by E_2, results that are both similar to present study. Expression of Vtg mRNA in the livers of cinnamon clownfish increased in the present study, which is in agreement with previous studies that found significantly increased expression of Vtg mRNA in the livers by E_2 in goldfish (Marlatt et al.,

2008) and tilapia (Davis et al., 2008; Esterhuyse et al., 2009). E_2 may first increase the plasma levels of E_2 and the expression of ER mRNA in the gonads and then induce ER and Vtg expressions in the liver (Flouriot et al., 1996; Bowman et al., 2002). Expression of ER mRNA was higher after treatment with high-dose E_2 at an early hour (1 μg/g) than after treatment with low-dose E_2 (0.1 μg/g), indicating that E_2 has a dose-dependent effect, in this study. This result agreed with the previous study in which liver ERα was induced in a dose dependent manner with a single E_2 injection (from 0.5 to 2.5 mg/kg) in male largemouth bass (Sabo-Attwood et al., 2004). In addition, gonadal histology was examined during sex change in cinnamon clownfish in previous study (An et al., 2010). As a results, the oocytes was found to had been developed and the testicular tissue were regressed from male to female.

Considering the previous studies and the present study, it is believed that the expression pattern of ER subtypes is dependent upon the maturity stage, hormone treatment, hormone concentration, and duration of hormone exposure.

Moreover, the ER and Vtg mRNA expressions were elevated after primary hepatic culture in cinnamon clownfish (Fig. 23). High levels of Vtg mRNA were detected in a high concentration of E_2 (1 μg/g), which agreed with Esterhuyse et al. (2009) who demonstrated that the expression of Vtg mRNA was high in a dose-dependent manner after treatment with E_2 (0.5, 1.0, and 60 μg/L E_2) in the livers of tilapia. Therefore, the study judged that the increased expressions of ER and Vtg in the gonads and livers may be because E_2 was a regulator of these genes in cinnamon clownfish.

In brief, this study found that: 1) increased ER and Vtg mRNA expressions during sex change from male to female in cinnamon clownfish, and 2) increased gene mRNA expression by E_2 injection in immature cinnamon clownfish, indicating that E_2 induces the increased expressions of ER and Vtg mRNA. As a result, it was concluded that E_2 is the regulator of ER and Vtg in cinnamon clownfish.

Chapter 5.

Molecular cloning and expression of caspase-3 in the protandrous cinnamon clownfish *Amphiprion melanopus* during sex change

1. Introduction

Apoptosis is a form of cell death characterized by a number of biochemical and morphological changes, including DNA fragmentation, formation of apoptotic bodies, and a minimal inflammatory response (Kerr et al., 1972; Hacker, 2000) in vertebrate. In most cases, the apoptotic process converges into the activation of caspases, a family of cysteine-dependent aspartate-specific proteases that are present in the cell as proenzymes (Earnshaw et al., 1999). Members of the caspase family share similarities in amino acid sequence and structure, and can be broadly divided into two groups: (1) those related to caspase-1 (caspases-1, 4, 5, 11, 12, 13, and 14), whose primary role appears to be in cytokine processing during inflammatory responses, and (2) apoptosis initiators or effectors (caspases-2, 3, 6, 7, 8, 9, and 10).

In particular, caspase-3 appears to be a key protease in the apoptotic pathway (Porter and Janicke, 1999) and the most characterized effector caspase. Activated caspase-3 stimulates apoptosis by cleaving and inactivating a number of molecules, leading to cellular characteristics and morphological changes typically seen in apoptotic cells (Fischer et al., 2003). The resultant caspase-3 activates an endonuclease (caspase-activated deoxyribonuclease), which is partially responsible for the low-molecular-weight pattern of DNA fragmentation observed during apoptosis (Enari et al., 1998; Sakahira et al., 1998). In addition, caspase-3 levels were reported to increase in follicles undergoing atresia (Boone and Tsang, 1998). Thus, caspase-3 seems to be involved in several cell death processes in the ovary.

Teleosts rely heavily on various environmental cues, such as temperature and

light, during spawning (Billard, 1986), and leads to gonadal regression, a process that may occur via the induction of apoptosis. In particular, apoptosis due to hormones and other chemicals that occurs during embryonic development and metamorphosis is involved in cell death (Corriero et al., 2009). Furthermore, testicular apoptosis is also known to occur during normal spermatogenesis in both mammals (Hong et al., 2008) and fish (Prisco et al., 2003), and it is thought to be essential for maintaining the correct ratio between sertoli cells and gametes during this process (Lee et al., 1997). During the gonadal development of juvenile zebrafish, all animals initially contain undifferentiated ovary-like gonads, regardless of their genotypic sex (Takahashi, 1977). Apoptosis mediates disappearance of oocytes within gonads as they change from ovary-like tissue to phenotypic testes in developing juvenile zebrafish (Uchida et al., 2002). There is evidence that gonadal apoptosis is mediated by both GTHs and locally produced hormones such as gonadal steroids (Billig et al., 1996) and peptides. In particular, GnRH was shown to induce apoptosis in the testis of mature goldfish (Andreu-Vieyra and Habibi, 2001). GnRH, not a circulating hormone, plays a central role in the regulation of gonadal maturation and reproduction in fish and other species (Kavanough et al., 2008). In gonad of gilthead seabream, changes in the endogenous GnRH transcripts were shown to be associated with sex change and gonadal differentiation, and treatment with GnRH stimulate apoptosis (Soverchia et al., 2007). There is evidence that steroid hormones may protect ovary and testis against apoptosis. In this context, in rainbow trout, apoptosis of preovulatory ovarian follicles can be suppressed by the administration of partially purified GTH, E_2, or epidermal growth factor (Janz and Van Der Kraak, 1997).

Social group of cinnamon clownfish typically consist of a mated adult pair and an immature individual, and the sexes of the fish are controlled by the social rank in the group. In general, the female is the largest of these fish and is dominant in the group. However, if a dominant female dies or is absent, the male partner undergoes a sex change to female (Godwin and Thomas, 1993; Godwin, 1994). Hence, cinnamon clownfish is a good model for studying the mechanism of sex change in

protandrous hermaphroditic fish. To date, researchers have investigated the changes in steroid hormones (Godwin and Thomas ,1993), the expression of maturation or differentiation genes (Kim et al., 2010), and the changes in gonads histology (An et al., 2010) that occur during sex change processes in cinnamon clownfish, but no studies have been reported on apoptosis involved in these processes.

To investigate the roles of caspase-3 in cinnamon clownfish sex change processes, cDNAs of caspase-3 was isolated from cinnamon clownfish and its mRNA level, activity, and proteins expression was studied during sex change process. In addition, the effects of GnRH analogue (GnRHa) on caspase-3 mRNA expression and activity was investigated in immature cinnamon clownfish.

2. Materials and methods

2.1. Experimental fish

This study was conducted on different subtypes of cinnamon clownfish, including mature males (I, 10.5 ± 1.2 g), males 90 days after removing females (II, 15.2 ± 0.9 g), and mature females (III, 22.2 ± 2.1 g). Fish were purchased from the CCORA (Jeju, Korea). Each group consisted of a mated pair (dominant female and male). Moreover, each group was reared in one of fifteen 50-L circulation filter tanks prior to inclusion in the experiments. The water temperature was maintained at 28 ± 0.5°C, and the photoperiod was a 12-h light:12-h dark. Fish were fed a commercial marine aquarium fish feed (Jeilfeed Co., Kyoungnam, Korea) twice a day. The experimental design used for sex change in the present study was next. Briefly, after random assignment of the groups, each consisting of a mated pair with a dominant female and a male, sex change was induced in each male fish by removing the paired female from each group and adding an immature clownfish for pairing with the male in experimental condition. The male fish was observed undergoing the sex change process from male to female at 90 days after removing the paired females. The process of sex change was divided into three developmental stages as follows: (I) mature male; (II) male at 90 days after removing female; and (III) mature female. The fish were anesthetized in tricaine methanesulfonate (MS-222; Sigma-Aldrich) and decapitated prior to tissue collection. Gonads from the fish at each gonad maturity stage were removed, immediately frozen in liquid nitrogen, and stored at −80°C until the total RNA was extracted for analysis.

2.2. cDNA cloning of caspase-3

Partial fragments of the caspase-3 gene were amplified as follows: Primers for caspase-3 were designed using highly conserved regions of European seabass *Dicentrarchus labrax* caspase-3 (**DQ345773**), large yellow croaker *Larimichthys*

crocea caspase-3 (**EU878546**), pufferfish *Takifugu rubripes* caspase-3 (**NM1032699**), and Atlantic salmon caspase-3 (**BT059710**), leading to caspase-3 forward primer (5′-CGA CCA GAC AGT GGA GCA AAT G-3′) and caspase-3 reverse primer (5′-A GA TCG GTG CCT CTG CAA G-3′).

Total RNA was extracted from the gonad by using the TRIzol kit (Gibco/BRL), which is free of DNA contamination. Reverse transcription (RT) was conducted using M-MLV reverse transcriptase (Promega), and PCR amplification was performed using 2×Taq Premix I (Solgent) according to the manufacturer's instructions. RT was carried out at 42°C for 50 min. PCR was subsequently performed for 40 cycles as follows: 1 cycle of denaturation at 94°C for 2 min, denaturation at 94°C for 20 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min, followed by 1 cycle of final extension at 72°C for 7 min. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide. The PCR products were purified and subsequently cloned into a pGEM-T Easy Vector (Promega). The colony formed by transformation was cultivated in DH5α cells (RBC Life Sciences). The plasmid DNA was subsequently extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo Genetech), and the insert DNA was separated using EcoRI (Fermentas). Based on the plasmid DNA, caspase-3 cDNA sequences were analyzed using an ABI DNA Sequencer (Applied Biosystems).

2.3. Rapid amplification of caspase-3 cDNA 3′ and 5′ ends (3′- and 5′-RACE)

Total RNA was extracted from gonads using a TRIzol kit (Gibco/BRL) 3 μg of total RNA as a template, and 3′-RACE cDNA and 5′-RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (SeeGene). First-strand cDNA synthesis was conducted using an oligo-d$(T)_{18}$ anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA T$(T)_{18}$-3′) and a CapFishing™ adaptor (SeeGene). Caspase-3-specific primers were selected from the PCR product obtained from RT-PCR. For 3′-RACE, the 5 μL PCR mixture contained 5 μL of 3′-RACE cDNA, 1 μL of 10 mM 3′-RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 mM

3′-RACE caspase-3-specific primer (5′-ATG ACT CCT GCG ATT ACA CGT GTC GTC-3′), and 25 μL of SeeAmp Taq Plus master mix (SeeGene). PCR was performed for 40 cycles as follows: 1 cycle of denaturation at 94°C for 5 min, denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 60 s, followed by 1 cycle of final extension at 72°C for 5 min. For 5′-RACE, the 50 μL PCR mixture contained 5 μL of 5′-RACE cDNA, 1 μL of 10 mM 5′-RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mM 5′-RACE caspase-3-specific primer (5′-TCA TGG TGT TCC TCC ACG AGT AGT AGC-3′), and 25 μL of SeeAmp Taq Plus master mix (SeeGene). PCR was performed using the protocol described above for 3′-RACE. Amplified PCR products were processed by electrophoresis using 1% agarose gels containing ethidium bromide. The transformation and sequencing were performed using the same method as described above in cDNA cloning of caspase-3 section.

2.4. Sequence data analyses of caspase-3

The predicted amino acid sequences of caspase-3 in cinnamon clownfish were deduced using Expasy Translate Tool (http://www.expasy.org) and that was modified from the methods of Monteiro et al. (2009). The nucleotide and protein full sequences were compared to sequences available in the GenBank database retrieved using the BLAST program, and multiple alignment were made using the CLUSTALW program. Caspase-3 domains and possible N-glycosylation sites were based on PROSITE predictions (www.expasy.org/prosite). The percentages of similarity and identity were calculated by pair-wise alignments by the program needle at www.ebi.ac.uk, with first and extending gap of 10 and 0.5, respectively.

2.5. Phylogenetic analysis of caspase-3

Caspase-3 phylogenetic analysis was conducted using known vertebrate caspase-3 amino acid sequences aligned using BioEdit software (Hall, 1999). The phylogenetic tree was constructed using the neighbor- joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics).

2.6. Production of caspase-3 polyclonal antibody

To obtain the antigen of the cinnamon clownfish caspase-3 antibody, a synthetic peptide was synthesized at Cosmo Genetech and was coupled to BSA. The peptide chain was designed against highly conserved regions of caspase-3 amino acid sequences of cinnamon clownfish and other teleosts and caspase family (Fig. 24; 182–198; CGIETDSGEDTTKIPVE-C). A rabbit was injected with 100 μg of BSA-conjugated synthetic peptide in Freund's complete adjuvant and boosted at 2-week intervals by subcutaneous injection of 200 μg of BSA-conjugated synthetic peptide in Freund's incomplete adjuvant. The rabbit was bled 1 week after the fifth injection. Antiserum was purified with affinity peptide column coupling using the same peptide as described above (Lu et al. 2000). This antibody recognizes full-length cinnamon clownfish caspase-3 that obtain bands in the 32 kDa. The titer and specificity of the purified antibody were tested with enzyme-linked immunosorbent assay (ELISA) and western blotting analyses.

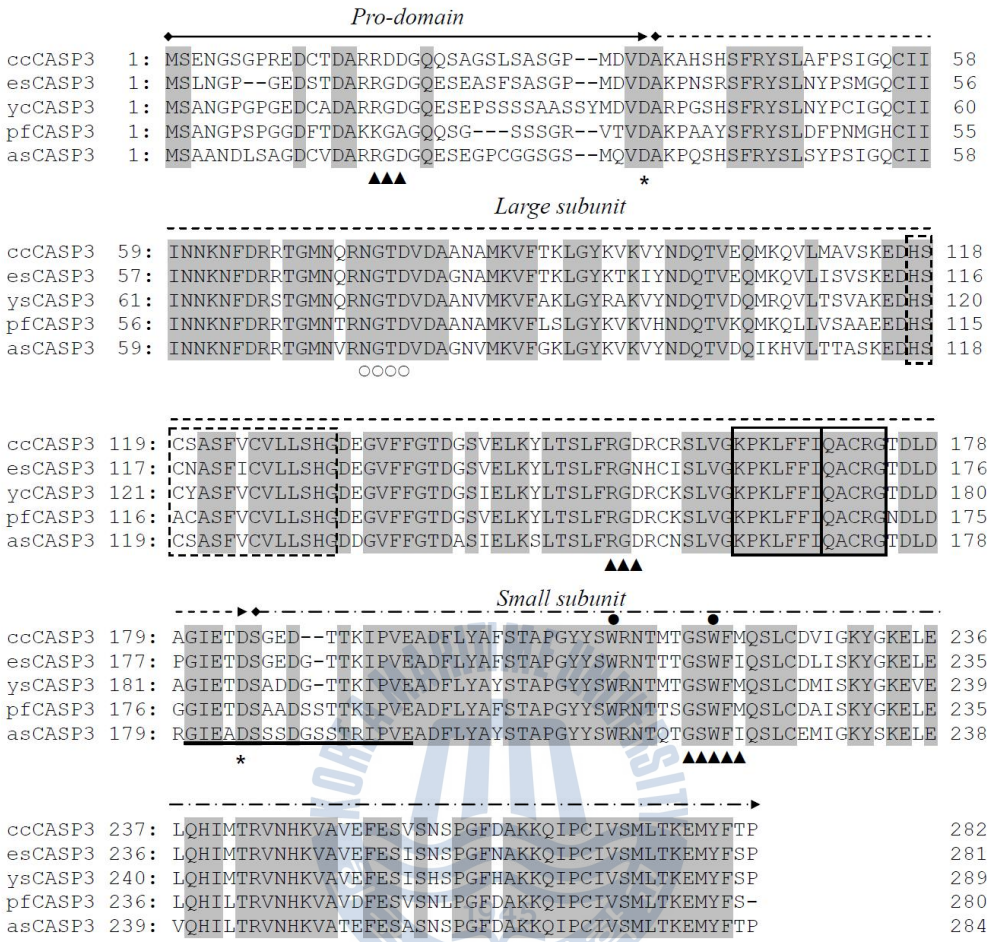

Fig. 24. Alignment of the amino acid sequences of caspase-3 from the cinnamon clownfish (ccCASP3, **JF345174**), European seabass (esCASP3, **DQ345773**), yellow croaker (ycCASP3, **EU878546**), pufferfish (pfCASP3, **NM001032699**), and Atlantic salmon (asCASP3, **BT059710**). The shades indicate amino acids that are identical to those in the first line, which correspond to the ccCASP3. The dashes indicate gaps inserted to enhance sequence similarity. The pentapeptide active-site motif (QACRG) and the caspase family signature are boxed in continuous and discontinuous lines, respectively. The cysteine active site of the caspase family is boxed in a continuous line, and N-glycosylation site (NGTD) is in open circles. The protein-binding domain (GSWFM) and RGD motifs are in filled triangles. The

putative cleavage site at the aspartic acid residues, which separates the pro-domain (right arrow) from the large subunit (right arrow) and from the small subunit (right arrow), is indicated with asterisks. The two tryptophan residues that undergo rearrangement when caspase-3 is activated are also indicated with filled circles. The synthesized peptide sequence for the antigen of the cinnamon clownfish caspase-3 antibody is in bold line in caspase-3 (aa, 180–195). The numbers indicate the amino acid positions, and the dashes indicate the gaps introduced to optimize similarity between sequences.

2.7. Direct ELISA assay of antibody

To demonstrate the specificity of cinnamon clownfish caspase-3 antibody, it was preincubated with purified antibody. The activity of the isolated antibody was measured by an indirect ELISA test (Magnadottir et al. 1999). Direct ELISA test was analysis on 3 times boosting (2, 4, and 6 weeks) and 2 times serum bleeding (3 and 5 weeks). The capture antibody titer was applied by adding antigen caspase-3-peptide to the appropriate wells (500 ng/well) of microtiter plates (96-well Nunc Maxisorp; Nunc, Roskilde, Denmark). Microtiter plates adding antigen caspase-3-peptide and coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$; 500 ng/well). The plate was incubated at 4°C overnight, subsequently washed three times with PBST (containing 0.05% Tween-20 in PBS), and well dried for 37°C 30 min. 200 μL of blocking solution was added to 5% skim milk in PBST in each well, and the plate was incubated at room temperature for 1 h. The plate was subsequently emptied and washed with PBST. Detection antibody was applied by adding caspase-3 peptide antibodies to appropriate wells (1 μg/mL, 100 μL/well). The microtiter plate was again incubated at room temperature for 2 h. Subsequently, the plate was emptied and washed three times with PBST. The secondary antibody was applied by adding horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (H/L) to appropriate wells. The plates were sealed and incubated at room temperature for 1 h. The plates were washed five times with PBST, and the peroxidase activity was revealed by adding 50 μL of substance D [*o*-Phenylenediamine dihydrochloride (OPD), 400 μg/mL, 0.03% H_2O_2, citrate buffer). The enzyme reaction was stopped after 30 min by addition of 100 μL solution (1 M NH_2SO_4), and the absorbance was read at 450 nm using a microplate reader.

2.8. Western blot analysis

Total protein isolated from the gonads during the sex changes in cinnamon clownfish was extracted using protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.1% SDS, 0.15 mg/mL PMSF), and 0.15 mg/mL leupeptin], sonicated, and quantified using the Bradford method (Bio-Rad). Total protein (30 μg) was loaded

per lane onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. For reference, a protein ladder (Fermentas) was also used. Samples were electrophoresed at 80 V until they moved through the stacking gel and at 150 V while they moved through the resolving gel, until the bromophenol blue dye front had run off the gel. The gels were immediately transferred to a 0.2 μm PVDF membrane (Bio-Rad) and run at 85 V for 1.5 h at 4°C. Thereafter, the membranes were blocked with 5% milk in TBS (pH 7.4) for 45 min, and were subsequently washed in TBS. The membranes were incubated with polyclonal rabbit antibody to caspase-3 (dilution 1:1,000); an internal control was β-tubulin (ab6046, Abcam). The blots were further treated with a HRP-conjugated anti-rabbit IgG secondary antibody (dilution 1:5,000, Bio-Rad) for 60 min. Protein bands were detected using both the standard ECL system and the more sensitive ECL system (ECL Advance; GE Life Sciences); exposure to autoradiography-sensitive film was performed for 5 min.

2.9. GnRH treatment

To investigate the roles of caspase-3 in the reproductive physiology of cinnamon clownfish, immature male fish was treated with GnRHa, and subsequently measured gonadal expression and activity of caspase-3. The fish were reared in 220-L circulation filter tanks in the laboratory, and were anesthetized with MS-222 (200 mg/l) prior to injection. GnRHa (des Gly^{10}-[$DAla^{6}$] LHRH ethylamide, Sigma-Aldrich) was dissolved in physiological saline (0.9% NaCl). Each fish was given an intraperitoneal injection of GnRHa [0.1 and 0.5 μg/g of fish body weight (BM)] at a volume of 10 μL/g BM, and sham group of fish was injected with a dissolved equal volume of 0.9% NaCl (10 μL/g BM). After injection, gonad samples were removed from five fish at each of the following time periods: 0, 6, 12, 24, and 48 h. During the experimental period, the water temperature and photoperiod were maintained at 28 ± 1°C and 12-h light:12-h dark ratio, respectively.

2.10. QPCR

QPCR was performed to determine the relative expression of caspase-3 mRNA in the gonads of cinnamon clownfish. Primers for QPCR were designed with reference to the known sequences of cinnamon clownfish as follows: caspase-3 forward primer (5′-CGA CCA GAC AGT GGA GCA AAT G-3′), caspase-3 reverse primer (5′-TAC CGA AGA ACA CGC CCT CAT C-3′), β-actin forward primer (5′ -GGA CCT GTA TGC CAA CAC TG-3′), β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′), GAPDH forward primer (5′-AGA AGG CCT CTG CTC ACT TG-3′), and GAPDH reverse primer (5′-TTG CTG ACA ACT GGG AGG GA-3′). PCR amplification was carried out using a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System and iQTM SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR was carried out as follows: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s. After the QPCR, the data from three replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. As an internal control, experiments were duplicated with β-actin and GAPDH; all data were expressed as change with respect to the corresponding β-actin and GAPDH calculated ΔCt levels. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR, QPCR data from three replicate samples were analyzed with the software of the cycler system to estimate the transcript copy numbers for each sample.

2.11. Histological analysis

The gonad tissues from each developmental group (mature male, male at 90 days after removing female, and mature female) for the analysis of gonads during sex change were fixed in Bouin's solution. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene, and embedded in paraffin. Sections (5-μm thick) were selected and stained with haematoxylin–eosin (H&E) for

observation under a light microscope (Leica DM 100, Germany), and images were captured with a digital camera (Leica DFC 290, Germany).

2.12. Caspase-3 activity assay

Caspase-3 activity of gonads was measured using a commercially available kit (Sigma-Aldrich), according to the manufacturer's protocol. Cinnamon clownfish gonads were lysed in lysis buffer after treatment with hexane fraction (HF) (25, 50, 100, or 200 μg/mL) for 24 h. Active caspase-3 was used as a positive control. All mixtures were incubated overnight in a humidified environment at 37°C. The amount of *p*-Nitroaniline (*p*-NA) released from the substrate was measured at 405 nm using a plate reader.

2.13. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc.). One-way ANOVA followed by Tukey's post hoc tests was used to compare the differences in the data ($P < 0.05$). All values are expressed as mean ± SE.

3. Results

3.1. Characterization of caspase-3 sequence

The sequences of cinnamon clownfish caspase-3 cDNA (**JF345174**) were obtained with RT-PCR using mRNA extracted from fish gonads (Fig. 24). For caspase-3, the cDNA sequence of 849 bp showed significant similarity with the caspase-3 of other vertebrates, as revealed by the BLAST results. Analysis in the PROSITE database predicted that the cinnamon clownfish caspase-3 fragment contained caspase family p20 (S^{52} to D^{176}) and p10 (G^{180} to P^{282}) domain profiles, a caspase family histidine active site (caspase family signature; H^{117}SCSASFVCVLLSHG), and a caspase family cysteine active site (K^{163}PKLFFIQACRG). An N-glycosylation site (N^{74}GTD) was also predicted. Furthermore, the pentapeptide active-site motif (Q^{168}ACRG) and the putative aspartic acid (Asp^{155}), which splits the large and small subunits, were conserved among all the sequences from the different species thatwere analyzed, as were the two subunits. The protein-binding domain GSWFM was also well conserved among the species analyzed, revealing only a conservative substitution in the last amino acid of the motif for Atlantic salmon. The two tryptophan residues that were previously shown to undergo rearrangement during activation of caspase-3, resulting in the reorganization of the active site (Park et al. 2004), were also conserved among the analyzed species, which suggests that the same process of reorganization can occur in fish. Furthermore, this results was shown that an integrin-recognition motif (RGD) near the active site was conserved in all analyzed species. The cinnamon clownfish caspase-3 sequence exhibited a very similar homology to these of other fish: 85% with European seabass, 85% with large yellow croaker, 84% with pufferfish, and 80% with Atlantic salmon.

3.2. Phylogenetic analysis

To reveal the molecular phylogenetic position of cinnamon clownfish caspase-3, a phylogenetic tree was constructed. As shown in Fig. 25, caspase-3 is clearly separated into two clusters in vertebrates. Phylogenetic analysis showed that the cinnamon clownfish caspase-3 was closely related to caspase-3 of other teleost. The teleost caspase-3 was grouped together, respectively.

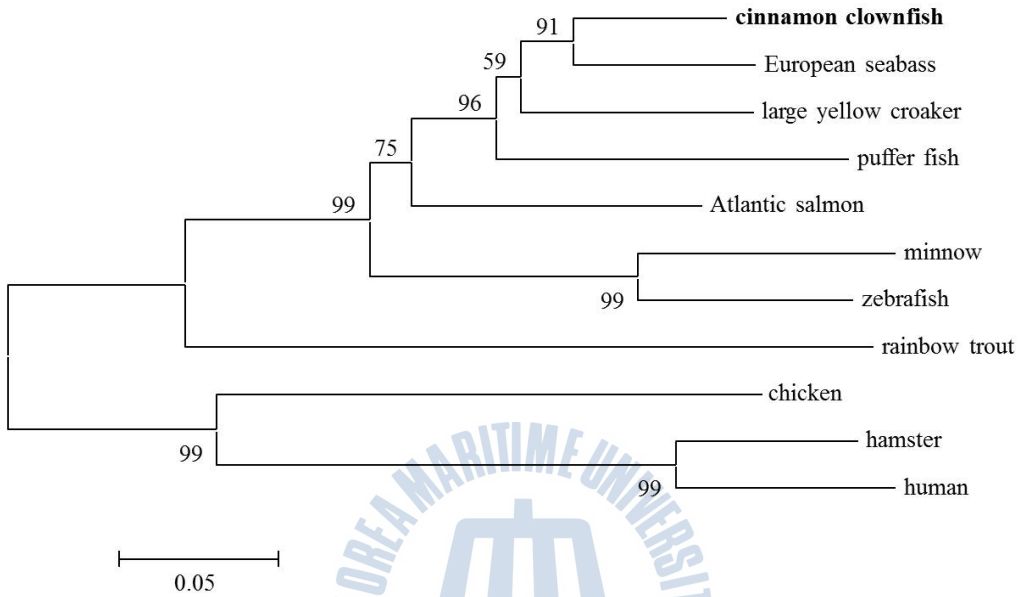

Fig. 25. Phylogenetic tree based on an amino acid alignment for caspase-3 in teleost fish. Bootstrap values (%) are indicated for 1,000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are as follows: caspase-3 cinnamon clownfish (**JF345174**), European seabass (**DQ345773**), large yellow croaker (**EU878546**), pufferfish (**NM001032699**), Atlantic salmon (**BT059710**), minnow (**GQ406344**), zebrafish (**AB047003**), rainbow trout (**FR751081**), chicken (**NM204725**), Chinese hamster (**FJ940732**), and human (**AJ413269**).

3.3. Direct ELISA assay for antibody characterization

On western blot analysis, a new polyclonal antibody to caspase-3 produced in this study recognized a band with a molecular mass of 32 kDa in the cinnamon clownfish ovary. Produced caspase-3 antibody have 34 kDa, as the titer and specificity of the purified caspase-3 antibody were tested with ELISA. The ELISA method developed in this work is based on the use of antibodies against a synthetic peptide from the cinnamon clownfish sequence. Prior to ELISA development and validation, the specificity of anti-caspase-3 antibodies was assessed. The antibodies were characterized by direct ELISA analysis using the caspase-3 peptide (Fig. 26). These results indicated that anti-caspase-3 antibodies cross-reacted with caspase-3 peptide (2, 4, and 6 weeks), and exhibited a decrease in the signal proportional to the dilution of the anticaspase- 3 antibodies in the range of 1:1,000, 1:5,000, and 1:10,000. Competitive ELISA development demonstrated the availability of a standard and an antibody specific of the protein.

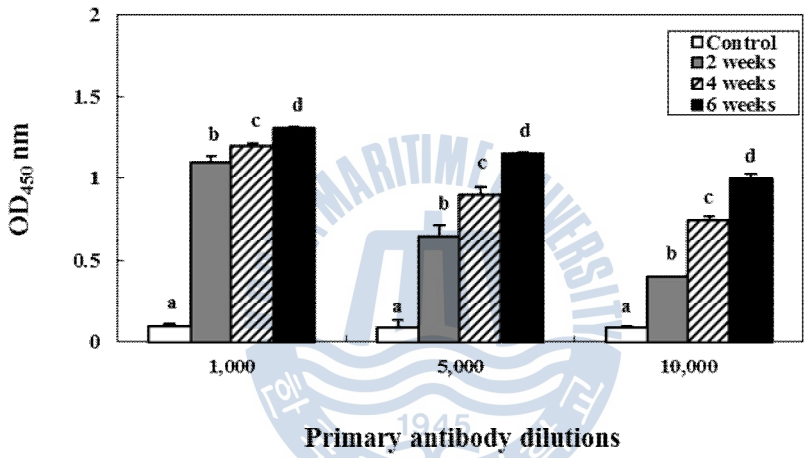

Fig. 26. Direct ELISA was performed with induced (2, 4, and 6 weeks) and non-induced (Control) samples from different fish species diluted to 1:5,000 and various dilutions (1,000, 5,000, and 10,000) of caspase-3 antibodies.

3.4. Expression and activity of caspase-3 during the sex change process

The caspase-3 mRNA and protein expression were detectable in gonads from developmental stages of the sex change (Fig. 27). The expression of caspase-3 was investigated in the gonads by mean of Western blot analysis, which revealed a protein with caspase-3 immunoreactivity of a size corresponding to the predicted size for cinnamon clownfish caspase-3 (32 kDa; Fig. 27A). The expression of this protein was correlated with the caspase-3 mRNA levels in cinnamon clownfish gonads ($P < 0.05$). The overall results demonstrate that the caspase-3 activity is higher in mature gonads, compared to the development gonad stage.

3.5. Histological analysis

The testis of mature male mainly consisted of testicular tissue, with oogonia and primary oocytes (Fig. 28A). And then the oocytes had developed and increased in size (Fig. 28B), the diameter of oocytes was increased, the testicular tissue was regressed, and finally the fish became females (Fig. 28C).

3.6. Time-related effect of GnRHa on the expression and activity of caspase-3

Treatment with 0.1 and 0.5 μg/g of GnRHa significantly increased caspase-3 mRNA level in cinnamon clownfish gonads (Fig. 29A) ($P < 0.05$). The GnRHa-induced caspase-3 mRNA was approximately 79.3 and 78.1 times higher (than the untreated control level) at 6 h (at 0.5 μg/g GnRHa) and 12 h (at 0.1 μg/g GnRHa), respectively. The activity of caspase-3 mRNA was maintained at 5.5 times more than the control level following injection with GnRHa (Fig. 29B).

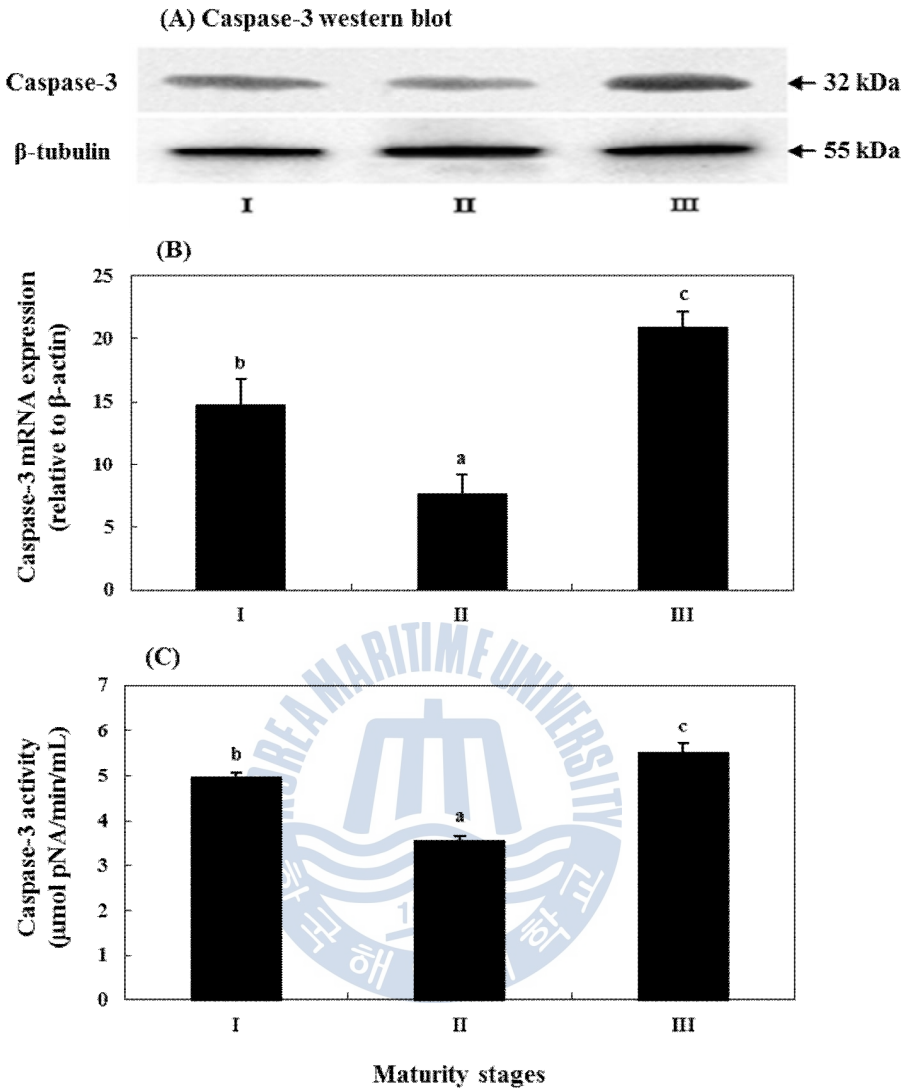

Fig. 27. Expression and activity of caspase-3 in gonads of cinnamon clownfish during the sex change (I, mature male; II, male at 90 days after removing female; III, mature female). (A) Western blot of caspase-3 protein expression in gonads, and β-tubulin (55 kDa) was the internal control. (B) Caspase-3 mRNA levels relative to β-actin mRNA levels in the gonads were analyzed with real-time PCR. (C) The activity of caspase-3 was also analyzed with a plate reader. Values with dissimilar letters are significantly different ($P < 0.05$). Values are expressed as mean ± SE ($n = 5$).

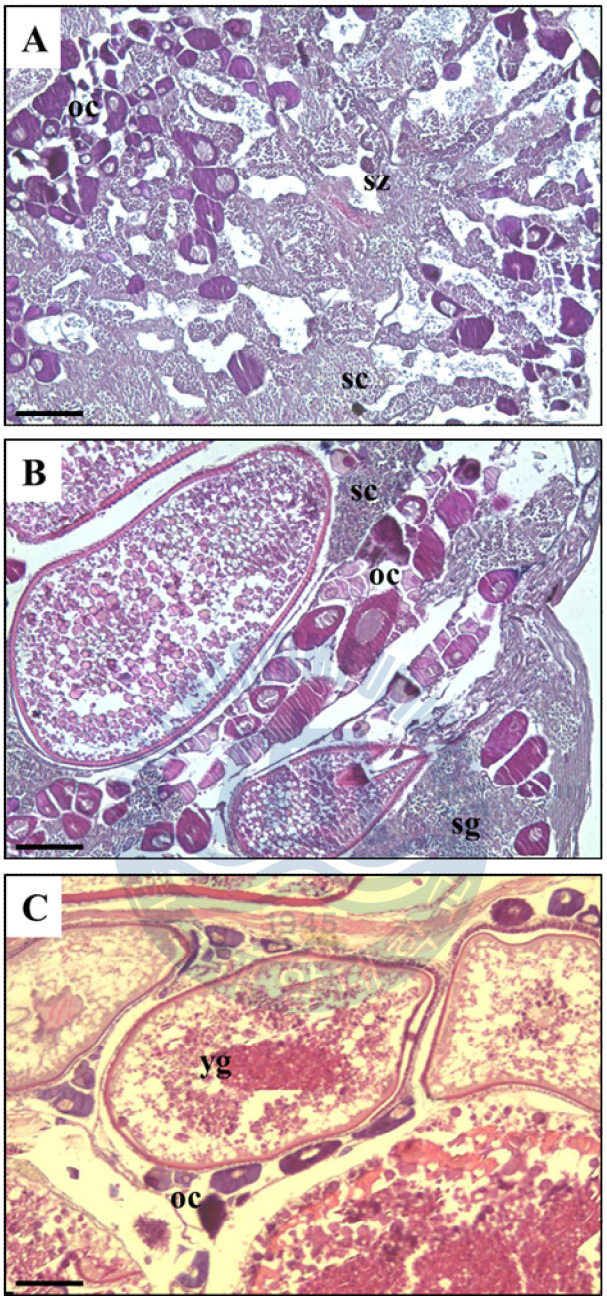

Fig. 28. Photomicrograph of gonad maturity stages during sex change in cinnamon clownfish. (A) Mature male (stage I), (B) male at 90 days after removing female (stage II), (C) mature female (stage III). oc, oocytes; sc, spermatocytes; sg, spermatogonia; yg, yolk granules. Scale bars = 100 μm.

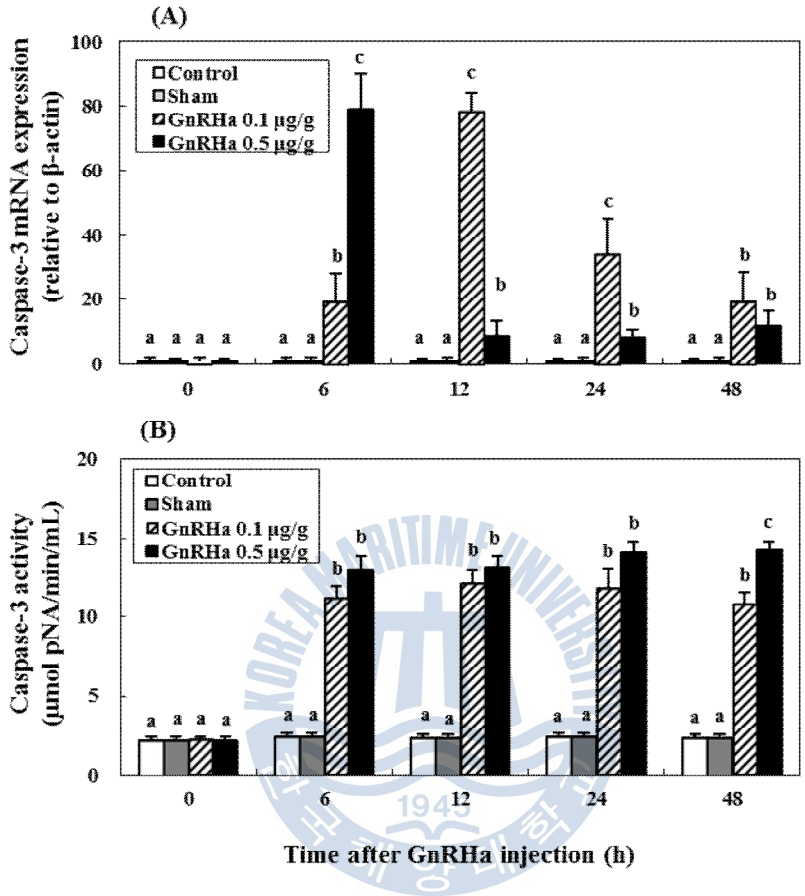

Fig. 29. Time-related effects of GnRHa (control, sham, 0.1, and 0.5 μg/g) on caspase-3 mRNA levels (A) and activity (B) in cinnamon clownfish gonads. Total RNA was extracted from gonads 0, 6, 12, 24, and 48 h after GnRHa treatment, and 3 μg was used for PCR. The expression level of each sample was normalized with respect to the β-actin and GAPDH signal, and is expressed as the relative expression level. Values with dissimilar letters are significantly different ($P < 0.05$) from each other. Values are expressed as mean ± SE ($n = 5$).

4. Discussion

The full-length caspase-3 was isolated from the gonads of cinnamon clownfish, and the changes in mRNA level, activity, and histological was investigated during the sex change from male to female. In this study further was investigated the effect of GnRHa on caspase-3 mRNA level and activity in immature cinnamon clownfish.

Caspase-3 cDNA contained 969 nucleotides, including an open reading frame predicted to encode a protein of 282 amino acids (Fig. 24). The results provide information on caspase family p20 and p10 domain profiles, and demonstrate that caspase family signature and cysteine active site are well conserved in the caspase-3 sequence. The pentapeptide active-site motif QACRG was also conserved and interrupted after its first amino acid (Q) by an intron, as in all species analyzed herein. It has been established previously that the active form of caspase-3 is generated after proteolytic cleavage of the proenzyme at aspartic acid residues (Wang and Keiser, 1998). In the presented alignment (Fig. 24), the cleavage sites of caspase-3, predicted at Asp^{36} (D^{36}) and Asp^{184} (D^{184}), separates the prodomain, large subunit, small subunits, and an RGD upstream of the active site. These sequences are conserved in all species analyzed except for the cinnamon clownfish sequence in which the aspartate residue is replaced by an aspartic acid (D^{155}). Aspartate-aspartate-X (DDX), located near the site of processing ($D^{28}DG$) that produces the p12 and p17 subunits, interacts with RGD peptides, leading to direct triggering of conformational changes that promote pro-caspase-3 auto processing and activation (Pasqualini et al., 1995).

In this study, caspase-3 mRNA expression level was low in the male fish during the sex change, from male to female, after the removal of the paired female (Fig. 27). Also, caspase-3 mRNA expression decrease during stage II, in which the developing ovarian tissue did not show activations of caspase-3. However, the expression level increased in fish during change from male to female. The caspase-3 mRNA level was highest in the mature female. This was correlated with

the observed increase in the activity of caspase-3 during the male-to-female sex change. The present study provides evidence that caspase-3 activity may be related to the sex change in clownfish. It can logically be postulated that caspase-3 may play a role in induction of testicular apoptosis in a process leading to the development of ovary and development of oocytes in protandrous cinnamon clownfish. Also, in Western blot analysis, the caspase-3 protein was similar to the mRNA expression and activity of caspase-3. These results are consistent with previous studies that demonstrated apoptosis during normal spermatogenesis and post-ovulatory regression in teleost gonad in rainbow trout (Uchida et al., 2002) and goldfish (Wood and Van Der Kraak, 2001). Thus, the present results provide a support for the hypothesis that caspase-3 is involved in the development and maturity of gonads by stimulating apoptosis during the sex change from male to female in cinnamon clownfish. In zebrafish development, oocyte apoptosis induces testicular and ovarian differentiation (Uchida et al., 2002). In such oocytes, a proapoptotic signaling pathway specific to males may be present and it may be stimulated by sex determining genes or changes in estrogen or thyroid hormone levels. In addition, gonadal histology was examined during sex change in cinnamon clownfish. As a result, it was found that the oocytes was developed and testicular tissue was regressed in the ovary as the cinnamon clownfish were changed from male to female (Fig. 28). This result was similar to the previous study (Godwin, 1994). This study therefore was suggested that gonad development and maturation were controlled by the pituitary-gonad axis through increases in the levels of caspase-3 mRNA in cinnamon clownfish.

The present study taken further steps to investigate the role of GnRHin regulation of apoptosis in cinnamon clownfish. Injection with GnRHa significantly increased caspase-3 mRNA expression in immature cinnamon clownfish gonad (Fig. 29). The observed GnRH-induced increase in caspase-3 mRNA is consistent with previous report in goldfish (Andreu-Vieyra and Habibi, 2001) and gilthead seabream (Soverchia et al., 2007). The stimulation of caspase-3 was observed at the highest dose of GnRHa tested presumably due to increasing proapoptotic proteins, opposing

the anti-apoptotic actions of GTH and growth factors (Parborell et al., 2002). Also, Andreu-Vieyra and Habibi (2001) was reported that the observed effect of GnRH on testicular apoptosis is significant, because local factors such as GnRH peptides are likely to play an important regulatory role in controlling testicular spermatogenesis during various reproductive cycles in goldfish. Recent studies have also suggested that GnRH induction of apoptosis correlates with an increased expression of the proapoptotic protein Bax, as well as with decreased basal testosterone production (Andreu-Vieyra et al., 2005). In addition, published studies have demonstrated that GnRH-induced apoptosis occurred only during the late stage of spermatogenesis (Chowdhury et al., 2008), and was mediated steroid hormone production as basal of T levels (Soverchia et al., 2007). The present results confirm previous observations that GnRH plays an important role in the control of gonadal development by controlling apoptotic pathways in the testis and ovary (Andreu-Vieyra et al., 2005; Soverchia et al., 2007). Furthermore, increased activity of caspase-3 was demonstrated during the male-to-female sex change process in clownfish. This evidence in this study also provided that GnRH agonists stimulate caspase-3 production which can in turn stimulate apoptosis. The findings facilitate better understanding of the mechanisms underlying sex change in cinnamon clownfish and other hermaphrodite teleost fish.

Chapter 6.

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

1. Introduction

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

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

sbGnRH neuronal cell bodies are localized in the POA, project their axons throughout various brain loci, have neuro-modulatory activities (Oka, 2009), and exert effects on pheromone production (Steven et al., 2003).

Recently, Kiss, a neuropeptide that regulates sexual differentiation and spawn time in vertebrates, and its receptor, GPR54, have been shown to play major roles in the central regulation of the HPG axis (Roa et al., 2011; Chang et al., 2012). Kiss, a member of the RF-amide peptide family, is located in the POA of the hypothalamus and regulates sexual maturation and regulation factors (Roa et al., 2011). Mammalian Kiss1 is suggested to be a key regulator of the HPG axis, and in teleosts, its ortholog is named Kiss1 (Kanda et al., 2008). In addition, the Kiss-GPR54 signal systemis one of the circuits regulating GnRH secretion in the hypothalamus (Colledge, 2009). According to a recent study performed in goldfish, both signal peptides could stimulate or inhibit the HPG axis dependent on the stage of gonadal development (Zmora et al., 2012). Many findings have led a number of authors to suggest an important role played by the Kiss system in the activation of the HPG axis in fish (Parhar et al., 2004; Filby et al., 2008; Kitahashi et al., 2009). The hypophysiotropic actions of Kiss1 was also confirmed *in vitro* on primary pituitary cell culture from goldfish (Yang et al., 2010), and results also showed that Kiss1 could stimulate prolactin and growth hormone secretion and gene expression in goldfish pituitaries (Yang et al., 2010). The synthesis and secretion of FSH and LH in the pituitary are stimulated by Kiss protein synthesis and secretion in the hypothalamus; in particular, Kiss plays an important role in regulating reproduction and stimulates LH secretion before reproduction and ovulation (Chang et al., 2012). In addition, LH stimulates the conversion of cholesterol to 17α-hydroxypregnenolone, the precursor of gonadal steroid hormones, which is secreted and affects the theca folliculi and granulosa cells during the final stage of maturation. The 17α-hydroxypregnenolone also plays an important role in the synthesis of sex hormones (estrogen and T), which interact with FSH (Dickey and Swanson, 2000; Yamato et al., 2010).

Estrogen is an essential steroid hormone in reproduction whose effects are

mediated by nuclear ERs; it plays important roles in sexual maturation and differentiation, which include oogenesis, vitellogenesis, and testicular development (Ishibashi and Kawashima, 2001). The induction of Vtg, a precursor yolk protein, in response to E_2 by GTH is well documented in several oviparous (egg-laying) fish species (Bowman et al., 2002; Davis et al., 2009).

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

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

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

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

2. Materials and methods

2.1. Experimental fish

The study was conducted with pairs of cinnamon clownfish artificially made [female, 8.4 ± 0.5 cm, 14.6 ± 0.5 g, gonadosomatic index (GSI; gonad weight/body weight × 100) = 0.15 ± 0.03; male, 6.5 ± 0.4 cm, 11.5 ± 0.3 g, GSI = 0.11 ± 0.03]. The fish were purchased from CCORA (Jeju, Korea). The water temperature was 27 ± 1°C, and the photoperiod was 12-h light:12-h dark, respectively. The fish were fed a commercial feed twice daily (at 09:00 and 17:00 h). The fish were reared under these conditions for 6 weeks.

2.2. Kiss treatment and sampling

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

All fish were anesthetized with MS-222 (Sigma-Aldrich) and decapitated prior to tissue collection. Pituitary, brain, gonad, and liver samples from the fish were removed, immediately frozen in liquid nitrogen, and stored at −80°C until the total RNA was extracted for analysis. The plasma samples were separated by centrifugation (4°C, 10,000 ×*g*, 5 min) and stored at −80°C until analysis. After being dissected and weighed, the gonads were fixed in Bouin's solution and subjected to histological observation. The GSI was calculated for each fish.

2.3. QPCR

QPCR was conducted to determine the relative mRNA expression levels of 3 GnRH types (sGnRH, sbGnRH, and cGnRH-II), the GTH subunits (GTHα, FSHβ, and LHβ), GTH receptors (FSHR and LHR), ER subtypes (ERα, ERβ1, and ERβ2), and Vtg using the total RNA extracted from cinnamon clownfish tissues. The primers for QPCR are shown in Table 3. These primers were designed for each gene using the Beacon Designer software (Bio-Rad). Primer alignments were performed with the BLAST database to ensure the specificity of primers. QPCR amplification was conducted similar to previous studies using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad). QPCR was performed in the following manner: 1 cycle of denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 20 s and annealing at 55°C for 20 s. Each reaction was run in triplicate to confirm consistency. The experiments were duplicated with β-actin as an internal control. The efficiencies of the reactions were determined by performing the QPCR. All data were expressed as changes with respect to the corresponding β-actin calculated ΔCt levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated as $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

Table 3. Primers used for QPCR amplification

Genes (accession no.)	Primer	DNA sequences
sGnRH	Forward	5′-GAA GAG AAG TGT GGG AGA G-3′
(**HQ883477**)	Reverse	5′-CGA AAG GAC TGG AAT CAT CT-3′
sbGnRH	Forward	5′-CTG CTG GTG GTG ATG ATG-3′
(**HQ883476**)	Reverse	5′-GGT CTC TCT TCC CTC CTG-3′
cGnRH-II	Forward	5′-AGC ACA TCA GAG ATT TCA GAG-3′
(**EU908057**)	Reverse	5′-CCA AGG CAT CCA AAA GAA TG-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′
FSHR	Forward	5′-CCT CTC ATT ACC GTG TCC GAC TC-3′
(**GU722648**)	Reverse	5′-GGG TGA AGA AGG CAT ACA GGA AGG-3′
LHR	Forward	5′-GGA AAC AGA AAT AGA GCC CAC TAC AG-3′
(**GU722649**)	Reverse	5′-CAC TTG ACG AAG GGG TTG TTA AGA C-3′
ERα	Forward	5′-CTG GGC TGG AGG TGC TGA TG-3′
(**HM185179**)	Reverse	5′-TCA ACA CAG TCG CCT TCG TTC C-3′
ERβ1	Forward	5′-GCT TCA GGC TAC CAC TAT-3′
(**HM185180**)	Reverse	5′-CGG CGG TTC TTG TCT ATA-3′
ERβ2	Forward	5′-GTC TCG GTT CCG TGA GCT GAA G-3′
(**HM185178**)	Reverse	5′-GCA CAG ATT GGA GTT GAG GAG GAT C-3′
Vtg	Forward	5′-CGA GAT TCT GAA ACA CCT G-3′
(**HM185181**)	Reverse	5′-GCT GAA ATA ATT CCA CAA ACT T-3′
β-actin	Forward	5′-GGA CCT GTA TGC CAA CAC TG-3′
(**JF273495**)	Reverse	5′-TGA TCT CCT TCT GCA TCC TG-3′

2.4. Western blot analysis

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

2.5. Histological analysis

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

2.6. Plasma parameter analysis

The plasma GnRH (E08810f), FSH (E15790Fh), LH (E15791Fh), E_2 (E1317Fh), 17α-hydroxypregnenolone (EQ027292FI), and 11-KT (E14106h) levels were analyzed using the immunoassay technique with the ELISA kit (Cusabio Biotech, Hubei, China). An anti-antibody specific to the antibodies of hormones (GnRH, FSH, LH, 17α-hydroxypregnenolone, E_2, and 11-KT) was pre-coated onto a microplate. There was 50 μL of plasma per well, and 50 μL of HRP-conjugate and 50 μL of an antibody was then added to each well. The samples were mixed well and then incubated for 2 h at 37°C. After the final wash, any remaining wash buffer was removed by aspirating or decanting. Then, 50 μL of substrate A and 50 μL of substrate B were added to each well, and the samples were incubated for 15 min at 37°C in the dark. After incubation, 50 μL of stop solution was added to each well. Finally, the optical density of each well was determined within 10 min using a microplate reader set to 450 nm.

2.7. Statistical analysis

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

10.0; SPSS Inc.). A one-way analysis of variance followed by Tukey's post hoc test was used to test for significant differences in the data ($p < 0.05$). The values are expressed as mean ± SE.

3. Results

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

The expression levels of 3 types of GnRH mRNA in the brains of paired cinnamon clownfish following Kiss injections are shown in Figs. 30 (female) and 31 (male).

In female cinnamon clownfish, treatment with 0.1 and 0.5 μg/g BM of Kiss significantly increased the mRNA levels of the 3 types of GnRH in the brain (Fig. 30) ($P < 0.05$). In particular, the level of sbGnRH mRNA was higher than that in the untreated controls and higher than the levels of the other GnRH forms at 6 weeks post-injection (Fig. 30C). In addition, following the Kiss injection, the plasma GnRH levels gradually increased to reach levels that were approximately 2.3-fold higher at 6 weeks than control (Fig. 30E). A western blot analysis revealed a protein with GnRH-specific immunoreactivity and a mass that corresponded to the predicted mass for cinnamon clownfish GnRH (52 kDa; Fig. 30A). The expression pattern of the protein resembled the pattern of the GnRH mRNA expressed in cinnamon clownfish brains.

Similar to the mRNA expression levels of the 3 types of GnRH in male cinnamon clownfish, the mRNA expression levels of the 3 types of GnRH in the brains of male cinnamon clownfish were significantly increased by treatment with 0.1 and 0.5 μg/g BM of Kiss (Fig. 31) ($P < 0.05$). The expression pattern of the protein resembled the pattern of the GnRH mRNA in cinnamon clownfish brains (Fig. 31A). In addition, following the Kiss injection, the plasma GnRH levels gradually increased to reach levels that were approximately 1.5-fold higher at 6 weeks than control (Fig. 31E).

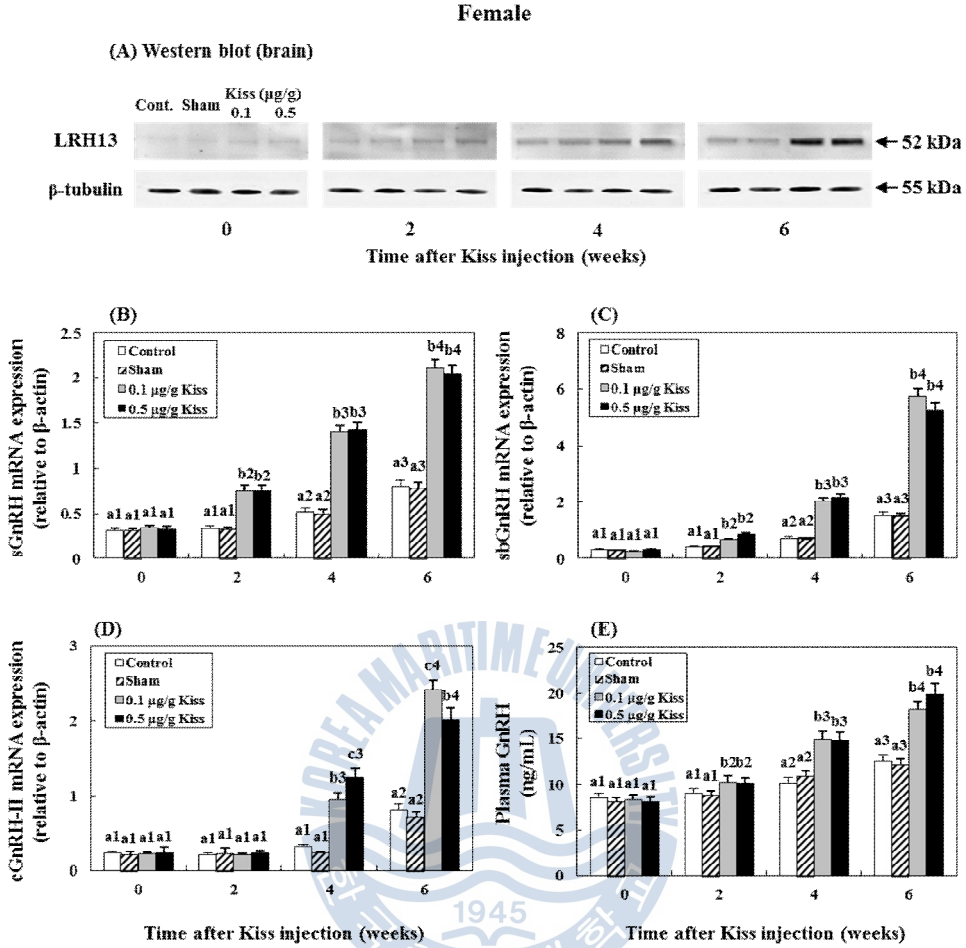

Fig. 30. Expression and activity of three types of GnRH in female cinnamon clownfish brains at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g). (A) Western blot using LRH13 (dilution 1:5,000; 52 kDa) to examine brain protein expression. The β-tubulin (55 kDa) was used as the internal control. (B) sGnRH, (C) sbGnRH, and (D) cGnRH-II mRNA levels relative to β-actin mRNA levels in the brain of cinnamon clownfish. (E) The activity of plasma GnRH of cinnamon clownfish was also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

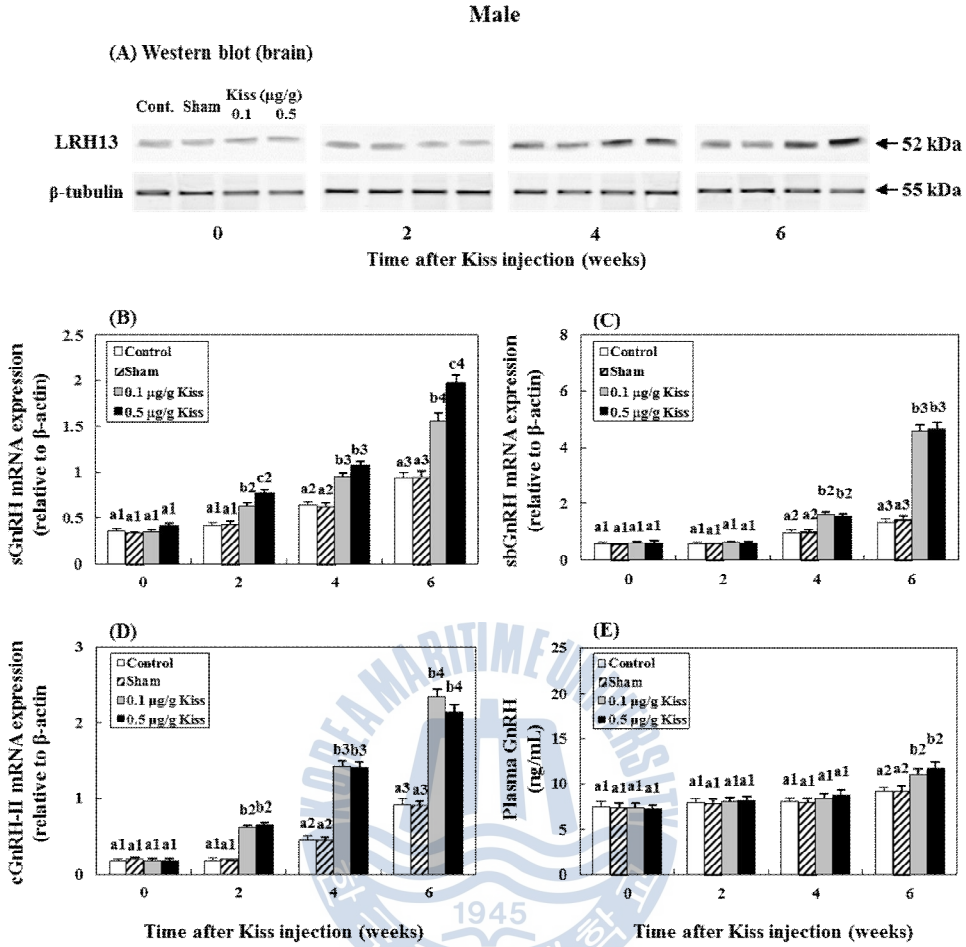

Fig. 31. Expression and activity of three types of GnRH in male cinnamon clownfish brains at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g). (A) Western blot using LRH13 (dilution 1:5,000; 52 kDa) to examine brain protein expression; β-tubulin (55 kDa) was used as the internal control. (B) sGnRH, (C) sbGnRH, and (D) GnRH-II mRNA levels relative to β-actin mRNA levels in the brain of cinnamon clownfish. (E) The activity of plasma GnRH of cinnamon clownfish at 2, 4, and 6 weeks after Kiss injection was also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

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

The mRNA expression levels of GTH subtypes (GTHα, FSHβ, and LHβ) in the pituitary of paired cinnamon clownfish following a Kiss injection are shown in Figs. 32 (female) and 33 (male). Treatment with 0.1 and 0.5 μg/g BM of Kiss significantly increased the GTH mRNA levels in the pituitary glands of female and male cinnamon clownfish (Figs. 32 and 33) ($P < 0.05$). In addition, a western blot analysis detected a GTHα protein with a size that corresponded to the predicted size for cinnamon clownfish (approximately 35 kDa), which exhibited similar mRNA expression across GTHα (Figs. 32A and 33A). In addition, following the Kiss injection, the plasma FSH and LH levels significantly increased and reached levels that were approximately 3.1- and 2.8-fold (females; Figs. 32D and F) and 2.9- and 1.8-fold (males; Figs. 33D and F) higher at 6 weeks than their control, respectively.

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

The mRNA expression levels of GTH receptors (FSHR and LHR) in the gonads following the Kiss injection are shown in Fig. 34 (females and males). Treatment with 0.1 and 0.5 μg/g BM of Kiss significantly increased the GTHR mRNA levels in the gonads of cinnamon clownfish ($P < 0.05$).

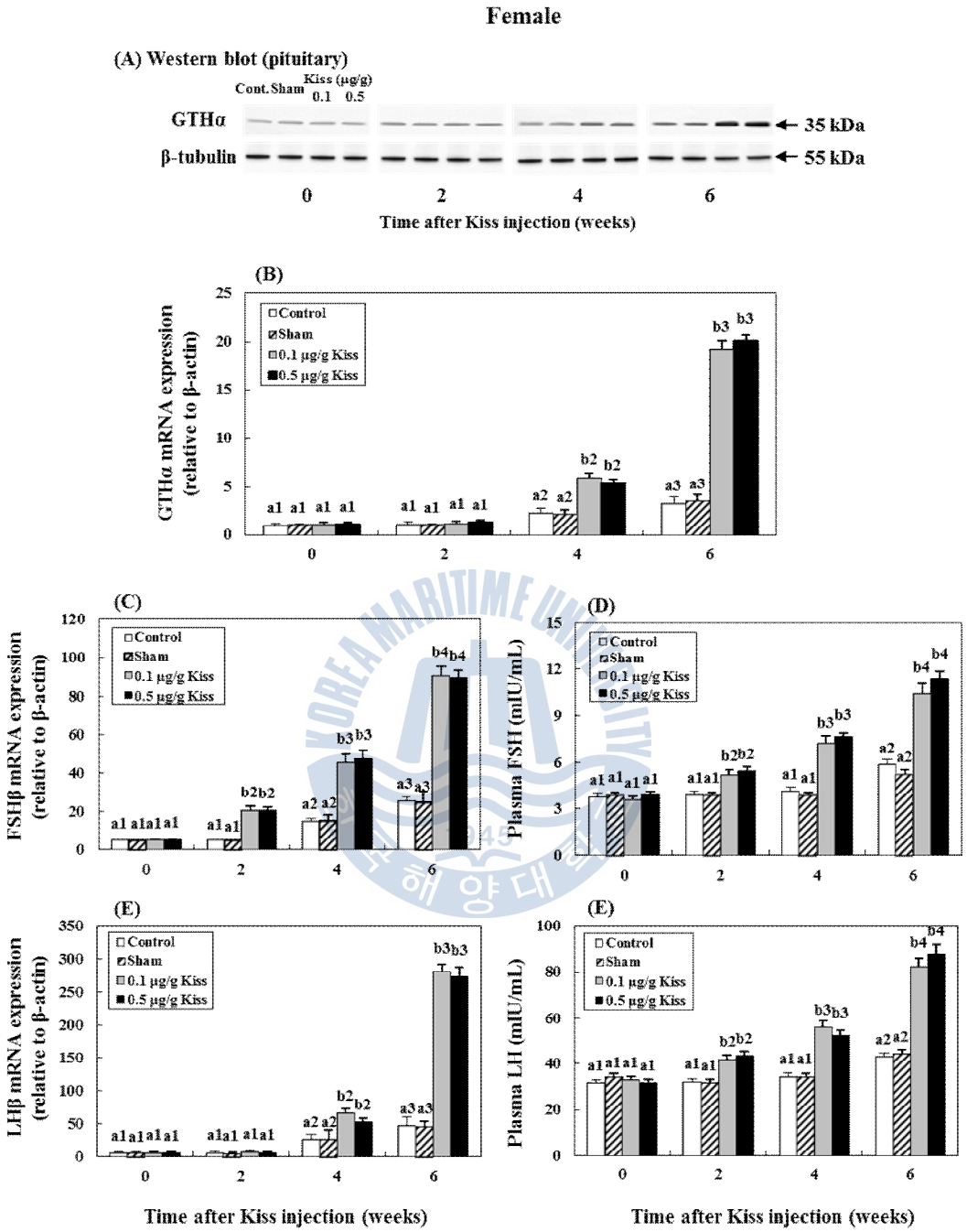

Fig. 32. Expression and activity of GTHs in female cinnamon clownfish pituitary glands at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g). (A)Western blot of GTHα (monoclonal rabbit antiserum; dilution, 1:4,000; 35 kDa) protein

expression in the pituitary of cinnamon clownfish. The β-tubulin (55 kDa) was used as the internal control. (B) GTHα, (C) FSHβ, and (E) LHβ mRNA levels relative to β-actin mRNA levels in the pituitary glands of cinnamon clownfish. The plasma (D) FSH and (F) LH activities of cinnamon clownfish at 2, 4, and 6 weeks after the Kiss injections were also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

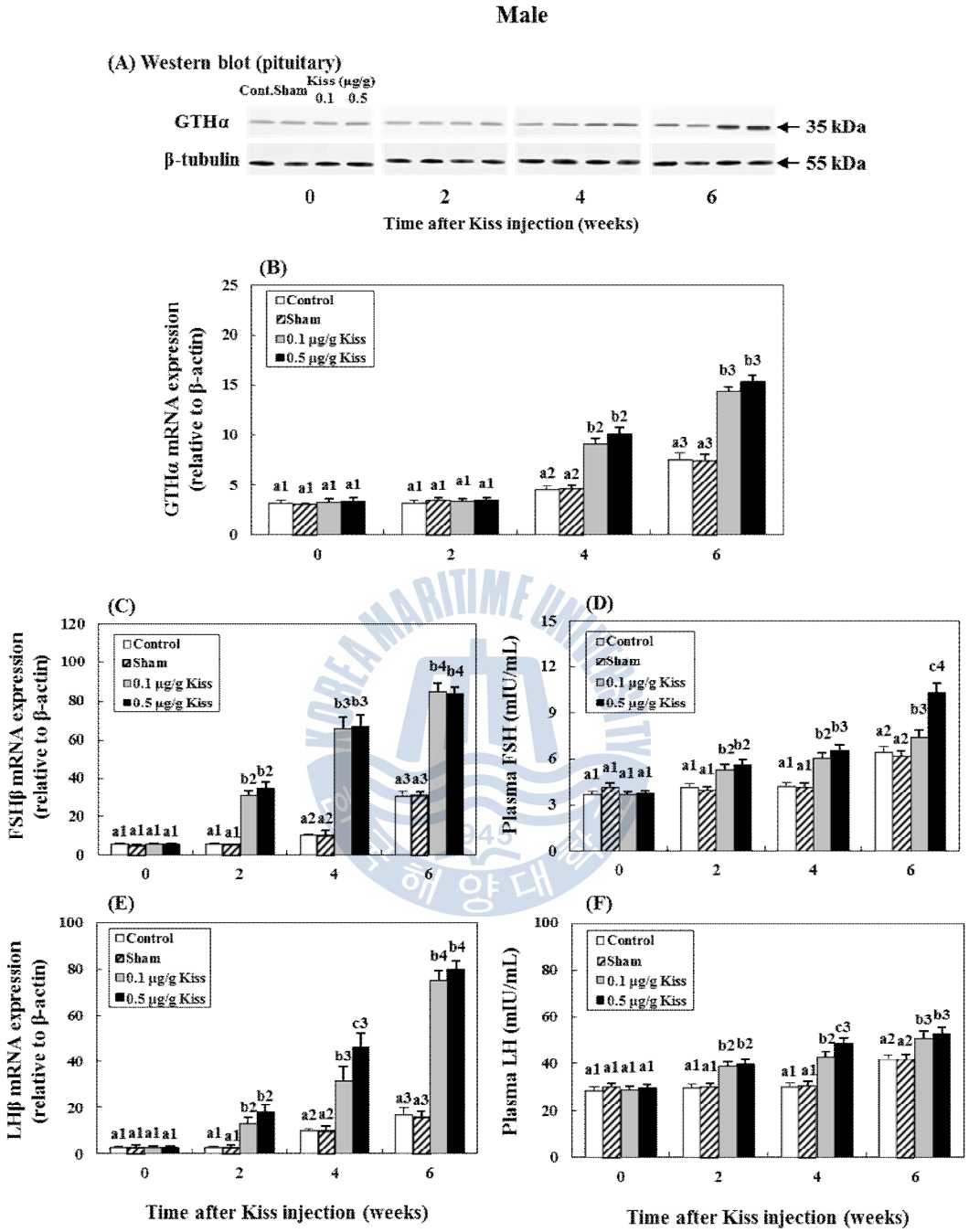

Fig. 33. Expression and activity of GTHs in male cinnamon clownfish pituitary glands at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g). (A) Western blot of GTHα (monoclonal rabbit antiserum; dilution, 1:4,000; 35 kDa) protein

expression in the pituitary of cinnamon clownfish. The β-tubulin (55 kDa) was used as the internal control. (B) GTHα, (C) FSHβ, and (E) LHβ mRNA levels relative to β-actin mRNA levels in the pituitary of cinnamon clownfish. The plasma (D) FSH and (F) LH activities of cinnamon clownfish at 2, 4, and 6 weeks after the Kiss injections were also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

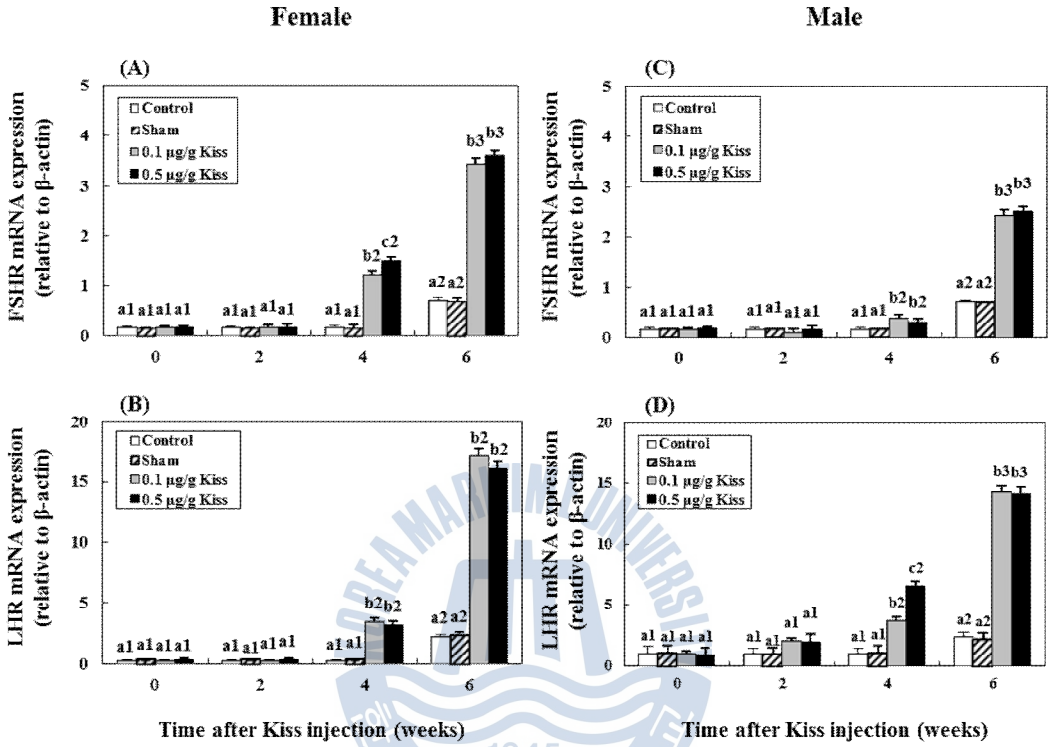

Fig. 34. Expression of GTHRs in cinnamon clownfish gonads [females (A and B) and males (C and D)] at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g). FSHR (A, C) and LHR (B, D) mRNA levels relative to β-actin mRNA levels in the gonads of cinnamon clownfish. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

3.4. Time course and dose-related effects of Kiss on ERs

The mRNA expression levels of ERs (ERα, ERβ1, and ERβ2) in the gonads following a Kiss injection are shown in Figs. 35 (female) and 36 (male). Treatment with 0.1 μg/g BM and 0.5 μg/g BM of Kiss significantly increased all ERs mRNA levels in the gonads of female and male cinnamon clownfish (Figs. 35 and 36) ($P < 0.05$). In addition, a Western blot analysis detected ERα and ERβ proteins with sizes that corresponded to the predicted sizes for cinnamon clownfish (approximately 66 kDa; ERα and 56 kDa; ERβ, respectively), which exhibited similar mRNA expressions across ERs.

Female

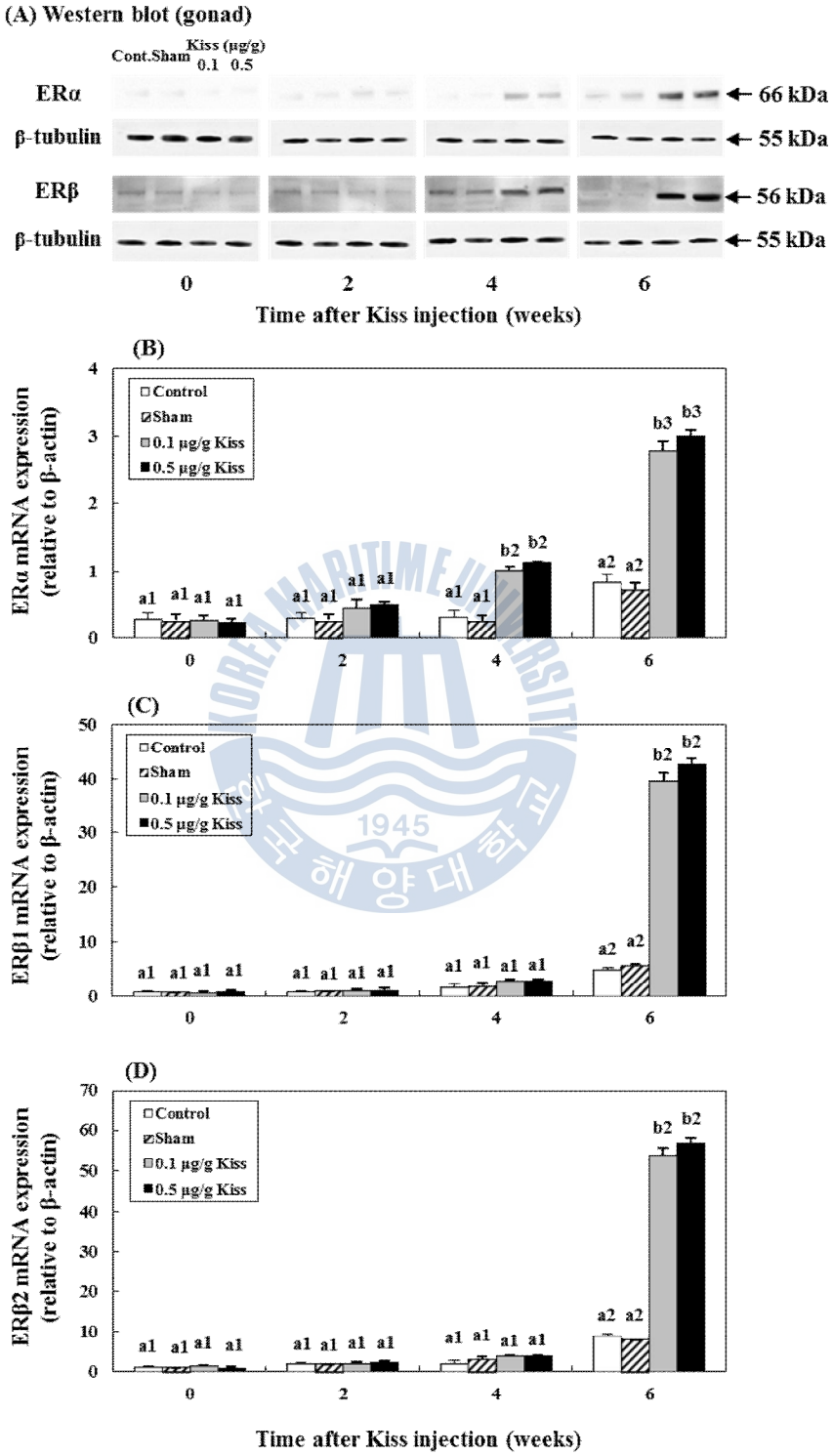

Fig. 35. Expression of ERs in female cinnamon clownfish gonads at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g). (A) Western blot using ERα (66 kDa) and ERβ (56 kDa) to examine protein expression in the gonads; β-tubulin (55 kDa) was the internal control. (B) ERα, (C) ERβ1, and (D) ERβ2 mRNA levels relative to β-actin mRNA levels in the gonads of cinnamon clownfish. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

Male

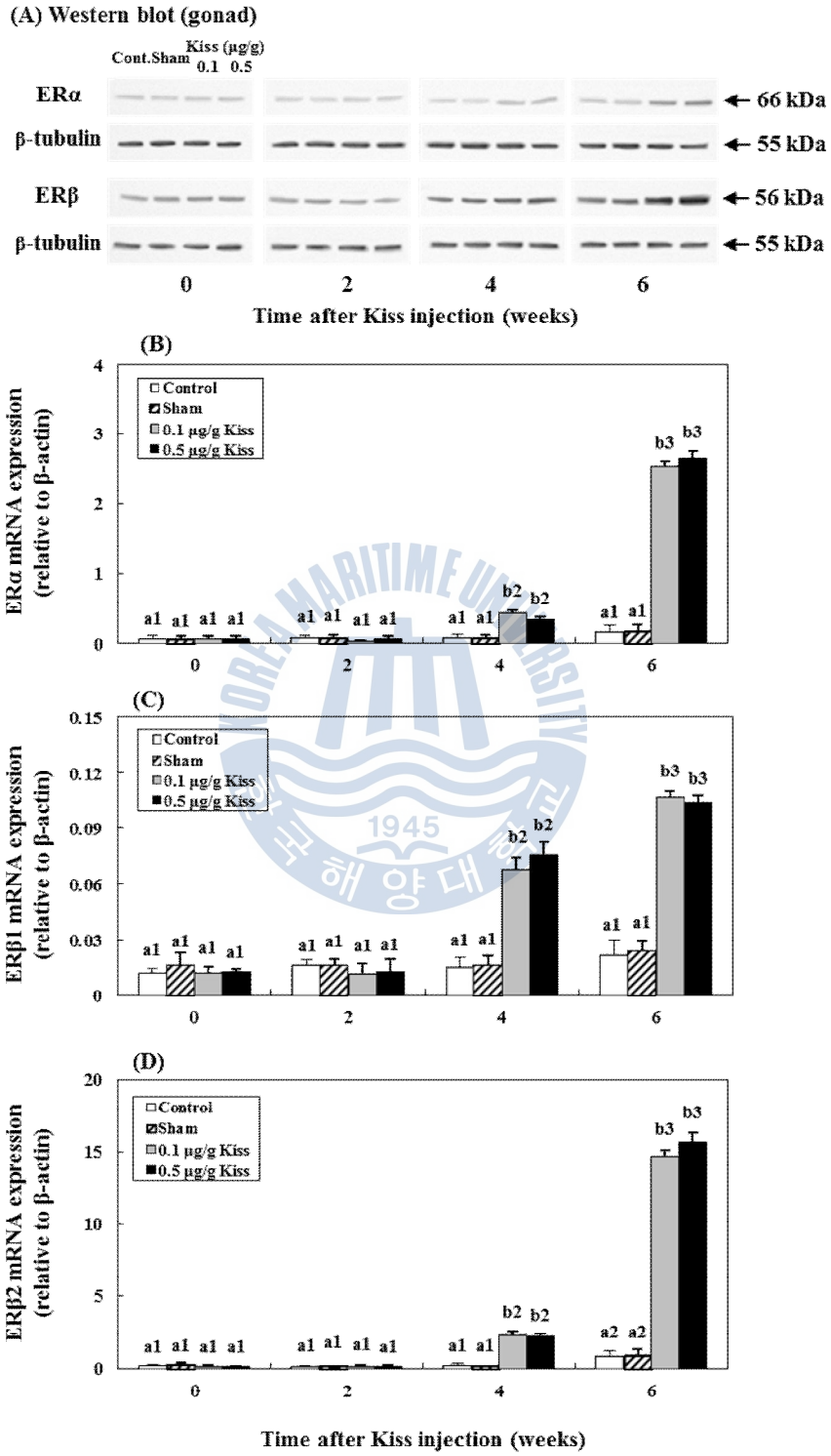

Fig. 36. Expression of ERs in male cinnamon clownfish gonads at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g). (A) Western blot using ERα (66 kDa) and ERβ (56 kDa) to examine protein expression in the gonads; β-tubulin (55 kDa) was the internal control. (B) ERα, (C) ERβ1, and (D) ERβ2 mRNA levels relative to β-actin mRNA levels in the gonads of cinnamon clownfish. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

3.5. Time course and dose-related effects of Kiss on Vtg

The liver expression levels of Vtg were measured following treatment with Kiss (Figs. 37A and B). Treatments with both doses of Kiss significantly increased the Vtg mRNA levels, particularly at 6 weeks after treatment with Kiss ($P < 0.05$).

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

The circulation level of 17α-hydroxypregnenolone was measured in female cinnamon clownfish following injection with Kiss (Fig. 37C). The plasma 17α -hydroxypregnenolone level was 22.4 ± 1.2 ng/mL at the beginning of the experiment. Most notably, the levels of 17α-hydroxypregnenolone levels increased to 58.2 ± 6.5 ng/mL at 6 weeks (0.5 μg/g of Kiss) ($P < 0.05$).

Following a Kiss injection in female, the plasma E_2 levels gradually increased to reach levels that were approximately 1.7-fold higher at 6 weeks than control (Fig. 38A). The plasma E_2 level was 393.2 ± 19.8 pg/mL at the beginning of the experiment. The levels of E_2 then increased to 781.1 ± 35.5 pg/mL at 6 weeks by treatment with Kiss ($P < 0.05$).

The plasma E_2 levels in male cinnamon clownfish following an injection with Kiss are shown in Fig. 38B. The plasma E_2 level was 344.5 ± 18.8 pg/mL at the beginning of the experiment. Most notably, the 11-KT levels increased to 522.3 ± 30.5 pg/mL at 6 weeks by treatment with Kiss ($P < 0.05$).

The 11-KT levels in male cinnamon clownfish following an injection with Kiss are shown in Fig. 38C. The plasma 11-KT level was 713.5 ± 39.8 pg/mL at the beginning of the experiment. Most notably, the 11-KT levels increased to 1,111.1 ± 55.5 pg/mL at 6 weeks by treatment with Kiss ($P < 0.05$).

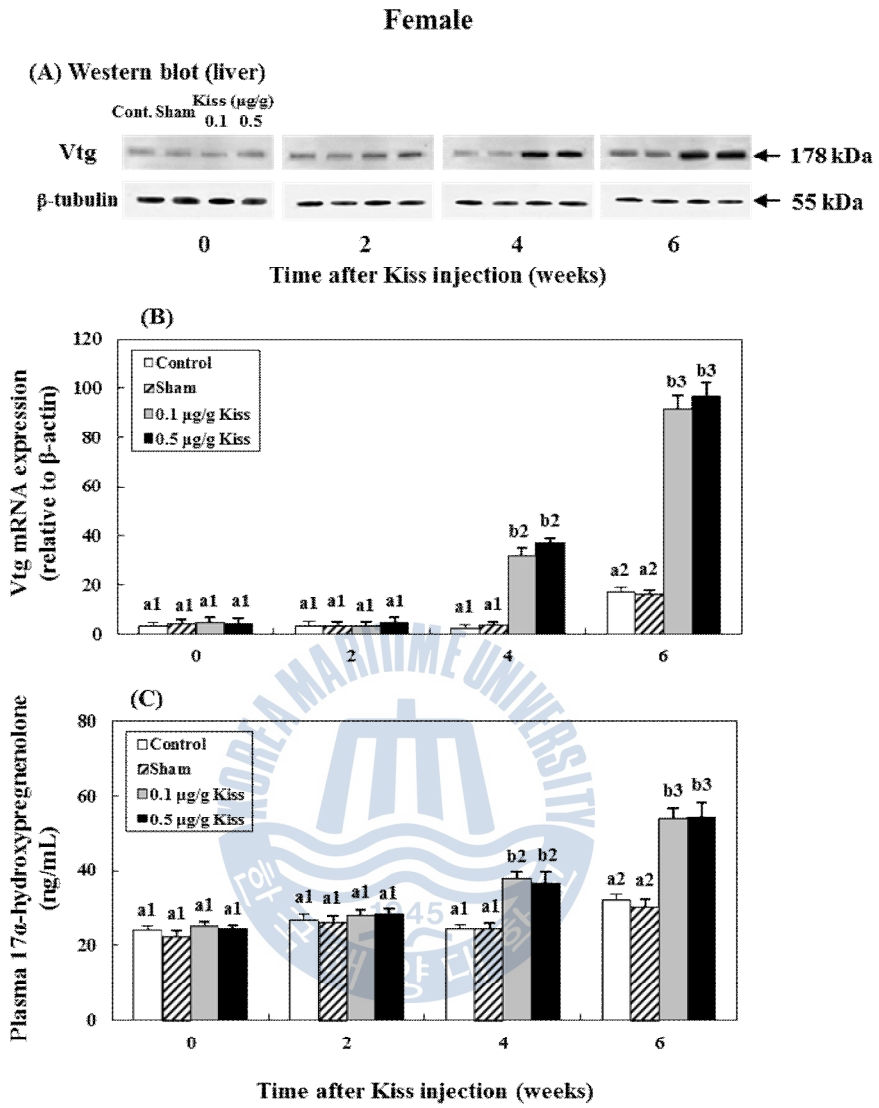

Fig. 37. Vtg expression in the liver and plasma 17α-hydroxypregnenolone levels of female cinnamon clownfish at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g). (A) Western blot of Vtg (monoclonal rabbit antiserum; dilution 1:5,000; 178 kDa) protein expression in the liver; β-tubulin (55 kDa) was the internal control. (B) VtgmRNA levels relative to β-actin mRNA levels in the livers of cinnamon clownfish. (C) The plasma 17α-hydroxypregnenolone concentrations of cinnamon clownfish at 2, 4, and 6 weeks after Kiss injection was also analyzed using a plate

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

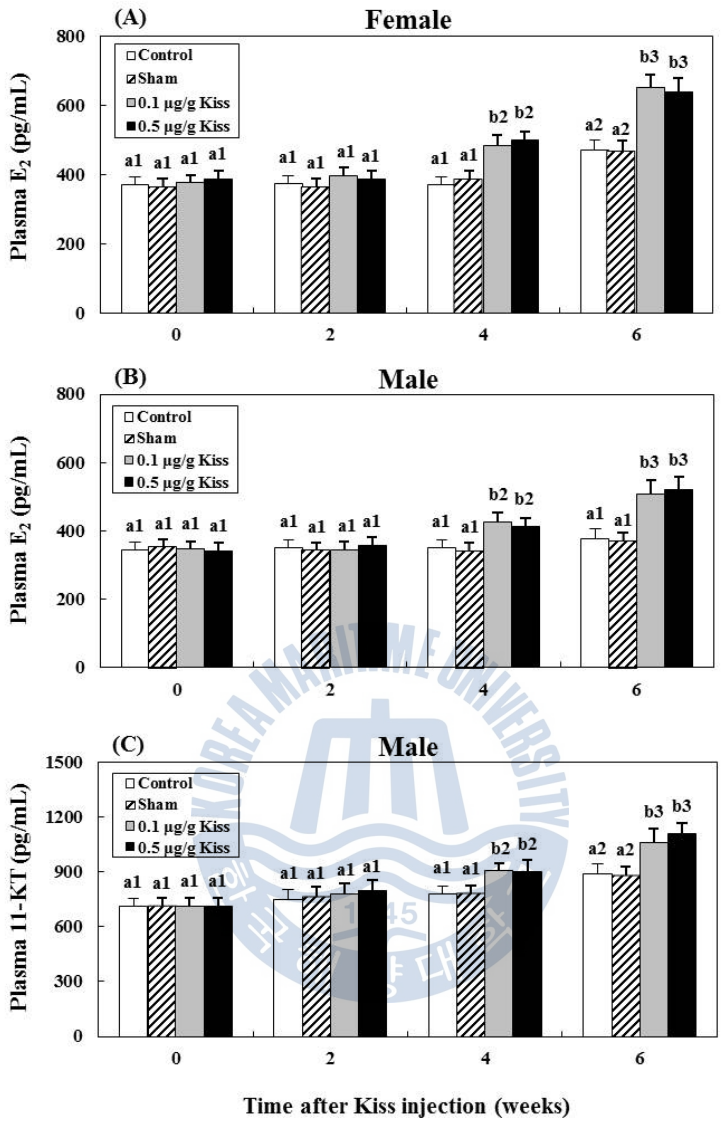

Fig. 38. Plasma E_2 (A, females; B, male) and 11-KT (C, males) levels of cinnamon clownfish at 2, 4, and 6 weeks after Kiss injection (0.1 and 0.5 μg/g) were also analyzed using a plate reader. Numbers indicate significant differences between different time points at the same Kiss concentration, and lowercase alphabet letters indicate significant differences between different Kiss concentrations at a single time point ($P < 0.05$). All values are mean ± SE ($n = 5$).

3.7. GSI and histological observations

The GSI of female cinnamon clownfish injected with Kiss were significantly greater than those of the control fish (Table 4), and the greatest effect was observed at the end of the experiment (6 weeks), at which point the Kiss injection group (0.1 and 0.5 μg/g) exhibited the greatest GSI (2.13 ± 0.22 and 2.42 ± 0.18 cm, respectively, for the 2 doses). Similar to the GSI of male cinnamon clownfish, the GSI of male cinnamon clownfish were greater than those of control fish (Table 5). The GSI was 0.11 ± 0.03 at the beginning of the experiment. Following the Kiss injection, the GSI increased to approximately 4.3-fold (GSI = 0.50 ± 0.07, Table 5) higher at 6 weeks than control ($P < 0.05$).

The gonads of females and males mainly consisted of testicular tissue with primary oocytes (Fig. 39A and E). In female cinnamon clownfish, the oocytes had developed and were largest in size (Fig. 39C, from the Kiss injection to 4 weeks), the diameter of the oocytes increased, the testicular tissue regressed, and finally, the fish became female (Fig. 39D, from the Kiss injection to 6 weeks). In contrast, the size of the testicular tissue and the primary oocytes were maintained in the gonads of males (Fig. 39F, from the Kiss injection to 4 weeks). Much of the testicular tissue then increased and contained spermatids (Fig. 39H, from the Kiss injection to 6 weeks).

Table 4. Changes of the GSI in female cinnamon clownfish

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

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

Table 5. Changes of the GSI in male cinnamon clownfish

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

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

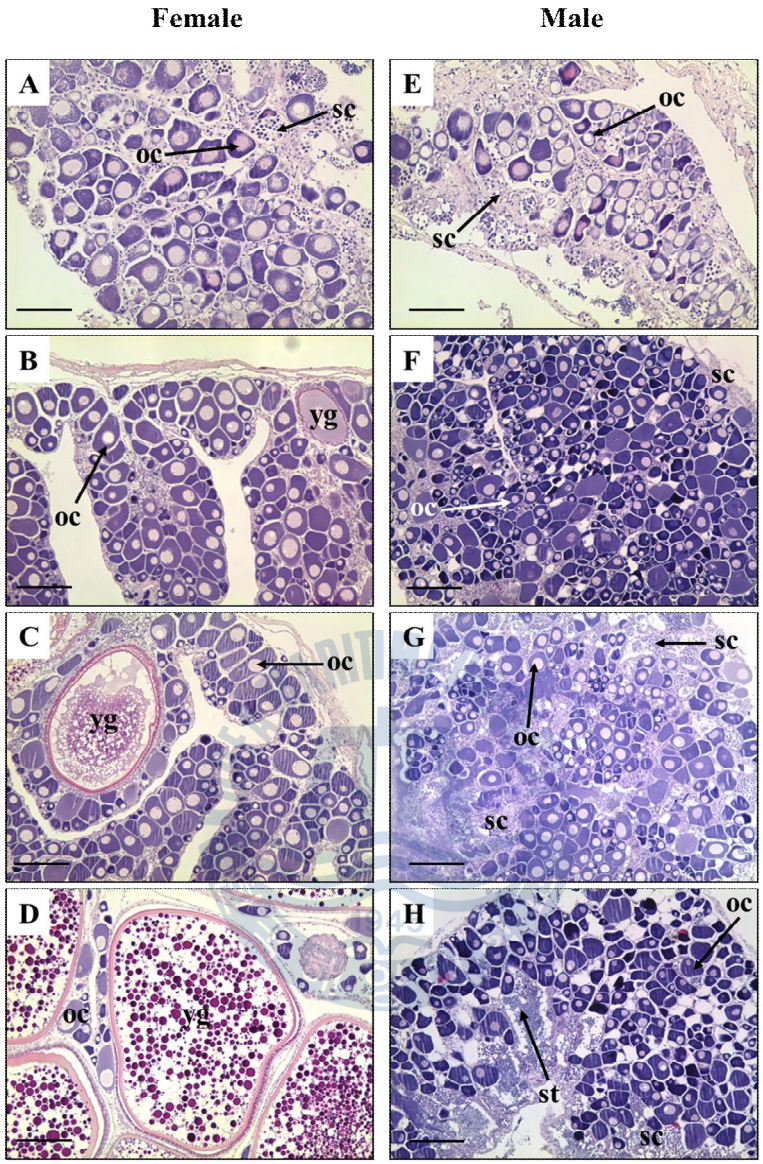

Fig. 39. Photomicrographs of cross sections of cinnamon clownfish after treatment with Kiss (0.5 μg/g). Females: (A) controls (un-injected control), (B) 2 weeks after treatment with Kiss, (C) 4 weeks after treatment with Kiss, and (D) 6 weeks after treatment with Kiss. Males: (E) controls (un-injected control), (F) 2 weeks after treatment with Kiss, (G) 4 weeks after treatment with Kiss, and (H) 6 weeks after treatment with Kiss. oc: oocytes, sc: spermatocytes, st: spermatids, yg: yolk granules. Scale bars = 10 μm.

4. Discussion

The effects of 6 weekly injections of Kiss on the regulation mechanism of sexual maturation in pairs of cinnamon clownfish, as assessed by studying the effects of Kiss on GnRHs, GTHs, GTH receptors, ERs, and Vtg mRNA expression, and the plasma concentrations of the precursor of gonadal steroid hormone, 17α-hydroxypregnenolone, E_2, and 11-KT was investigated. In addition, the maturity of female and male gonads was also examined using histological analyses after the injection of Kiss and investigated the effects of Kiss on the development of oocytes and spermatocytes.

This study was investigated the effects of HPG axis activity and the process of sexual maturation after weekly injections of Kiss (0.1 and 0.5 μg/g of body weight) in each pair of cinnamon clownfish over a 6-week period. The mRNA expression levels was observed and the 3 types of GnRH (sGnRH, sbGnRH, and cGnRH-II) in the hypothalamus, as well as plasma GnRH concentrations, increased significantly in all experimental groups, but there were no significant differences between the different Kiss treatment concentrations (Figs. 30 and 31). In particular, the expression of GnRH mRNA and the plasma GnRH levels increased significantly after 2 or 4 weeks of Kiss treatment and showed the greatest increase at 6 weeks. According to the histological observations of Irwig et al. (2004), Kiss is located with GnRH neurons in the hypothalamus. The interaction between GnRH and Kiss is a function of the GnRH regulator, which regulates gonadal steroid hormones. These hormones interact with Kiss1 and are conveyed to the brain as a feedback mechanism. In addition, Kiss administration now offers promising and efficient stimulation of hypothalamic GnRH release, as observed in previous studies in fish, including the medaka *Oryzias latipes* (Zhao and Wayne, 2012), and yellowtail kingfish *Seriola lalandi* (Nocillado et al., 2012). Similar to the previous study, the results of this study was demonstrated high GnRH expression as a result of Kiss treatment. This study have considered the possibility that Kiss increases the

expression of GnRH, and the activity of the HPG axis plays an important role in the sexual maturation of teleosts. In particular, this study observed higher mRNA expression level and secretion of GnRH in females (Fig. 30) than in males (Fig. 31) during Kiss treatment, which is consistent with a previous study indicating that Kiss influences GnRH secretion to a greater extent in females than that in males (Cheng et al., 2010; Pita et al., 2011). Kiss is distributed largely in the anteroventral periventricular nucleus (AVPV) of the POA, more so in females than that in males (Bleier et al., 1982), which is why—with respect to GnRH secretion—Kiss is believed to affect the maturation of females to a greater extent than that of males.

In this study, GTH changes was investigated according to neuron expression levels of GnRH, the secretion of which was in turn stimulated by Kiss. We observed that the levels of GTHs (GTHα, FSHβ, and LHβ) in the pituitary, plasma FSH and LH, and GTH receptors (FSHR and LHR) increased significantly in all experimental groups (Figs. 32–34). GTH levels in the pituitary increased with Kiss injections; increased GTH stimulates germ intracellular cAMP, which regulates the expression of GTH receptors (Vischer et al., 2003). In addition, FSH regulates the early phases of gametogenesis, such as vitellogenesis and spermatogenesis, and LH stimulates the final maturation stages, such as ovulation and spermatogenesis (Swanson et al., 2003). In the present study, Kiss treatment in cinnamon clownfish significantly increased FSH and LH levels at 6 weeks. In the present study, the FSH and LH levels of cinnamon clownfish after Kiss treatment increased significantly, with the highest value at 6 weeks. The results of this study were similar to previous reports that FSH and LH expression increased with Kiss injections in the adult female zebrafish (Kitahashi et al., 2009), goldfish (Li et al., 2009), and male European sea bass (Felip et al., 2009) because of stimulation of the HPG axis. In addition, LH secretion in the pituitary cells of goldfish (Chang et al., 2012) increased after Kiss treatment (*in vitro*). In rodents, Kiss1 neurons have been found to express both estrogen and androgen receptors, which explains how gonadal steroids can have both stimulatory and inhibitory effects on Kiss1 expression (Smith et al., 2005; Kauffmann et al., 2007). Furthermore, in sheep,

both positive and negative feedback of sex steroids are mediated by Kiss neurons (Estrada et al., 2006). It was proposed that Kiss1 neural sensitivity to estrogen not only provides a critical estradiol-dependent amplification mechanism to activate GnRH neurons and complete the onset of puberty, but also subsequently facilitates the regular preovulatory GnRH/LH surge in adult females (Clarkson et al., 2010). Recent work in female zebrafish has similarly shown that Kiss2 neurons of the ventral hypothalamus are estrogen-sensitive (Servili et al., 2011), with estradiol treatment causing a significant increase in both Kiss1 and Kiss2 mRNA expression in the brain of juvenile zebrafish (Servili et al., 2011). It was hypothesized that Kiss directly influences pituitary cells.

Kiss interacts with various steroid hormones in the gonads (Colledge, 2009), and with E_2 and ERs to synthesize combined Vtg in the liver, thus affecting oocyte maturation (Bowman et al., 2002; Davis et al., 2009). In this study, the expression levels of ERα, ERβ1, Ersβ2, and Vtg mRNAs, and the plasma concentrations of 17α-hydroxypregnenolone and E_2, increased at 6 weeks (Figs. 35, 37, and 38A). Choi et al. (2007) reported that GnRH treatment increased gonadal ER and that plasma E_2 and E_2 regulated the development and maturation of oocytes. Similarly, in this study, Kiss treatment regulated ER mRNA expression and the plasma E_2 level, thus playing an important role in the sexual maturation of cinnamon clownfish was hypothesized. Plasma 17α-hydroxypregnenolone levels were significantly higher in the Kiss treatment group than that in the other groups (Fig. 37B). 17α-Hydroxypregnenolone is generated fromplasma steroids during steroidogenesis by stimulating LH and FSH (Hu et al., 2001), is involved in sexual maturation along with progesterone and 17-hydroxyprogesterone, and plays a role in the formation of the egg yolk (Canario and Scott, 1988). Thus, this study has hypothesized that increasing FSH and LH with the concentrations of 17α-hydroxypregnenolone stimulates the formation of the yolk and affects final maturation. This result was supported by histological observations: mature oocyte cells were observed in many Kiss-treated female gonad tissues, in which accelerated sexual maturation was confirmed (Fig. 39D).

The expression levels of ERα, ERβ1, and Ersβ2 and the plasma concentrations of E_2 and 11-KT, which acts as an androgen and determines the degree of male maturation was also determined. In this study, the expression levels of ERα, ERβ1, and Ersβ2, and plasma concentrations of E_2 increased at 6 weeks (Figs. 36 and 38B). The results of this study were similar to previous reports that E_2 promotes early spermatogonia renewal, albeit in some species, such as medaka (Song and Gutzeit, 2003), and the gilthead seabream (Kadmon et al., 1985). Similarly, it was hypothesized that Kiss treatment regulated ERs mRNA expression and plasma E_2 level, thus playing an important role in the sexual maturation of cinnamon clownfish. In addition, plasma 11-KT levels were increased by Kiss treatment in the male cinnamon clownfish (Fig. 38C). Sex steroids are known to be involved in the progression of spermatogenesis. In general, levels of androgens, particularly 11-KT, are shown to increase gradually as spermatogenesis progresses (Schulz et al., 2010). Selvaraj et al. (2013) reported that circulating levels of 11-KT in Kiss-treated fish increased in correlation with an increase in the GSI and progression of spermatogenesis, suggesting their involvement in the acceleration of spermatogenesis in Kiss-treated fish.

After processing, many mature spermatocytes were observed in the gonadal tissue of Kiss-treated male cinnamon clownfish at 6 weeks. Therefore, Kiss induced testes development in cinnamon clownfish (Fig. 39H).

In summary, Kiss, which is located in the POA cells of the hypothalamus, stimulated the secretion of GnRH in female and male cinnamon clownfish and played an important role in PG axis activity. Kiss also affected the sexual maturation of cinnamon clownfish, and which made it possible to hypothesize that Kiss can be widely distributed in the hypothalamus and plays an important role in the HPG axis activity and the final maturation of the gonads. In this study, Kiss affected the activity of the HPG axis in the cinnamon clownfish, a teleost that can undergo sex changes. Therefore, this study have been considered that the basic processes involved in teleost sexual maturation.

Chapter 7.

General Discussion

Teleosts are among the most diverse species and display a wide range of plasticity in reproduction and gonadal development. There are both hermaphroditic and gonochoristic species that reproduce once a year, once in a lifetime or several times per year. While reproduction of gonochoristic teleosts has been studied extensively over many decades, much less information is available on reproductive biology of hermaphroditic species. Among hermaphroditic teleosts more information is available on reproductive endocrinology of protogynous hermaphrodite teleosts than protandrous species that begins life as a male and then changes into a female at a later stage. Cinnamon clownfish *Amphiprion melanopus* is a protandrous hermaphroditic teleosts, and study of its reproductive biology as a model species would be important to understand evolution and diversity of reproductive endocrinology among teleosts. To better investigate control of reproduction of cinnamon clownfish, after the groups consisting of a mated pair (dominant female and male) were made, sex change was induced in male fish by removing the female from each group and adding the immature clownfish. After 60, 90, and 120 days after removal of the female, the male fish underwent sex change from male to female.

I. GnRHs play pivotal roles in the control of reproduction and gonadal maturation in teleost fish. Fish have multiple GnRH genes that encode structurally distinct peptides. The full-length cDNAs of three molecular forms of prepro-GnRH (sGnRH, sbGnRH, and cGnRH-II) was isolated in the gonads of cinnamon clownfish. Gonadal GnRH variant mRNA levels and circulating levels of E_2 was subsequently investigated during sex change from male to female. Since changes in GnRH variant expression was observed at different stages of gonadal development, the effects of GnRH peptides were investigated on sGnRH, sbGnRH, and cGnRH-II

mRNA levels, *in vivo* and *in vitro* in immature cinnamon clownfish gonads. The deduced amino acid sequences of the three prepro-GnRHs were found to be highly similar to those of other species. Also, the expression levels of sGnRH, sbGnRH, and cGnRH-II mRNA were higher in mature testes and ovaries, as compared to the levels in gonads at earlier stages of maturity. These results support previous evidence indicating that the level of GnRH mRNA decreases in switching testes compared to mature testes, and that the level of GnRH mRNA is generally highest in the mature ovaries of the gilthead seabream (Soverchia et al., 2007) and black porgy (An et al., 2008a). GnRH transcript level was gradually increased during the stages of ovarian development. The present results suggests that the three GnRH forms might be important players in paracrine control of ovarian development and sex-change process in cinnamon clownfish and other group of sequential hermaphrodites. The high levels of GnRH in mature testes and ovaries may help to maintain the appropriate ratio of Sertoli cells to gametes by stimulating apoptosis in the testes and ovaries. Similarly, the levels of the three prepro-GnRH mRNA species and the plasma E_2 levels increased after injection of the three GnRH variants. The levels of sbGnRH gene expression increased significantly following injection of the three GnRHs. These findings support the hypothesis that especially sbGnRH plays important roles in the regulation of gonadal development and sex change in cinnamon clownfish. These findings support the hypothesis that GnRH peptides play important roles in the regulation of the HPG axis and are probably involved in paracrine control of gonadal development and sex change in cinnamon clownfish.

II. GTHs are the key regulators of reproduction in vertebrates. The molecular characterization of GTH and GTHRs were provided to previous study (An et al., 2010). Here, a method to produce biologically active rGTHs, rLH and rFSH, which was used to provide novel information on autoregulation of LH and FSH-induced responses is described. Given the size of these fish, it would be very difficult to extract and purify pituitary LH and FSH, and production of rLH and rFSH are the

best approach. The present study is also important as it validate previously described approach to produce single chain recombinant LH and FSH (Kobayashi et al., 2010). So, the present study investigated autoregulatory effects of GTHs, using produced rGTHs (rFSH and rLH) in cinnamon clownfish. Experiments were carried out to investigate the actions of cinnamon clownfish rFSH and rLH on expression of GTH subunits (FSH and LH), GTHRs (FSHR and LHR), and Vtg mRNA *in vivo* and *in vitro*. Plasma E_2 level was also measured in immature fish following treatments with rFSH and rLH. The results demonstrate increasing levels of GTHs, GTHRs, Vtg mRNA levels, as well as plasma E_2 levels following injection with rFSH and rLH. The results indicate that single chain rLH and rFSH are biologically active and exert specific autoregulatory actions by upregulation of GTH subunits in the pituitary and gonads. The present results demonstrate that rFSH has greater potency in up regulating FSHβ transcript level as well as FSHR in cinnamon clownfish. Likewise, rLH was found to have greater potency in up regulating LHβ transcript level as well as LHR. These observations provide novel information on homologous upregulatory mechanisms underlying GTH-mediated control of reproduction in cinnamon clownfish. The results was hypothesized to have provided indirect evidence for LH and FSH receptor specificity. The observed autoregulatory mechanism may be particularly important during ovulation to facilitate maximum gonadotropic response to LH and FSH by upregulating LHR and FSHR in gonadal tissue as well as increasing production of GTHs in cinnamon clownfish. The findings support the hypothesis that LH and FSH stimulate reproduction, in part, by autoregulatory mechanisms leading to upregulation of GTH receptors and GTH hormone production in cinnamon clownfish. The results provide a framework for better understanding of the mechanisms of GTH-mediated control of reproduction in cinnamon clownfish and other vertebrates.

III. In the present study, we investigated the expression pattern of ER and Vtg mRNA in the gonads and liver during sex change in cinnamon clownfish. We divided gonadal development during the sex change from male to female into 3

stages (mature male, male at 90 days after removing female, and mature female) and investigated ER and Vtg mRNA expressions during the sex change. Also, to understand the role of ER and Vtg genes in the gonads and livers, the expression of ER subtypes and Vtg mRNA and plasma E_2 levels after E_2 treatment (*in vivo* and *in vitro* experiments) was examined in the gonads and livers of cinnamon clownfish. With female, the ER and Vtg mRNA expressions increased. In western blot analysis, ERα protein was detected only in the ovaries of female cinnamon clownfish, and plasma E_2 was highest in females during the sex change. Generally, ER and Vtg genes are involved in development and maturity of oocytes in fish, as well as E_2 (Nagahama et al., 1995; Tyler and Sumpter, 1996). It has been reported that ER and Vtg are involved in maturity of oocytes after synthesis of Vtg by binding E_2 and ER in the liver (Flouriot et al., 1996; Bowman et al., 2002; Davis et al., 2009), so it was judged that oocytes developed as a result of increasing ER and Vtg mRNA levels during sex change to female in cinnamon clownfish. Also, to understand the effect of E_2, we investigated the ER and Vtg mRNA expression patterns in the gonads and liver, and the changes in plasma E_2 level after E_2 injection. E_2 treatment increased both mRNA expression levels of ER and Vtg and plasma E_2 levels. Among ER subtypes, ERα had the highest levels after E_2 injection, and previous studies demonstrated that ERα was important for the synthesis of Vtg after E_2 injection (Sabo-Attwood et al., 2004; Marlatt et al., 2008). In the present study, ERα mRNA was highest, and it was thought that ERα affected Vtg synthesis in cinnamon clownfish. In addition, this result judged that the increased expressions of ER and Vtg in the gonads and livers may be because E_2 was a regulator of these genes in cinnamon clownfish. The present study describes the molecular characterization of ER subtypes and the interactions between ER and Vtg after E_2 treatment in cinnamon clownfish.

IV. The caspase-3 appears to be a key protease in the apoptotic pathway. We identified caspase-3 cDNAs from the ovaries of the protandrous cinnamon clownfish, and investigated its mRNA and proteins, and activity levels, and

histological during the sex change from male to female (I, mature male; II, male at 90 days after removing of the female; and III, mature female). In this study further was investigated the effect of GnRHa on caspase-3 mRNA level and activity in immature cinnamon clownfish. The nucleotide sequence of the caspase-3 cDNA was 969 base pairs in length with ORF encoding peptides of 282 amino acids. The caspase-3 mRNA and protein, and activity levels in stages of the mature gonad are higher than those of the development gonad stage. These results are consistent with previous studies that demonstrated apoptosis during normal spermatogenesis and postovulatory regression in teleost gonad in rainbow trout (Uchida et al., 2002) and goldfish (Wood and Van Der Kraak, 2001). It can logically be postulated that caspase-3 may play a role in induction of testicular apoptosis in a process leading to the development of ovary and development of oocytes in protandrous cinnamon clownfish. As a result, it was found that the oocytes were developed and testicular tissue was regressed in the ovary as the cinnamon clownfish during changed from male to female. This result was similar to the previous study (Godwin, 1994). This study therefore was suggested that gonad development and maturation were controlled by the pituitary-gonad axis through increases in the levels of caspase-3 mRNA in cinnamon clownfish. To understand the effect of GnRH on gonad apoptosis, we examined expression of genes caspase-3 mRNA and activity level in immature cinnamon clownfish gonads after GnRHa. The stimulation of caspase-3 was observed at the highest dose of GnRHa tested presumably due to increasing proapoptotic proteins, opposing the anti-apoptotic actions of gonadotropin hormones and growth factors (Parborell et al., 2002). The findings support the hypothesis that caspase-3 expression is associated with both testicular and ovarian development, and suggests that it may play a role in the control of ovarian development in cinnamon clownfish. Also, we demonstrate that GnRH agonists stimulate caspase-3 production which can in turn stimulate apoptosis. The present study provides a framework for better understanding of the role of caspase-3 during sex change processes in fish.

V. Kiss have been recognized as potent regulators of reproduction in teleosts,

and Kiss is suggested to be a key regulator of the HPG. However, its regulatory role on reproduction in fish remains unclear. Therefore, aim of this study was investigated the effects of 6 weekly injections of Kiss on the regulation mechanism of sexual maturation in pairs of cinnamon clownfish, as assessed by studying the effects of Kiss on GnRHs, GTHs, GTH receptors, ERs, and Vtg mRNA expression, and the plasma concentrations of the precursor of gonadal steroid hormone, 17α-hydroxypregnenolone, E_2, and 11-KT. In addition, the maturity of female and male gonads was also examined using histological analyses after the injection of Kiss and investigated the effects of Kiss on the development of oocytes and spermatocytes. The expression levels of HPG axis genes increased after the Kiss injection. In addition, the levels of plasma 17α-hydroxypregnenolone, E_2, and 11-KT increased. According to the histological observations of Irwig et al. (2004), Kiss is located with GnRH neurons in the hypothalamus. The interaction between GnRH and Kiss is a function of the GnRH regulator, which regulates gonadal steroid hormones. These hormones interact with Kiss1 and are conveyed to the brain as a feedback mechanism. It have been considered the possibility that Kiss increases the expression of GnRH, and the activity of the HPG axis plays an important role in the sexual maturation of teleosts. Furthermore, Kiss interacts with various steroid hormones in the gonads (Colledge, 2009), and with E_2 and ERs to synthesize combined Vtg in the liver, thus affecting oocyte maturation (Bowman et al., 2002; Davis et al., 2009). These results support the hypothesis that Kiss play important roles in the regulation of the HPG axis and are most likely involved in gonadal development and sexual maturation in cinnamon clownfish.

In conclusion, this study showed that changes in the expression of HPG axis gene (GnRHs, GTHs, GTHRs, ERs, and Vtg) and apoptosis related gene (caspase-3), Kiss, such as sex maturation-related gene, as well as in the levels of plasma GnRH, FSH, LH, E_2, caspase-3 levels during sex change from male to female and hormone treatment (3 types of GnRHs, GnRHa, E_2, and Kiss). As the results, in cinnamon clownfish, the 3 type of GnRHs (sGnRH, sbGnRH, and

cGnRH-II) mRNA expression levels were found to be higher in mature testes and ovaries, as compared to gonads at different stages of maturity. The levels of sbGnRH gene expression increased significantly following injection of the three GnRHs. These findings support the hypothesis that especially sbGnRH plays important roles in the regulation of gonadal development and sex change in cinnamon clownfish. Also, present study provides information on the production of biologically active single chain recombinant cinnamon clownfish LH and FSH, which were subsequently used in experiments that provide valuable insight into autoregulatory mechanisms of gonadotropin control of reproduction in cinnamon clownfish. Furthermore, caspase-3 production was incrase during the male-to-female sex change process in clownfish. GnRH agonists stimulate caspase-3 production which can in turn stimulate apoptosis. Kiss, which is located in the POA cells of the hypothalamus, stimulated the secretion of GnRH in female and male cinnamon clownfish and played an important role in PG axis activity. Kiss also affected the sexual maturation of cinnamon clownfish, and Kiss was hypothesized to have widely distributed in the hypothalamus and plays an important role in the HPG axis activity and the final maturation of the gonads.

In summary, these findings support the hypothesis that HPG axis gene (GnRHs, GTH, ERs, and Vtg), caspase-3, and Kiss play important roles in the regulation of the hypothalamic-pituitary-gonadal axis and are probably involved in paracrine control of gonadal development and sex change in cinnamon clownfish. The finding will enhance understanding of hormonal control of reproduction in protandrous cinnamon clownfish which is used here as a suitable model to investigate reproductive endocrinology in a protandrous hermaphroditic teleost. In addition, this study have been considered the basic processes involved in teleost sexual maturation.

Acknowledgements

해양과학 연구에 대한 큰 꿈을 갖고 해양분자환경생리학 연구실에 입실하여 10여년 만에 연구의 결과를 맺게 되었습니다. 그동안 아낌없는 조언과 관심을 주신 분들께 박사과정을 마치면서 진심으로 감사의 마음을 전하고자 합니다. 먼저, 학부 시절부터 분자생물학을 접하며 많은 과학적 지식을 가르쳐 주시고 아낌없는 조언을 해주신 최철영 교수님께 깊은 감사의 마음을 전해드립니다. 그리고 전공 분야와 관련하여 다양한 학문적 소양뿐만 아니라 따뜻한 조언을 주신 박인석 교수님과 조성환 교수님, 공사다망하신 와중에도 박사학위 논문을 애정으로 봐주시고 세심한 부분까지 다듬어 주신 국립수산과학원 김봉석 박사님과 많은 조언을 아끼지 않으신 한국해양과학기술원 최영웅 박사님께도 감사의 말씀을 올립니다. 대학교 입학 후, 학문적 기초를 만들어 주신 강효진, 노일, 서영완, 안종웅, 이경은, 이호진, 임선영 교수님들과 부경대학교 장영진 교수님, 강릉대학교 진덕희 교수님, 박흠기 교수님께도 진심으로 감사드립니다. 해양분자환경생리학 연구실에서 오랜 시간 같이 생활하며 의지할 수 있었던 영재 오빠, 지용이, 진아 후배님들과, 졸업 후에도 많은 관심과 격려를 아끼지 않은 조필규 박사님, 민병화 박사님, 신현숙 박사님, 박찬흠 선배님께도 감사드립니다. 마지막으로 항상 무한한 사랑으로 저를 믿고 아껴주시는 아버지와 어머니, 그리고 여동생 나윤이와 많은 관심과 애정을 주시는 친지 분들께 이 논문을 바칩니다. 논문이 완성되기까지 도와주신 소중한 분들에게 다시 한 번 감사의 말씀을 올립니다.

References

Adams, B.A., Tello, J.A., Erchegyi, J., Warby, C., Hong, D.J., Akinsanya, K.O., Mackie, G.O., Vale, W., Rivier, J.E., Sherwood, N.M., 2003. Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate *Ciona intestinalis*. Endocrinology 144, 1907–1919.

Adams, B.A., Vickers, E.D., Warby, C., Park, M., Fischer, W.H., Craig, A.G., Rivier, J.E., Sherwood, N.M., 2002. Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid *Coregonus clupeaformis*. Biol. Reprod. 67, 232–239.

Amano, M., Urano, A., Aida, K., 1997. Distribution and function of gonadotropin-releasing hormone (GnRH) in the teleost brain. Zool. Sci. 14, 1–11.

An, K.W., Lee, J., Choi, C.Y., 2010. Expression of three gonadotropins and gonadotropin receptor mRNA during male-to-female sex change in the cinnamon clownfish *Amphiprion melanopus*. Comp. Biochem. Physiol. A 156, 407–415.

An, K.W., Lee, K.-Y., Yun, S.G., Choi, C.Y., 2009. Molecular characterization of gonadotropin subunits and gonadotropin receptors in black porgy *Acanthopagrus schlegeli*: effects of estradiol-17β on mRNA expression profiles. Comp. Biochem. Physiol. B 152, 177–188.

An, K.W., Nelson, E.R., Habibi, H.R., Choi, C.Y., 2008a. Molecular characterization and expression of three GnRH forms mRNA during gonad sex-change process, and effect of GnRHa on GTH subunits mRNA in the protandrous black porgy *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol. 159, 38–45.

An, K.W., Nelson, E.R., Jo, P.G., Habibi, H.R., Shin, H.S., Choi, C.Y., 2008b. Characterization of estrogen receptor β2 and expression of the estrogen receptor subtypes α, β1, and β2 in the protandrous black porgy *Acanthopagrus schlegeli* during the sex change process. Comp. Biochem. Physiol. B 150, 284–291.

Andersson, E., Fjelldal, P.G., Klenke, U., Vikingstad, E., Taranger, G.L., Zohar, Y., Stefansson, S.O., 2001. Three forms of GnRH in the brain and pituitary of the turbot *Scophthalmus maximus*: immunological characterization and seasonal variation. Comp. Biochem. Physiol. B 129, 551–558.

Ando, H., Urano, A., 2005. Molecular regulation of gonadotropin secretion by gonadotropin-releasing hormone in salmonid fishes. Zool. Sci. 22, 379–389.

Andreu-Vieyra, C.V., Buret, A.G., Habibi, H.R., 2005. Gonadotropin-releasing hormone induction of apoptosis in the testes of goldfish *Carassius auratus*. Endocrinology 146, 1588–1596.

Andreu-Vieyra, C.V., Habibi, H.R., 2000. Factors controlling ovarian apoptosis. Can. J. Physiol. Pharmacol. 78, 1003–1012.

Andreu-Vieyra, C.V., Habibi, H.R., 2001. Effects of salmon GnRH and chicken GnRH-II on testicular apoptosis in goldfish *Carassius auratus*. Comp. Biochem. Physiol. B 129, 483–487.

Andrews, W.V., Mauer, R., Conn, P.M., 1988. Stimulation of rat luteinzing hormone β-messenger RNA levels by gonadotropin-releasing hormone. J. Biol. Chem. 263, 13755–13761.

Auchus, R.J., Fuqua, S.A., 1994. Hormone-nuclear receptor interactions in health and disease. The estrogen receptor. Baillières Clin. Endocrinol. Metab. 8, 433–449.

Billard, R., 1986. Spermatogenesis and spermatology of some teleost fish species. Reprod. Nutr. Dev. 26, 877–890.

Billig, H., Chun, S.Y., Eisenhauer, K., Hsueh, A.J., 1996. Gonadal cell apoptosis, hormone-regulated cell demise. Hum. Reprod. 2, 103–117.

Bleier, R., Byne, W., Siggelkow, I., 1982. Cytoarchitectonic sexual dimorphisms of the medial preoptic and anterior hypothalamic areas in guinea pig, rat, hamster, and mouse. J. Comp. Neurol. 212, 118–130.

Boone, D.L., Tsang, B.K., 1998. Caspase-3 in the rat ovary: localization and possible

role in follicular atresia and luteal regression. Biol. Reprod. 58, 1533–1539.

Bouma, J., Nagler, J.J., 2001. Estrogen receptor-a protein localization in the testis of the rainbow trout *Oncorhynchus mykiss* during different stages of the reproductive cycle. Biol. Reprod. 65, 60–65.

Bowman, C.J., Kroll, K.J., Gross, T.G., Denslow, N.D., 2002. Estradiol-induced gene expression in largemouth bass *Micropterus salmoides*. Mol. Cell. Endocrinol. 196, 67–77.

Canario, A.V.M., Scott, A.P., 1988. Structure–activity relationships of C21 steroids in an *in vitro* oocyte maturation bioassay in rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 71, 338–348.

Chang, C.F., Lau, E.F., Lin, B.Y., 1995. Estradiol-17β suppresses testicular development and stimulates sex change in protandrous black porgy, *Acanthopagrus schlegeli*. Fish Physiol. Biochem. 14, 481–488.

Chang, J.P., Johnson, J.D., Sawisky, G.R., Grey, C.L., Mitchell, G., Booth, M., Volk, M.M., Parks, S.K., Thompson, E., Goss, G.G., Klausen, C., Habibi, H.R., 2009. Signal transduction in multifactorial neuroendocrine control of gonadotropin secretion and synthesis in teleosts-studies on the goldfish model. Gen. Comp. Endocrinol. 161, 42–52.

Chang, J.P., Mar, A.,Wlasichuk, M., Wong, A.O.L., 2012. Kisspeptin-1 directly stimulates LH and GH secretion from goldfish pituitary cells in a Ca^{2+}-dependent manner. Gen. Comp. Endocrinol. 179, 38–46.

Chang, X., Kobayashi, T., Todo, T., Ikeuchi, T., Yoshiura, Y., Kajiura-Kobayashi, H., Morry, C., Nagahama, Y., 1999. Molecular cloning of estrogen receptors α and β in the ovary of a teleost fish, the tilapia *Oreochromis niloticus*. Zool. Sci. 16, 653–658.

Cheng, G., Coolen, L.M., Padmanabhan, V., Goodman, R.L., Lehman, M.N., 2010. The kisspeptin/neurokininB/dynorphin (KNDy) cell population of the arcuate

nucleus: sex differences and effects of prenatal testosterone in sheep. Endocrinology 151, 301–311.

Choi, C.Y., An, K.W., Jo, P.G., Kang, D.Y., Chang, Y.G., 2007. Effects of gonadotropin-releasing hormone analog (GnRHa) on steroidogenic factor-1 (SF-1) and estrogen receptor beta (ER beta) gene expression in the black porgy *Acanthopagrus schlegeli*. Comp. Biochem. Physiol. B 147, 82–86.

Choi, C.Y., Habibi, H.R., 2003. Molecular cloning of estrogen receptor α and expression pattern of estrogen receptor subtypes in male and female goldfish. Mol. Cell. Endocrinol. 204, 169–177.

Chowdhury, I., Tharakan, B., Bhat, G.K., 2008. Caspase—an update. Comp. Biochem. Physiol. B 151, 10–27.

Clarkson, J., Han, S.-K., Liu, X., Lee, K., Herbison, A.E., 2010. Neurobiological mechanisms underlying Kisspeptin activation of gonadotropin-releasing hormone (GnRH) neurons at puberty. Mol. Cell. Endocrinol. 324, 45–50.

Colledge, W.H., 2009. Kisspeptins and GnRH neuronal signaling. Trends Endocrinol. Metab. 20, 115–121.

Colombo, G., Chicca, M., 2003. Immunocytochemical studies on the pituitary gland of *Anguilla anguilla* L., in relation to early growth stages and diet-induced sex differentiation. Gen. Comp. Endocrinol. 131, 66–67.

Corriero, A., Medina, A., Mylonas, C.C., Bridges, C.R., Santamaria, N., Deflorio, M., Losurdo, M., Zupa, R., Gordin, H., Gandara, F., Belmonte, R.A., Pousis, C., Metrio, G., 2009. Proliferation and apoptosis of male germ cells in captive Atlantic bluefin tuna *Thunnus thynnus* L. treated with gonadotropin-releasing hormone agonist (GnRHa). Anim. Reprod. Sci. 116, 257–346.

Davis, L.K., Pierce, A.L., Hiramatsu, N., Sullivan, C.V., Hirano, T., Grau, E.G., 2008. Genderspecific expression of multiple estrogen receptors, growth hormone receptors, insulin-like growth factors and vitellogenins, and effects of

17β-estradiol in the male tilapia *Oreochromis mossambicus*. Gen. Comp. Endocrinol. 156, 544–551.

Davis, L.K., Visitacion, N., Riley, L., Hiramatsu, N., Sullivan, C., Hirano, T., Gordon Grau, E., 2009. Effects of *o*,*p*'-DDE, heptachlor, and 17β-estradiol on vitellogenin gene expression and the growth hormone/insulin-like growth factor-I axis in the tilapia *Oreochromis mossambicus*. Comp. Biochem. Physiol. C 149, 507–514.

Dickey, J.T., Swanson, P., 2000. Effects of salmon gonadotropin-releasing hormone on follicle-stimulating hormone secretion and subunit gene expression in coho salmon *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 118, 436–449.

Earnshaw, W.C., Martins, L.M., Kaufmann, S.H., 1999. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 68, 383–424.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., Nagata, S., 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor. ICAD Nature 391, 43–50.

Esterhuyse, M.M., Venter, M., Veldhoen, N., Helbing, C.C., van Wyk, J.H., 2009. Characterization of Vtg-1 mRNA expression during ontogeny in *Oreochromis mossambicus*. J. Steroid Biochem. 117, 42–49.

Estrada, K.M., Clay, C.M., Pompolo, S., Smith, J.T., Clarke, I.J., 2006. Elevated Kiss1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/luteinizing hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. J. Neuroendocrinol. 18, 806–809.

Felip, A., Zanuy, S., Pineda, R., Pinilla, L., Carrillo, M., Tena-Sempere, M., Gomez, A., 2009. Evidence for two distinct kiss genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing

activities in fish and mammals. Mol. Cell. Endocrinol. 312, 61–71.

Fernald, R.D., White, R.B., 1999. Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front. Neuroendocrinol. 20, 224–240.

Filby, A.L., Thorpe, K.L., Tyler, C.R., 2006. Multiple molecular effect pathways of an environmental estrogen in fish. J. Mol. Endocrinol. 37, 121–134.

Filby, A.L., van Aerle, R., Duitman, J., Tyler, C.R., 2008. The Kisspeptin/ Gonadotropin releasing hormone pathway and molecular signaling of puberty in fish. Biol. Reprod. 78, 278–289.

Finn, R.N., Kristoffersen, B.A., 2007. Vertebrate vitellogenin gene duplication in relation to the "3R hypothesis": correlation to the pelagic egg and the oceanic radiation of teleosts. PLoS ONE 169, 1–12.

Fischer, U., Janicke, R.U., Schulze-Osthoff, K., 2003. Many cuts to ruin: a comprehensive update of caspase substrates. Cell. Death. Differ. 10, 76–100.

Flouriot, G., Pakdel, F., Valotaire, Y., 1996. Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. Mol. Cell. Endocrinol. 124, 173–183.

Gen, K., Okuzawa, K., Senthilkumaran, B., Tanaka, H., Moriyama, S., Kagawa, H., 2000. Unique expression of gonadotropin-I and -II subunit genes in male and female red seabream *Pagrus major* during sexual maturation. Biol. Reprod. 63, 308–319.

Godwin, J., 1994. Histological aspects of protandrous sex change in the anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). J. Zool. Lond. 232, 199–213.

Godwin, J.R., Thomas, P., 1993. Sex change and steroid profiles in the protandrous anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). Gen. Comp. Endocrinol. 91, 144–157.

Gothilf, Y., Elizur, A., Zohar, Y., 1995. Three forms of gonadotropin-releasing

hormone in gilthead seabream and striped bass: physiological and molecular studies. In: Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. Austin, TX: Fish Symp. 95, 52–54.

Guiguen, Y., Jalabert, B., Thouard, E., Fostier, A., 1993. Changes in plasma and gonadal steroid hormones in relation to the reproductive cycle and the sex inversion process in the protandrous seabass *Lates calcarifer*. Gen. Comp. Endocrinol. 92, 327–338.

Guilgur, L.G., Moncaut, N.P., Canário, A.V.M., Somoza, G.M., 2006. Evolution of GnRH ligands and receptors in gnathostomata. Comp. Biochem. Physiol. A 144, 272–283.

Guilgur, L.G., Orití, G., Strobl-Mazzulla, P.H., Fernandino, J.I., Mranda, L.A., Somoza, G.M., 2007. Characterization of the cDNAs encoding three GnRH forms in the pejerrey fish *Odontesthes bonariensis* (Atheriniformes) and the evolution of GnRH precursors. J. Mol. Evol. 64, 614–627.

Habibi, H.R., Andreu-Vieyra, C.V., 2007. Hormonal regulation of follicular atresia in the teleost fish. In: Babin, P.J., Cerda, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer Pub. Co., pp. 231–250.

Habibi, H.R., Matsoukas, J., 1999. Gonadotropin-releasing hormone: structural and functional diversity. In: Matsoukas, J., Mavromoustakos, T. (Eds.), Bio-active Peptides in Drug Discovery and Design: Medical Aspects Volume 22 in Biomedical and Health Research. IOS Press, pp. 247–255.

Habibi, H.R., Van der Kraak, G., Fraser, R., Peter, R.E., 1989. Effect of a teleost GnRH analog on steroidogenesis by the follicle-enclosed goldfish oocytes, *in vitro*. Gen. Comp. Endocrinol. 76, 95–105.

Hacker, G., 2000. The morphology of apoptosis. Cell. Tissue Res. 301, 5–17.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and

analysis program for Windows 95/98/NT. Nucleic. Acids. Symp. Ser. 41, 95–98.

Halm, S., Martínez-Rodríguez, G., Rodríguez, L., Prat, F., Mylonas, C.C., Carrillo, M., Zanuy, S., 2004. Cloning, characterization, and expression of three oestrogen receptors (ERα, ERβ1, and ERβ2) in the European sea bass *Dicentrarchus labrax*. Mol. Cell. Endocrinol. 223, 63–75.

Hassin, S., Holland, M.C.H., Zohar, Y., 2000. Early maturity in the male striped bass *Morone saxatilis*: follicle-stimulating hormone and luteinizing hormone gene expression and their regulation by gonadotropin-releasing hormone analogue and testosterone. Biol. Reprod. 63, 1691–1697.

Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P., 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptor in teleosts. Proc. Natl. Acad. Sci. U. S. A. 97, 10751–10756.

Hiramatsu, N., Matsubara, T., Fujita, T., Sullivan, C.V., Hara, A., 2006. Multiple piscine vitellogenins: biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. Mar. Biol. 149, 35–47.

Holland, M.C.H., Gothilf, Y., King, J.A., Okuzawa, K., Elizur, A., Zohar, Y., 1998. Levels of the native forms of GnRH in the pituitary of the gilthead seabream *Sparus aurata* at several characteristic stages of the gonadal cycle. Gen. Comp. Endocrinol. 112, 394–405.

Hong, I.S., Cheung, A.P., Leung Peter, C.K., 2008. Gonadotropin-releasing hormones I and II induce apoptosis in human granulosa cells. J. Clin. Endocrinol. Metab. 93, 3179–3185.

Hu, M.-C., Chiang, E.F.-L., Tong, S.-K., Lai, W., Hsu, N.-C., Wang, L.C.-K., Chung, B.-C., 2001. Regulation of steroidogenesis in transgenic mice and zebrafish. Mol. Cell. Endocrinol. 171, 9–14.

Huang, J.D., Chang, C.F., 2002. Cloning and expression of estrogen receptors in the protandrous black porgy *Acanthopagrus schlegeli*: Implication of sex

change mechanism. Mar. Biotechnol. 4, 236–246.

Hutchinson, T.H., Pickford, D.B., 2002. Ecological risk assessment and testing for endocrine disruption in the aquatic environment. Toxicology 182, 383–387.

Irwig, M.S., Fraley, G.S., Smith, J.T., Acohido, B.V., Popa, S.M., Cunningham, M.J., 2004. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KISS-1 mRNA in the male rat. Neuroendocrinology 80, 264–272.

Ishibashi, O., Kawashima, H., 2001. Cloning and characterization of the functional promoter of mouse estrogen receptor beta gene. Biochim. Biophys. Acta 1519, 223–229.

Janz, D.M., Van Der Kraak, G., 1997. Suppression of apoptosis by gonadotropin, 17β-estradiol, and epidermal growth factor in rainbow trout pre-ovulatory ovarian follicles. Gen. Comp. Endocrinol. 105, 186–193.

Jeffries, K.M., Jackson, L.J., Ikonomou, M.G., Habibi, H.R., 2010. Presence of natural and anthropogenic organic contaminants and potential fish health impacts along two river gradients in Alberta, Canada. Environ. Toxicol. Chem., 29, 2379–2387.

Jeffries, K.M., Nelson, E.R., Jackson, L.J., Habibi, H.R., 2008. Basin-wide impacts of compounds with estrogen-like activity on longnose dace *Rhinichthys cataractae* in two prairie rivers of Alberta, Canada. Environ. Toxicol. Chem. 27, 2042–2052.

Kadmon, G., Yaron, Z., Gordon, H., 1985. Sequence of gonadal events and estradiol levels in *Sparus auratus* (L.) under two photoperiod regimes. J. Fish Biol. 26, 609–620.

Kamei, H., Ohira, T., Yoshiura, Y., Uchida, N., Nagasawa, H., Aida, K., 2003. Expression of a biologically active recombinant follicle stimulating hormone of Japanese eel *Anguilla japonica* using methylotropic yeast, *Pichia pastoris*. Gen. Comp. Endocrinol. 134, 244–254.

Kanda, S., Akazome, Y., Matsunaga, T., Yamamoto, N., Yamada, S., Tsukamura,

H., Maeda, K., Oka, Y., 2008. Identification of Kiss1 product kisspeptin and steroidsensitive sexually dimorphic kisspeptin neurons in medaka *Oryzias latipes*. Gen. Comp. Endocrinol. 149, 2467–2476.

Kauffmann, A.S., Clifton, D.K., Steiner, R.A., 2007. Emerging ideas about kisspeptin GPR54 signaling in the neuroendocrine regulation of reproduction. Trends Neurosci. 30, 504–511.

Kavanough, S.I., Nozaki, M., Sower, S.A., 2008. Origins of GnRH in vertebrates: identification of a novel GnRH in a basal vertebrate, the sea lamprey. Endocrinology 149, 3860–3869.

Kerr, J.F., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239–257.

Kim, N.N., Jin, D.H., Lee, J., Kil, G.-S., Choi, C.Y., 2010. Upregulation of estrogen receptor subtypes and vitellogenin mRNA in cinnamon clownfish *Amphiprion melanopus* during the sex change: Profiles on effects of 17β -estradiol. Comp. Biochem. Physiol. B 157, 198–204.

Kim, N.N., Shin, H.S., Habibi, H.R., Lee, J., Choi, C.Y., 2012. Expression profiles of three types of GnRH during sex-change in the protandrous cinnamon clownfish *Amphiprion melanopus*: Effects of exogenous GnRHs. Comp. Biochem. Physiol. B 161, 124–133.

Kitahashi, T., Ogawa, S., Parhar, I.S., 2009. Cloning and expression of Kiss2 in the zebrafish and medaka. Gen. Comp. Endocrinol. 150, 821–831.

Kitahashi, T., Ogawa, S., Soga, T., Sakuma, Y., Parhar, I., 2007. Sexual maturation modulates expression of nuclear receptor types in laser-captured single cells of the cichlid *Oreochromis niloticus* pituitary. Endocrinology 148, 5822–5830.

Klein, J., Lobel, L., Pollak, S., Lustbader, B., Ogden, R.T., Sauer, M.V., Lustbader, J.W., 2003. Development and characterization of a long-acting recombinant hFSH agonist. Hum. Reprod. 18, 50–56.

Ko, H., Park, W.D., Kim, D.J., Kobayashi, M., Sohn, Y.C., 2007. Biological activities of recombinant Manchurian trout FSH and LH: their receptor specificity, steroidogenic and vitellogenic potencies. J. Mol. Endocrinol. 38, 99–111.

Kobayashi, M., Hayakawa, Y., Park, W., Banba, A., Yoshizaki, G., Kumamaru, K., Kagawa, H., Kaki, H., Nagaya, H., Sohn, Y.C., 2010. Production of recombinant Japanese eel gonadotropins by baculovirus in silkworm larvae. Gen. Comp. Endocrinol. 167, 379–386.

Kobayashi, M., Morita, T., Ikeguchi, K., Yoshizaki, G., Suzuki, T., Watabe, S., 2006. *in vivo* biological activity of recombinant goldfish gonadotropins produced by baculovirus in silkworm larvae. Aquaculture 256, 433–442.

Krust, A., Green, S., Argos, P., Bumar, V., Walter, J.M.B., Chabon, P., 1986. The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocoticoid receptors. EMBO J. 5, 891–897.

Kumar, R.S., Trant, J.M., 2004. Hypophyseal gene expression profiles of FSH-β, LH-β, and glycoprotein hormone-α subunits in *Ictalurus punctatus* throughout a reproductive cycle. Gen. Comp. Endocrinol. 136, 82–89.

Kumar, V., Green, S., Stack, S., Berry, M., Jin, J.R., Chambon, P., 1987. Functional domains of the human estrogen receptor. Cell 51, 941–951.

Kwok, H.F., So, W.K., Wang, Y., Ge, W., 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone receptors—evidence for their distinct functions in follicle development. Biol. Reprod. 72, 1370–1381.

Lee, J., Richburg, J.H., Younkin, S.C., Boekelheide, K., 1997. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology 138, 2081–2088.

Lee, Y.H., Du, J.L., Shih, Y.S., Jeng, S.R., Sun, L.T., Chang, C.F., 2004. *in vivo* and *in vitro* sex steroids stimulate seabream gonadotropin-releasing hormone content and release in the protandrous black porgy *Acanthopagrus schlegeli*.

Gen. Comp. Endocrinol. 139, 12–19.

Lee, Y.H., Du, J.L., Yen, F.P., Lee, C.Y., Dufour, S., Huang, J.D., Sun, L.T., Chang, C.F., 2001a. Regulation of plasma gonadotropin II secretion by sex steroids, aromatase inhibitors, and antiestrogens in the protandrous black porgy, *Acanthopagrus schlegeli* Bleeker. Comp. Biochem. Physiol. B 129, 399–406.

Lee, Y.H., Du, J.L., Yueh, W.S., Lin, B.Y., Huang, J.D., Lee, C.Y., Lee, M.F., Lau, E.L., Lee, F.Y., Morrey, C., Nagahama, Y., Chang, C.F., 2001b. Sex change in the protandrous black porgy *Acanthopagrus schlegeli*: A review in gonadal development, estradiol, estrogen receptor, aromatase activity and gonadotropin. J. Exp. Zool. 290, 715–726.

Lee, Y.H., Lee, F.Y., Tacon, P., Du, J.L., Chang, C.N., Jeng, S.R., Tanaka, H., Chang, C.F., 2000. Profiles of gonadal development, sex steroids, aromatase activity, and gonadotropin II in the controlled sex change of protandrous black porgy *Acanthopagrus schlegeli* Bleeker. Gen. Comp. Endocrinol. 119, 111–120.

Leung, P.C.K., Steele, G.L., 1992. Intracellular signaling in the gonads. Endocrinol. Rev. 13, 476–498.

Li, S., Zhang, Y., Liu, Y., Huang, X., Huang,W., Lu, D., Zhu, P., Shi, Y., Cheng, C., Liu, X., Lin, X., 2009. Structural and functional multiplicity of the kisspeptin/ GPR54 system in goldfish *Carassius auratus*. J. Endocrinol. 201, 407–418.

Lu, R., Wang, W., Uzzau, S., Vigorito, R., Zielke, H.R., Fasano, A., 2000. Affinity purification and partial characterization of the zonulin/zonula occludens toxin (Zot) receptor from human brain. J. Neurochem. 74, 320–326.

Ma, C.H., Dong, K.W., Yu, K.L., 2000. cDNA cloning and expression of a novel estrogen receptor β-subtype in goldfish *Carassius auratus*. Biochim. Biophys. Acta. 1490, 145–152.

Magnadottir, B., Jonsdottir, H., Helgason, S., Bjornsson, B., Jorgensen, T., Pilstrom, L., 1999. Humoral immune parameters in Atlantic cod *Gadus morhua* (L.) I: the

effects of environmental temperature. Comp. Biochem. Physiol. B 122, 173–180.

Marlatt, V.L., Martyniuk, C.J., Zhang, D., Xiong, H., Watt, J., Xia, X., Moon, T., Trudeau, V.L., 2008. Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17β-estradiol action in the neuroendocrine axis of male goldfish. Mol. Cell. Endocrinol. 283, 38–48.

Menuet, A., Pellegrini, E., Anglada, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66, 1881–1892.

Meucci, V., Arukwe, A., 2006. Transcriptional modulation of brain and hepatic estrogen receptor and P450-arom iso-types in juvenile Atlantic salmon *Salmo salar* after waterborne exposure to the xenoestrogen, 4-nonylphenol. Aquat. Toxicol. 77, 167–177.

Millar, R.P., 2003. GnRH II and type II GnRH receptors. Trends Endocrinol. Metab. 14, 35–43.

Millar, R.P., Lu, Z.L., Pawson, A.J., Flanagan, C.A., Morgan, K., Maudsley, S.R., 2004. Gonadotropin-releasing hormone receptors. Endocr. Rev. 25, 235–275.

Miwa, S., Yan, L., Swanson, P., 1994. Localization of two gonadotropin receptors in the salmon gonad by *in vitro* ligand autoradiography. Biol. Reprod. 50, 629–642.

Monteiro, S.M., dos Santos, N.M., Calejo, M., Fontainhas-Fernandes, A., Sousa, M., 2009. Copper toxicity in gills of the teleost fish *Oreochromis niloticus*: effects in apoptosis induction and cell proliferation. Aquat. Toxicol. 94, 219–228.

Nabissi, M., Soverchia, L., Polzonetti-Magni, A.M., Habibi, H.R., 2000. Differential splicing of three gonadotropin-releasing hormone transcripts in the ovary of seabream *Sparus aurata*. Biol. Reprod. 62, 1329–1334.

Nagahama, Y., 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38, 217–229.

Nagahama, Y., Yoshikuni, M., Yamashita, M., Tokumoto, T., Katsu, Y., 1995. Regulation of oocyte growth and maturation in fish. Curr. Top. Dev. Biol. 30, 103–145.

Nelson, E.R., Habibi, H.R., 2010. Functional significance of nuclear estrogen receptor subtypes in the liver of goldfish. Endocrinology 151, 1668–1676.

Nelson, E.R., Wiehler, W.B., Cole, W.C., Habibi, H.R., 2007. Homologous regulation of estrogen receptor subtypes in goldfish *Carassius auratus*. Mol. Reprod. Dev. 74, 1105–1112.

Nocillado, J.N., Biran, J., Lee, Y.Y., Levavi-Sivan, B., Mechaly, A.S., Zohar, Y., Elizur, A., 2012. The kiss2 receptor (Kiss2r) gene in southern bluefin tuna, *Thunnus maccoyii*, and in yellowtail kingfish *Seriola lalandi* - functional analysis and isolation of transcript variants. Mol. Cell. Endocrinol. 362, 211–220.

Oka, Y., 2009. Three types of gonadotropin-releasing hormone neurons and steroidsensitive sexually dimorphic kisspeptin neurons in teleosts. J. Neuroendocrinol. 21, 334–338.

Pakdel, F., Feon, S., Le Gac, F., Le Menn, F., Valotaire, Y., 1991. *In vivo* estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. Mol. Cell. Endocrinol. 75, 205–212.

Pakdel, F., Metivier, R., Flouriot, G., Valotaire, Y., 2000. Two estrogen receptor (ER) isoforms with different estrogen dependencies are generated from the trout ER gene. Endocrinology 141, 571–580.

Parborell, F., Pecci, A., Gonzalez, O., Vitale, A., Tesone, M., 2002. Effects of a gonadotropin-releasing hormone agonist on rat ovarian follicle apoptosis: regulation by epidermal growth factor and the expression of Bcl-2-related genes. Biol. Reprod. 67, 81–486.

Parhar, I.S., Ogawa, S., Sakuma, Y., 2004. Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein coupled receptor (GPR54) during maturation in cichlid fish. Gen. Comp.

Endocrinol. 145, 3613–3618.

Park, M.K., Wakabayashi, K., 1986. Preparation of a monoclonal antibody to common amino acid sequence of LHRH and its application. Endocrinol. Jpn. 33, 257–272.

Pasqualini, R., Koivunen, E., Ruoslahti, E., 1995. A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins. J. Cell. Biol. 130, 1189–1196.

Pati, D., Habibi, H.R., 1998. Presence of salmon GnRH and compounds with GnRH-like activity in the ovary of goldfish. Endocrinology 139, 2015–2024.

Pati, D., Habibi, H.R., 2000. Direct action of GnRH variants on goldfish oocyte meiosis and follicular steroidogenesis. Mol. Cell. Endocrinol. 16075–16088.

Pati, D., Habibi, H.R., 2002. The involvement of protein kinase c and arachidonic acid pathways in the gonadotropin-releasing hormone regulation of oocyte meiosis and follicular steroidogenesis in the goldfish ovary. Biol. Reprod. 66, 813–822.

Patiño, R., Xia, Z., Gale, W.L., Wu, C., Maule, A.G., Chang, X., 2000. Novel transcripts of the estrogen receptor α gene in channel catfish. Gen. Comp. Endocrinol. 120, 314–325.

Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50, 465–495.

Pinto, P.I.S., Teodosio, H.R., Galay-Burgos, M., Power, D.M., Sweeney, G.E., Canario, A.V. M., 2006. Identification of estrogen-responsive genes in the testis of seabream *Sparus auratus* using suppression subtractive hybridization. Mol. Reprod. Dev. 73, 318–329.

Pita, J., Barrios, V., Gavela-Pérez, T., Martos-Moreno, G.Á., Muñoz-Calvo, M.T., Pozo, J., Rovira, A., Argente, J., Soriano-Guillén, L., 2011. Circulating kisspeptin levels exhibit sexual dimorphism in adults, are increased in obese prepubertal girls and do not suffer modifications in girls with idiopathic central precocious puberty. Peptides 32, 1781–1786.

Porter, A.G., Janicke, R.U., 1999. Emerging roles of caspase-3 in apoptosis. Cell. Death Differ. 6, 99–104.

Powell, J.F., Zohar, Y., Elizur, A., Park, M., Fischer, W.H., Craig, A.G., Rivier, J.E., Lovejoy, D.A., Sherwood, N.M., 1994. Three forms of gonadotropin-releasing hormone characterized from brains of one species. Proc. Natl. Acad. Sci. USA. 91, 12081–12085.

Prisco, M., Liguoro, A., Comitato, R., Cardone, A., D'Onghia, B., Ricchiari, L., Angelini, F., Andreuccetti, P., 2003. Apoptosis during spermatogenesis in the spotted ray *Torpedo marmorata*. Mol. Reprod. Dev. 64, 341–348.

Roa, J., Navarro, T.M., Tena-Sempere, M., 2011. Kisspeptins in reproductive biology: consensus knowledge and recent developments. Biol. Reprod. 85, 650–660.

Rocha, A., Gómez, A., Zanuy, S., Cerdá-Reverter, J.M., Carrillo, M., 2007. Molecular characterization of two sea bass gonadotropin receptors: cDNA cloning, expression analysis, and functional activity. Mol. Cell. Endocrinol. 272, 63–76.

Rogers, S.A., Llewellyn, L., Wigham, T., Sweeney, G.E., 2000. Cloning of the Atlantic salmon *Salmo salar* estrogen receptor-α gene. Comp. Biochem. Physiol. B 125, 379–385.

Ryffel, G.U., 1978. Synthesis of vitellogenin, an attractive model for investigating hormone-induced gene activation. Mol. Cell. Endocrinol. 12, 213–221.

Sabo-Attwood, T., Kroll, K.J., Denslow, N.D., 2004. Differential expression of largemouth bass *Micropterus salmoides* estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol. Cell. Endocrinol. 218, 107–118.

Sakahira, H., Enari, M., Nagata, S., 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391, 96–99.

Sambroni, E., Gac, F.L., Breton, B., Lareyre, J., 2007. Functional specificity of the rainbow trout *Oncorhynchus mykiss* gonadotropin receptors as assayed in a mammalian cell line. J. Endocrinol. 195, 213–228.

Sawaguchi, S., Kagawa, H., Ohkubo, N., Hiramatsu, N., Sullivan, C.V., Matsubara, T., 2006. Molecular characterization of three forms of vitellogenin and their yolk protein products during oocyte growth and maturation in red seabream *Pagrus major*, a marine teleost spawning pelagic eggs. Mol. Reprod. Dev. 73, 719–736.

Schulz, R.W., de França, L.R., Lareyre, J.J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

Selvaraj, S., Ohga, H., Nyuji, M., Kitano, H., Nagano, N., Yamaguchi, A., Matsuyama, M., 2013. Subcutaneous administration of Kiss1 pentadecapeptide accelerates spermatogenesis in prepubertal male chub mackerel *Scomber japonicus*. Comp. Biochem. Physiol. A 166, 228–236.

Senthilkumaran, B., Okuzawa, K., Gen, K., Ookura, T., Kagawa, H., 1999. Distribution and seasonal variations in levels of three native GnRHs in the brain and pituitary of perciform fish. J. Neuroendocrinol. 11, 181–186.

Servili, A., Le Page, Y., Leprince, J., Caraty, A., Escobar, S., Parhar, I.S., Seong, J.Y., Vaudry, H., Kah, O., 2011. Organisation of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. Gen. Comp. Endocrinol. 1, 1527–1540.

Shahjahan, M., Hamabata, T., Motohashi, E., Doi, H., Ando, H., 2010. Differential expression of three types of gonadotropin-releasing hormone genes during the spawning season in grass puffer *Takifugu niphobles*. Gen. Comp. Endocrinol. 167, 153–163.

Smith, J.T., Dungan, H.M., Stoll, E.A., Gottsch, M.L., Braun, R.E., Eacker, S.M., Clifton, D.K., Steiner, R.A., 2005. Differential regulation of Kiss1 mRNA expression by sex steroids in the brain of the male mouse. Gen. Comp. Endocrinol. 146, 2976–2984.

So, W.K., Kwok, H.F., Ge, W., 2005. Zebrafish gonadotropins and their receptors:

II. Cloning and characterization of zebrafish follicle stimulating hormone and luteinizing hormone subunits their spatial–temporal expression patterns and receptor specificity. Biol. Reprod. 72, 1382–1396.

Socorro, S., Power, D.M., Olsson, P.E., Canario, A.V.M., 2000. Two estrogen receptors expressed in the teleost fish *Sparus aurata*: cDNA cloning, characterization and tissue distribution. J. Endocrinol. 166, 293–306.

Song, M., Gutzeit, H.O., 2003. Effect of 17α-ethynyl estradiol on germ cell proliferation in organ and primary culture of medaka *Oryzias latipes* testis. Develop. Growth Differ. 45, 327–337.

Sorensen, P.W., Stacey, N.E., Chamberlain, K.J., 1989. Differing behavioral and endocrinological effects of two female sex pheromones on male goldfish. Horm. Behav. 23, 317–332.

Soverchia, L., Carotti, M., Andreu-Vieyra, C., Mosconi, G., Cannella, N., Habibi, H., Polzonetti-Magni, A.M., 2007. Role of gonadotropin-releasing hormone (GnRH) in the regulation of gonadal differentiation in the gilthead seabream *Sparus aurata*. Mol. Reprod. Dev. 74, 57–67.

Soverchia, L., Ruggeri, B., Palermo, F., Mosconi, G., Cardinaletti, G., Scortichini, G., Gatti, G., Polzonetti-Magni, A.M., 2005. Modulation of vitellogenin synthesis through estrogen receptor beta-1 in goldfish *Carassius auratus* juveniles exposed to 17β-estradiol and nonylphenol. Toxicol. Appl. Pharmacol. 209, 236–243.

Stacey, N.E., Kobayashi, M., 1996. Androgen induction of male sexual behaviors in female goldfish. Horm. Behav. 30, 434–445.

Steven, C., Lehnen, N., Kight, K., Ijiri, S., Klenke, U., Harris, W.A., Zohar, Y., 2003. Molecular characterization of the GnRH system in zebrafish *Danio rerio*: cloning of chicken GnRH-II, adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II. Gen. Comp.

Endocrinol. 133, 27–37.

Swanson, P., Dickey, J.T., Campbell, B., 2003. Biochemistry and physiology of fish gonadotropins. Fish Physiol. Biochem. 28, 53–59.

Takahashi, H., 1977. Juvenile hermaphroditism in the zebrafish, Brachy *Danio rerio*. Bull. Fac. Fish Hokkaido Univ. 28, 57–65.

Taranger, G.L., Carrillo, M., Schulz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.A., Dufour, S., Karlsen, Ø., Norberg, B., Andersson, E., Hansen, T., 2010. Control of puberty in farmed fish. Gen. Comp. Endocrinol. 165, 483–515.

Tchoudakova, A., Pathak, S., Callard, G.V., 1999. Molecular cloning of an estrogen receptor β subtype from the goldfish *Carassius auratus*. Gen. Comp. Endocrinol. 113, 388–400.

Tyler, C.R., Sumpter, J.P., 1996. Oocyte growth and development in teleosts. Rev. Fish Biol. Fish. 6, 287–318.

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J. Exp. Biol. 205, 711–718.

Um, H.N., Han, J.M., Hwang, J.I., Hong, S.I., Vaudry, H., Seong, J.Y., 2010. Molecular coevolution of kisspeptins and their receptors from fish to mammals. Ann. N.Y. Acad. Sci. 1200, 67–74.

Uzbekova, S., Lareyre, J.J., Guiguen, Y., Ferrière, F., Bailhache, T., Breton, B., 2001. Expression of sGnRH mRNA in gonads during rainbow trout gametogenesis. Comp. Biochem. Physiol. B 129, 457–465.

Vischer, H.F., Granneman, J.C., Linskens, M.H., Schulz, R.W., Bogerd, J., 2003. Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. J. Mol. Endocrinol. 31, 133–140.

Wang, H., Keiser, J.A., 1998. Molecular characterization of rabbit CPP32 and its function in vascular smooth muscle cell apoptosis. Am. J. Physiol. 274, H1132–H1140.

Wang, H., Tan, J.T.T., Emelyanov, A., Korzh, V., Gong, Z., 2005. Hepatic and extrahepatic expression of vitellogenin genes in the zebrafish *Danio rerio*. Gene 356, 91–100.

Weltzien, F.A., Andersson, E., Andersen, Ø., Shalchian-Tabrizi, K., Norberg, B., 2004. The brain–pituitary–gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). Comp. Biochem. Physiol. A 137, 447–477.

White, S.A., Kasten, T.L., Bond, C.T., Adelman, J.P., Fernald, R.D., 1995. Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. Proc. Natl. Acad. Sci. U. S. A. 92, 8363–8367.

Wood, A.W., Van Der Kraak, G.J., 2001. Apoptosis and ovarian function: novel perspectives from the teleosts. Biol. Reprod. 64, 264–271.

Wu, C., Patino, R., Davis, K.B., Chang, X., 2001. Localization of estrogen receptor alpha and beta RNA in germinal and nongerminal epithelia of the channel catfish testis. Gen. Comp. Endocrinol. 124, 12–20.

Xia, Z., Patino, R., Gale, W.L., Maule, A.G., Densmore, L.D., 1999. Cloning, *in vitro* expression, and novel phylogenetic classification of a channel catfish estrogen receptor. Gen. Comp. Endocrinol. 113, 360–368.

Yamato, S., Nakagawaa, S., Yamazakia, N., Aketob, T., Tachikawac, E., 2010. Simultaneous determination of pregnenolone and 17α-hydroxypregnenolone by semi-micro high-performance liquid chromatography with an immobilized cholesterol oxidase as a pre-column reactor: application to bovine adrenal fasciculata cells. J. Chromatogr. B 3358–3362.

Yang, B., Jiang, Q., Chan, T., Ko, W.K.W., Wong, A.O.L., 2010. Goldfish kisspeptin: molecular cloning, tissue distribution of transcript expression, and stimulatory effects on prolactin, growth hormone and luteinizing hormone secretion and gene expression via direct actions at the pituitary level. Gen. Comp. Endocrinol. 165, 60–71.

Zhang, D., Trudeau, V.L., 2006. Integration of membrane and nuclear estrogen receptor signaling. Comp. Biochem. Physiol. A 144, 306–315.

Zhao, Y., Wayne, N.L., 2012. Effects of kisspeptin1 on electrical activity of an extrahypothalamic population of gonadotropin-releasing hormone neurons in medaka *Oryzias latipes*. PLoS One 7, e37909.

Zmora, N., Kumar, S., Kazeto, Y., Trant, J.M., 2003. Production of channel catfish *Ictalurus punctatus* recombinant gonadotropins using the S2 drosophila cell line system. Fish Physiol. Biochem. 28, 475–477.

Zmora, N., Stubblefield, J., Zulperi, Z., Biran, J., Levavi-Sivan, B., Antonio Muñoz-Cueto, J., Zohar, Y., 2012. Differential and gonad stage-dependent roles of kisspeptin 1 and kisspeptin 2 in reproduction in the modern teleosts, Morone species. Biol. Reprod. 86, 1–12.

Zohar, Y., Munoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165, 438–455.