Studies on the GnRH Receptor System in the Reproduction of Chub Mackerel (Scomber japonicus)

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Studies on the GnRH Receptor System in the Reproduction of Chub Mackerel (*Scomber japonicus*)

Dissertation submitted in accordance with the requirements for the degree of Doctor of Philosophy

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ABBREVIATIONS

ANOVA	analysis of variance
AP	adaptor primer
BPG	brain-pituitary-gonad
cAMP	cyclic adenosine monophosphate
CEB	cerebellum
CHO	Chinese hamster ovary
cmPE	chub mackerel pituitary extract
CRE-Luc	cAMP response element-Luciferase
Ct	threshold cycle
DMSO	dimethyl sulfoxide
dph	days post hatch
D-PBS	Dulbecco's phosphate-buffered saline
dscDNA	double strand cDNA
*ef1*α	elongation factor 1 alpha
ES	early spermatogenesis
EV	early vitellogenesis
FBS	fetal bovine serum
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
FSHβ	follicle stimulating hormone β
GSP	gene-specific primer
GnRH	gonadotropin releasing hormone
GnRHa	gonadotropin releasing hormone analogue
GnRHR	gonadotropin releasing hormone receptor
GPCR	G protein-coupled receptor
GtH	gonadotropin
GtHR	gonadotropin receptor

ABBREVIATIONS

HYP	hypothalamus
IM	Immature
Kiss	kisspeptin
KissR	kisspeptin receptor
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LHβ	luteinizing hormone β
LS	late spermatogenesis
LV	late vitellogenesis
MAPK	mitogen-activated protein kinase
MB.T	midbrain tegmentum
MO.SC	medulla oblongata and spinal cord
MS	mid spermatogenesis
MV	mid vitellogenesis
OB	olfactory bulb
ORF	open reading frame
PBS	phosphate-buffered saline
pCRE	cAMP response element promoter
PIT	pituitary
PKA	protein kinase A/cAMP
PKC	protein kinase C
POA	preoptic area
qPCR	quantitative real-time PCR
RACE	rapid amplification of cDNA ends
SEM	standard errors of the mean
SP	spermiation
TM	transmembrane

PREFACE

Fisheries is one of the primary industries and is also one of the primary sources of food in the world. Studies on endocrine mechanisms in fish regulating reproductive processes such as puberty, contribute to the development and management of the aquaculture industry. Puberty in fish is the time through which an organism becomes capable of reproduction. The timing of puberty, however, varies with species. Typically, larger fishes such as the blue fin tuna (*Thunnus orientalis*), require many years to attain puberty. On the other hand, one common problem for some farmed fishes, such as European eel (*Anguilla anguilla*), is the inability to reproduce under farming conditions. Thus, an understanding on the mechanisms involved in pubertal process which could help in modifying the timing of puberty, could be very beneficial in the farming of many commercially important fish species.

Brain-Pituitary-Gonad (BPG) axis

Reproductive processes in fish are regulated by several endocrine hormones in the BPG axis (Fig. 1). Various physiological factors (age, growth, and energy) and environmental factors (photoperiod, temperature, and feed)

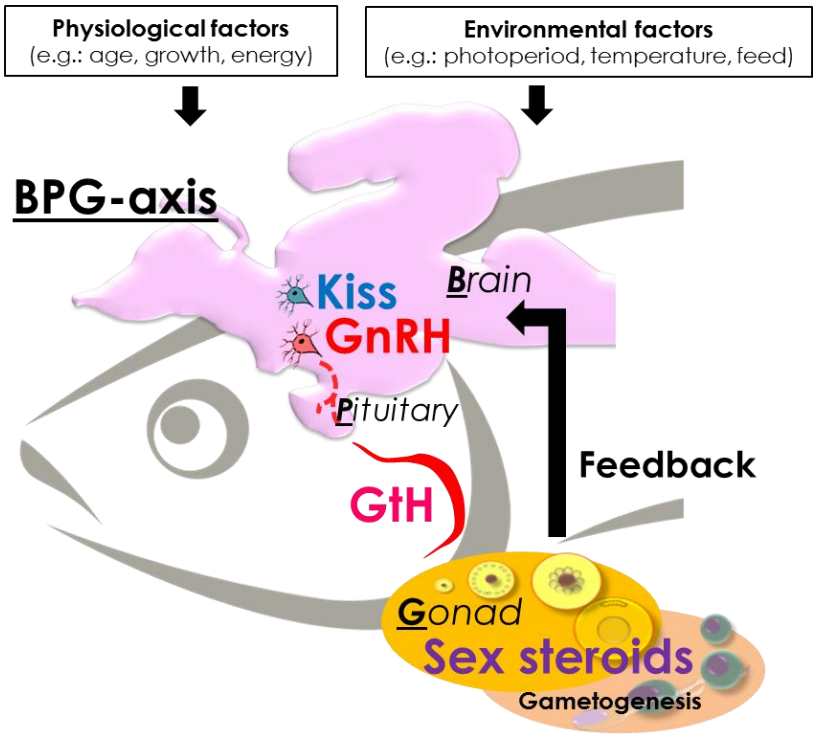


Figure 1. Schematic representation of the brain-pituitary-gonad (BPG) axis, involved in the regulation of gametogenesis in fishes. Various physiological factors (age, growth, and energy) and environmental factors (photoperiod, temperature, and feed) plus the endocrine feedback from gonads, could activate gonadotropin-releasing hormone (GnRH) in the brain. In turn, GnRH stimulates the synthesis and release of gonadotropins (GtHs) comprised of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in the pituitary. Actions of GnRH are mediated by binding with GnRH receptors. GtHs stimulate gametogenesis via FSH and LH receptors, in part by stimulating sex steroid production in the gonads. In addition, Kisspeptin (Kiss) may have a role in the brain for the activation of the BPG axis.

plus the endocrine feedback from gonads, could activate gonadotropin-releasing hormone (GnRH) in the brain. Kisspeptin acts through the kisspeptin receptor (Kiss1R/GPR54), expressed primarily by GnRH neurons. These GnRH neurons control the reproductive process in vertebrates by stimulating the synthesis and release of pituitary gonadotropins (GtHs) (see reviews by Lethimonier et al., 2004; Kah et al., 2007; Weltzien et al., 2004). Pituitary GtHs are composed of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which further stimulate the synthesis of sex steroids, responsible for gonadal growth progression and maturation (see review Nagahama and Yamashita, 2008; Schulz et al., 2001; Yaron et al., 2003). Actions of GnRHs are mediated by binding with GnRH receptors.

Gonadotropin releasing hormone receptors (GnRHRs)

Presently, the GnRH family includes 30 different forms, representing 15 vertebrate and 15 invertebrate species; eight of these forms have been identified in fish (Roch et al., 2011). These GnRH isoforms are further classified as GnRH1, GnRH2, or GnRH3, based on phylogenetic analysis and neuroanatomical distribution (Fernald and White, 1999). GnRH1 is the species-specific hypophysiotropic and reproductively relevant isoform mainly distributed in the neuronal population of the preoptic area (POA) and

hypothalamus. There are different GnRH1 forms found in fish which include the mammalian form (mGnRH) and various fish-specific peptides such as catfish (cGnRH), medaka (mdGnRH), seabream (sbGnRH), whitefish (whGnRH) and herring (hrGnRH). The GnRH2 form exists in the midbrain tegmentum region, and is represented by chicken GnRH-II (cGnRH-II) in all vertebrates examined to date. On the other hand, GnRH3 (salmon GnRH; sGnRH) is a teleost-specific form expressed in neuronal populations in the olfactory bulb, terminal nerve ganglion region, and POA (Kah et al., 2007; Lethimonier et al., 2004).

GnRHRs belong to the rhodopsin family of G-protein coupled receptors that contains seven transmembrane (TM) domains (Stojilkovic et al., 1994; Lethimonier et al., 2004). GnRHRs are highly conserved through evolution as demonstrated by the cloning of a functional GnRHR from invertebrate species such as octopus (Kanda et al., 2006) and three functional GnRHRs from the tunicate (Kusakabe et al., 2003; Sakai et al., 2010; Tello et al., 2005). The presence of these several forms of GnRHs in a single species is usually associated with the corresponding receptor subtypes. There have been an increasing reports in most vertebrates of atleast two GnRHR types, Type I and Type II (Hildahl et al., 2011; Kah et al., 2007; Lethimonier et al., 2004; Millar et al., 2001; Okubo et al., 2001; Wang et al., 2001). The first ever GnRHR was cloned from the αT3 gonadotrope cell line of the mouse

(Reinhart et al., 1992; Tsutsumi et al., 1992). On the other hand, the first teleost GnRHR was cloned from the pituitary of African catfish (Tensen et al., 1997). Successive reports of teleost GnRHR were then identified (Hildahl et al., 2011; Kah et al., 2007; Lethimonier et al., 2004) including up to 5 GnRHRs encoded in the genome of two pufferfish species (*Fugu rubripes* and *Tetraodon nigroviridis;* Ikemoto and Park, 2005) and the European seabass (Moncaut et al., 2005). However, understanding the cell/tissue distribution, regulation and function of various GnRHRs are still not clear.

Chub mackerel (*Scomber japonicus*)

The chub mackerel (*Scomber japonicus*), is a coastal pelagic fish that belongs to the order Perciformes, family Scombridae (Fig. 2). This species is one of the most important commercial marine food fishes in Japan. Chub mackerel is mainly caught by purse seine in the East China Sea, along the coast of Japan Sea, and on the Pacific Coast of Japan. Due to the increasing demand, cultivation of chub mackerel has initiated in southwestern Japan by catching young to adult fish from the wild and being reared to sea cages for a period of time (Matsuyama et al., 2005).

Chub mackerel is a multiple spawner with an asynchronous-type ovary (Asano and Tanaka, 1989; Murua and Sabarido-Rey, 2003). Like many other

Figure 2. Experimental fish, chub mackerel

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Family: Scombridae

Genus: *Scomber*

Species: *Scomber japonicus* (Houttuyn, 1782)

Japanese name: Masaba (マサバ)

farmed fish species, female chub mackerel fails to complete the reproductive cycle in captivity without any external stimulation. To solve the problem, GnRH analogue (GnRHa) is injected intramuscularly at 400µg/kg body weight for induced spawning (Shiraishi et al., 2008). In addition, the experimental rearing system of this species has already been established allowing for fish sampling at different reproductive stages throughout the year to clearly understand the neuroendocrine mechanisms regulating reproduction in fish (Murata et al., 2005; Shiraishi et al., 2005). These features make chub mackerel a suitable experimental fish model to elucidate the mechanism of pubertal onset

In chub mackerel, the key molecular elements in the BPG axis have already been isolated, namely kisspeptins (Selvaraj et al., 2010), kisspeptin receptors (Ohga et al., 2013), GnRHs (Selvaraj et al., 2009), GtHs (Nyuji et al., 2012a, 2012b; Ohga et al., 2012), GtH receptors (Nyuji et al., 2013), and sex steroids (Matsuyama et al., 2005). However, studies on GnRH receptor in chub mackerel are lacking.

Research objectives

Based on the above background, the present research has been performed to clearly understand the role of GnRH/GnRH receptor system in

the regulation of reproductive cycle in chub mackerel. This dissertation is divided into two chapters as follows:

Chapter 1: Identification and characterization of pituitary GnRH receptor involved in the puberty of a commercial scombroid fish, chub mackerel.

Chapter 2: Regulation of LH by GnRHs and expression variation of *cmgnrhr1* mRNA levels in the pituitary of chub mackerel.

CHAPTER 1

Identification and characterization of pituitary GnRH receptor involved in the puberty of a commercial scombroid fish, chub mackerel

1. INTRODUCTION

Puberty is the process through which an organism becomes capable of reproduction (Taranger et al., 2010). The start of puberty in teleosts is associated with the onset of spermatogenesis (i.e., the appearance of type B spermatogonia or primary spermatocytes) in males and is marked by the onset of vitellogenesis (i.e., the appearance of yolk globules in the cytoplasm) in females (Holland et al., 2000; Matsuyama et al., 1991; Okuzawa, 2002). The brain–pituitary–gonad (BPG) axis is a classical neuroendocrine hormonal pathway regulating reproductive processes, including puberty (see review by Weltzien et al., 2004). Kisspeptins have emerged as upstream regulators of the reproductive BPG axis in vertebrates (see review by Tena-Sempere et al., 2012). Kisspeptins act through the kisspeptin receptor (Kiss1R/GPR54), expressed primarily by GnRH neurons. These GnRH neurons control the reproductive process in vertebrates by stimulating the synthesis and release of pituitary gonadotropins (GtHs) (see reviews by Lethimonier et al., 2004; Kah et al., 2007; Weltzien et al., 2004). Pituitary GtHs are composed of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which further stimulate the synthesis of sex steroids, responsible for gonadal growth progression and maturation (see review Nagahama and Yamashita, 2008; Schulz et al., 2001; Yaron et al., 2003). The actions of GnRH are mediated by

binding with GnRH receptors (GnRHRs) on the plasma membranes of gonadotroph cells in the pituitary (see review by Hapgood et al., 2005).

GnRHRs belong to the rhodopsin β subfamily of G-protein-coupled receptors (GPCRs), which contain seven transmembrane (TM) domains (Stojilkovic et al., 1994). In mammals, only one or two GnRHRs have been detected, primarily in the pituitary and extra-pituitary tissues of several species (see review by Hapgood et al., 2005). In teleosts, as many as five GnRHRs have been isolated, mainly in the brain and pituitary of two pufferfish species (*Fugu rubripes* and *Tetraodon nigroviridis*; Ikemoto and Park, 2005) and the European seabass (*Dicentrarchus labrax*; Moncaut et al., 2005). Likewise, mammals possess one or two variants of GnRH, while teleosts have two to three GnRH variants (see review by Millar et al., 2004). Increasingly, vertebrate studies show at least two GnRHR classifications; however, no well-established nomenclature has been created to classify its receptors. Several investigators proposed different nomenclatures for GnRHRs, composed of two (Flanagan et al., 2007; Hildahl et al., 2011; see review by Lethimonier et al., 2004), three (see reviews by Levavi-Sivan and Avitan, 2005; and Millar et al., 2004), or four types (Ikemoto et al., 2004, 2005; see review by Kim et al., 2011) divided into different subtypes.

GnRHRs are a special type of GPCR, capable of activating multiple signaling pathways such as mitogen-activated protein kinase (MAPK), protein

kinase A (PKA), and protein kinase C (PKC) pathways (see reviews by Kraus et al., 2001; Naor, 2009). Previous studies have investigated the ability of GnRHRs to couple to the inositol phosphate (IP) second-messenger pathway in fish, for instance in medaka (*Oryzias latipes*; Okubo et al., 2001) and in lamprey (*Petromyzon marinus*; Joseph et al., 2012).

The chub mackerel (*Scomber japonicus*) is a coastal pelagic fish that belongs to the order Perciformes, family Scombridae. This species is an important commercial marine food fish in Japan and is closely related to the blue fin tuna (*Thunnus orientalis*). Typically, fish as large as the bluefin tuna require many years to attain puberty. Shortening this period would be beneficial to the aquaculture industry. However, it is costly and burdensome to conduct studies using larger fish, because they require more food and space. These problems can be alleviated using smaller fish, such as the chub mackerel. Experimental rearing techniques have already been established for egg development and larval and juvenile growth of cultured chub mackerel (Murata et al., 2005; Shiraishi et al. 2005). These features make chub mackerel a suitable experimental fish model to elucidate the mechanism of pubertal onset In chub mackerel, the key molecular elements in the BPG axis have already been isolated, namely kisspeptins (Selvaraj et al., 2010), kisspeptin receptors (Ohga et al., 2013), GnRHs (Selvaraj et al., 2009), GtHs (Nyuji et al., 2012a, 2012b; Ohga et al., 2012), GtH receptors (Nyuji et al., 2013), and sex

steroids (Matsuyama et al., 2005). However, it is essential to study the functional GnRH receptor in this fish to clearly understand the regulation of puberty in chub mackerel.

In the present study, the author isolated one type of GnRH receptor in the pituitary of chub mackerel and determined its binding efficiency to each GnRH decapeptides.

2. MATERIALS AND METHODS

2.1. Cloning of pituitary GnRH receptor

Total RNA from the brain and pituitaries of adult chub mackerel were extracted using ISOGENE (Nippon Gene, Japan). First strand cDNA library from the pituitaries was prepared for partial cloning using Superscript III RTase (Invitrogen, Carlsbad, CA, USA) and Oligo-dT primers (Sigma-Aldrich, St. Louis, USA), following the manufacturer's protocol for reverse transcription. Double strand cDNA (dscDNA) library from the brains was synthesized using Marathon cDNA amplification Kit (Clontech, Mountain View, CA, USA) for 3′ and 5′ RACE PCR. Degenerate primers were designed from the conserved regions of various teleost fish GnRHRs, namely Japanese eel, *Anguilla japonica* [AB041327], medaka, *Oryzias latipes* [NM_001104882; NM_001104922], Nile tilapia, *Oreochromis niloticus*

[AB111356; XM_019360053], Orange-spotted grouper, *Epinephelus coioides* [DQ536435], spotted Green pufferfish, *Tetraodon nigroviridis* [AB212824; AB212820], striped bass, *Morone saxatilis* [AF218841], and zebrafish, *Danio rerio* [EF571595; NM_001144979; NM_001144980; NM_001177450]. The list of primers used is presented in Table 1. PCRs were performed in a final volume of 10µl containing 5µl 2x Amplitaq Gold PCR master mix (Applied Biosystems, Branchburg, NJ, USA), 0.5µl of each forward and reverse primers, 3.5µl PCR-grade water and 0.5µl of synthesized cDNA. Thermal cycling consisted of initial denaturation at 95˚C for 9 min, followed by 35 cycles at 94˚C for 1 min, 60˚C for 1min, 72˚C for 1min. PCR products were purified from 1.5% agarose gel using Geneclean II kit (MP-Biomedicals, Santa Ana, CA, USA) and were sublconed into pGEM-T Easy Vector (Promega, Madison, WI, USA). Plasmid DNA with insert cDNA was sequenced using a CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Villepinte, France) and CEQ 8000 Genetic Analysis System. Gene specific primers (GSPs) were used for sequencing 3′ end, 5′ end, and full-length cDNAs which were carried out using 1µl of template dscDNA in a final volume of 10µl containing 0.2µl of each 10µM primers, 7.2µl PCR-grade water, 1µl of 10x cDNA PCR Reaction Buffer (Clontech), 0.2µl dNTP Mix (Takara, Bio Inc.,

Table 1. List of degenerate, specific and adaptor primers used in the chub mackerel *gnrhr1* cloning.

Purpose		**Primer sequence (5'-3')**
Partial cloning	Sense	TGRTGACMTTYRTSGTGATGC
	Antisense	CCAGTACCAVAKBCCCAGCAG
	Sense	TGGAACRTSACRGTSCAGTG
	Antisense	GCAGGTARTAYGGHGTCCAGC
3'RACE	Sense	CCATGTTCACTTTCTCCTGCC
	Sense	CACCCCAATGAGTCTAGCAGCTCTG
5'RACE	Antisense	CAAGAACAGGCAGGAGAAAGTG
	Antisense	CACAGCCAGGTTGCAAAAGGC
ORF	Sense	TGTTTCAGAAAATGAACGCCACTCTC
cloning	Antisense	TTCCCTCACATGATGCTTTCAGAGC

R=A+G, M=A+C, Y=C+T, S=G+C, V=G+A+C, K=G+T, B=G+T+C

Otsu, Shiga, Japan) and 50x Advantage 2 Polymerase Mix (Clontech). Reaction conditions for PCR were 94˚C for 30sec; 5cycles at 94˚C for 5sec,

72˚C for 2min; 5cycles at 94˚C for 5sec, 70˚C for 2min; 25cycles at 94˚C for 5sec, 68˚C for 2min.

2.2. Phylogenetic and sequence identity analyses

The Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST) was used for homology searches of the chub mackerel pituitary GnRHR. The amino acid sequences of different precursors of GnRHRs were obtained from GenBank (www.ncbi.nlm.nih.gov). The list of GnRHRs were as follows: African cichlid (*Haplochromis burtoni*); Atlantic croaker (*Micropogonias undulatus*); black porgy (*Acanthopagrus schlegelii*); common octopus (*Octopus vulgaris*); European seabass (*Dicentrarchus labrax*); greater amberjack (*Seriola dumerili*); house mouse (*Mus musculus*); human (*Homo sapiens*); medaka; Nile tilapia; pejerrey (*Odontesthes bonariensis*); red seabream (*Pagrus major*); Rhesus monkey (*Macaca mulatta*); and striped bass. TM domains were predicted using the HMMTOP 2.0 server (Tusnády and Simon, 1998; 2001). Phylogenetic tree was constructed by the neighbour-joining method using MEGA6 software (Tamura et al., 2013). Common octopus GnRHR was used as a reference for clustering GnRHRs. Amino acid sequence identity of chub mackerel pituitary GnRHR to other types of receptors were calculated by BioEdit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.3. Synthetic peptides

Three GnRH synthetic decapeptides corresponding to chub mackerel (cm) GnRH1 (cmGnRH1; seabream form; Glp-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2), cmGnRH2 (chicken-II form; Glp-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2) and cmGnRH3 (salmon form; Glp-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2) were purchased in Sigma, Life Science (Hokkaido, Japan) with purities of 90.3%, 84.4% and 95.2% respectively. GnRH analogue (GnRHa; D-Ala^6, des-Gly^{10})-LHRH ethylamide was obtained from Sigma Aldrich (St. Louis, USA) with a purity of 97%. The peptides were analysed by analytical HPLC and mass spectrometry. Each stock peptide was initially dissolved to 10^{-3} M with 1% of final concentration using dimethyl sulfoxide (DMSO) and further diluted with 99% of final concentration using ultra-pure water.

2.4. Transfection and luciferase reporter gene assay

The ORF of chub mackerel pituitary GnRHR containing lobster L21 sequence (which enhances translation efficiency) at the N-terminal, was subcloned into the *Nhe1* and *Xho1* sites of the expression vector pcDNA3.1 (Invitrogen). The cDNA construct was verified by sequencing. Transient transfection and cell culture conditions were followed according to Nyuji et al. (2013). Chinese hamster ovary (CHO) cells were grown at 37˚C in Ham's F-

12 medium (Life Technologies, Baltimore, MD) supplemented with 10% fetal bovine serum (Gibco, NY, USA), 1% HT supplement (Gibco), 50U/ml penicillin (Nakarai Tesque, Kyoto, Japan), and 50µg/ml streptomycin (Nakarai Tesque). One day before transfection, the cells were seeded into 6 well plates. Cotransfection of pc-cmGnRHR1 (1µg/well), pCRE (1µg/well; Agilent, CA), and pRL-TK (8ng/well; Promega) was carried out with X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). After transfection for 24 h, cells were removed and plated in 96-well plates (3×10^4 cells/well). After 44 h, cells were incubated for 6 h with vehicle of decreasing concentrations (concentrations from 10^{-6} to 10^{-12} M; 10-fold dilution) of either synthetic peptide. Luciferase activity in the cell extract was measured using a Dual-Luciferase reporter system (Promega) in Lumat LB95701 luminometer (Berthold Technologies, Bad Wildbad, Germany). The EC50 values were calculated from concentration response curves by means of computerized nonlinear curve fitting with Prism 4 (Graphpad Software, San Diego, CA, USA).

3. RESULTS

3.1. Sequence and phylogenetic analysis of pituitary GnRHR cDNA

The author has identified one GnRHR in the pituitary of chub mackerel named as cmGnRHR1. The sequence was submitted to GenBank with the accession number KX153188. The ORF encodes 1284 bp in length with 428 amino acids and contains seven TM domains shown in Fig. 3. Sequence alignment of deduced chub mackerel GnRHR amino acid sequence and representatives from other types of GnRHRs are shown in Fig 4. Phylogenetic analysis showed two separate groups; Type I and Type II GnRHRs. These types were further subdivided into Type 1A, Type 1B (mammalian GnRHRs), Type IIA (tetropod GnRHRs) and Type IIB as consolidated by Hildahl et al., (2011). Common octopus GnRHR was used as an outgroup. The cmGnRHR1 amino acid sequence was clustered with other teleost type IIB GnRHRs (Fig. 5).

3.2. Comparison of GnRHR amino acid sequence of chub mackerel with other receptors

The amino acid sequence identity of cmGnRHR1 ranges from 59-86.8% when compared with other Type IIB GnRHRs. The greater amberjack (order Perciformes) showed the highest sequence identity of 86.8%, while

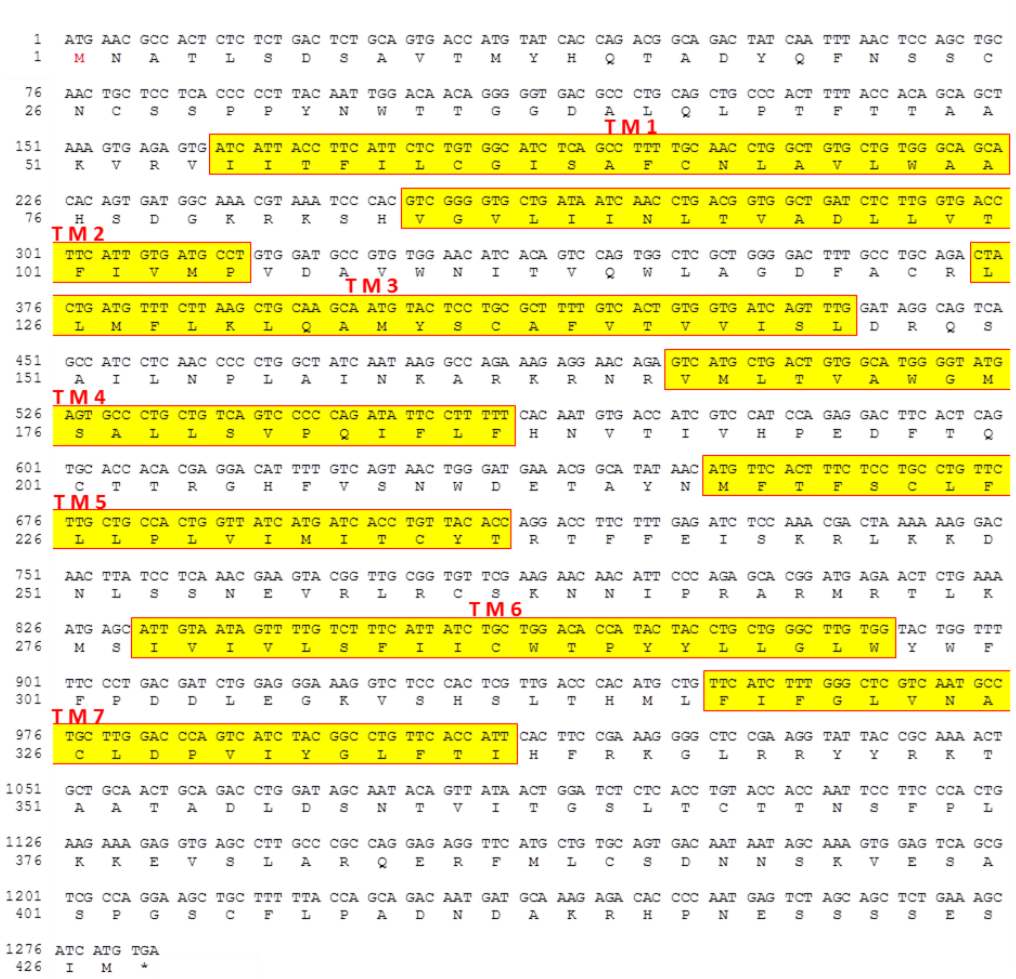


Figure 3. Chub mackerel pituitary GnRHR (*cmgnrhr1*) open reading frame (ORF) nucleotide and amino acid sequences arranged and translated using Life science tools (http://www.fr33.net/translator.php). Transmembrane (TM) domains were predicted using the HMMTOP 2.0 server (Tusnády and Simon, 1998; 2001). The 7 TM domain regions are boxed. The stop codon (TGA) is indicated by an asterisk (*).

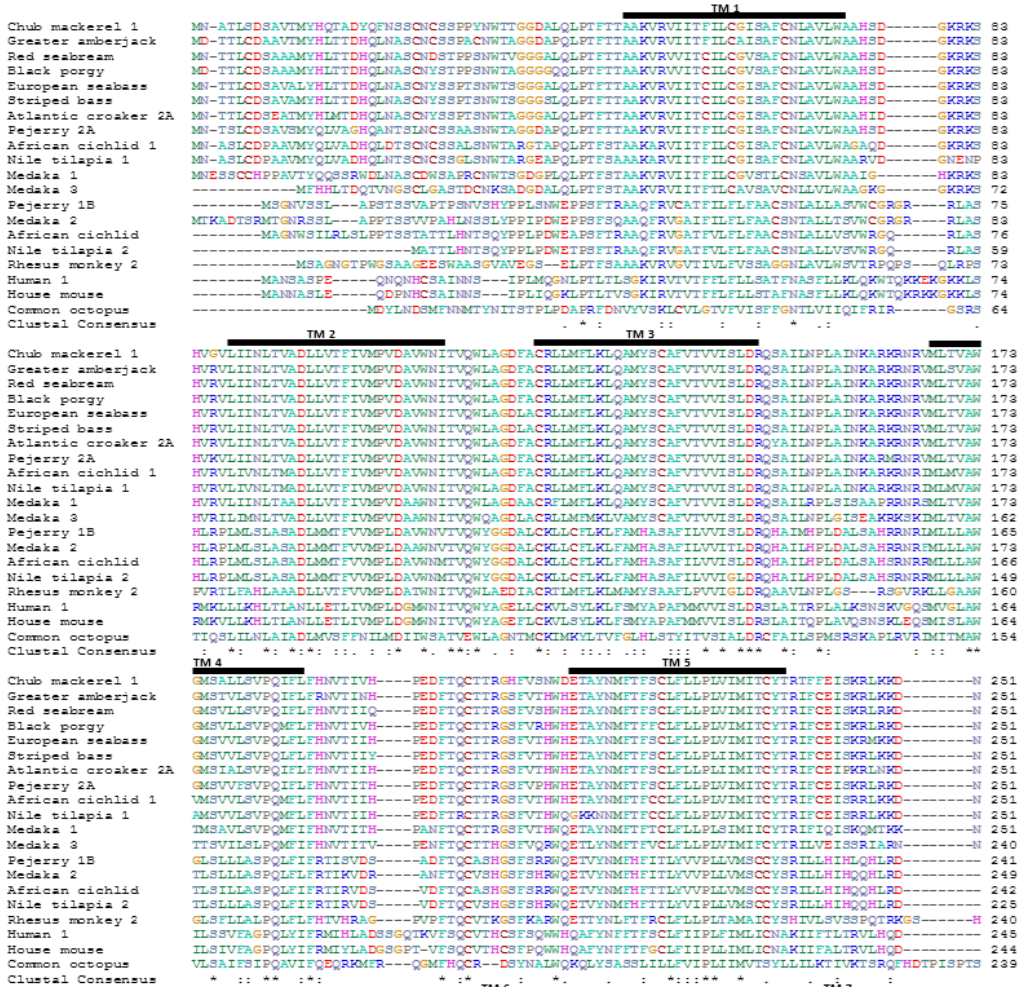


Figure 4. Sequence alignment of deduced chub mackerel GnRHR amino acid sequence and representatives from other types of GnRHRs. The putative 7 transmembrane domains are indicated. ClustalW using BioEdit Sequence Alignment Editor version 7.1.30 software (Hall, 1999) was used.

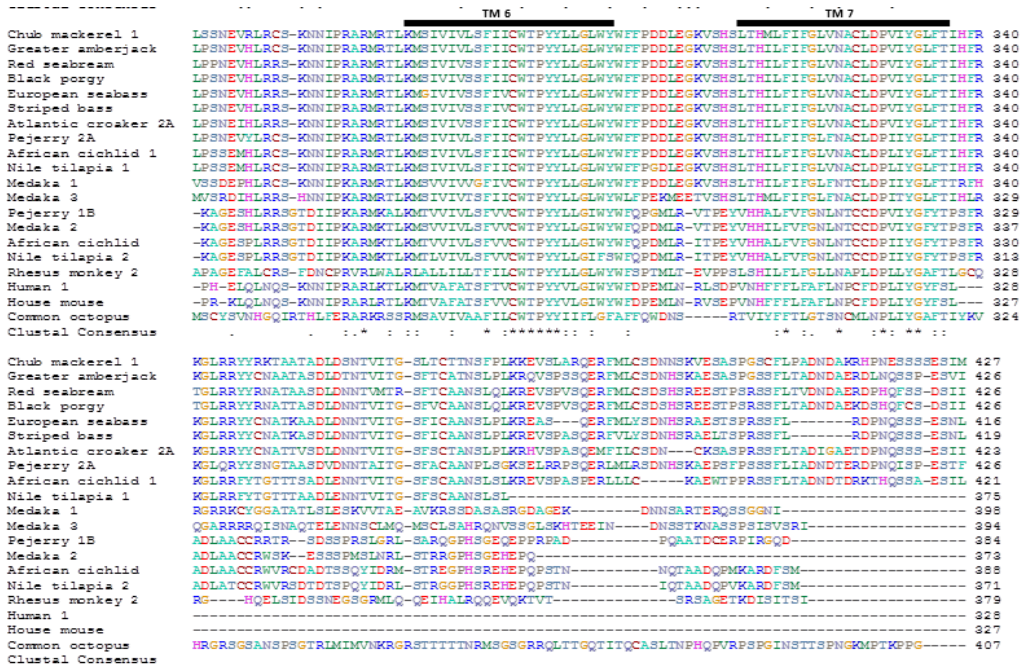


Figure 4. (continued)

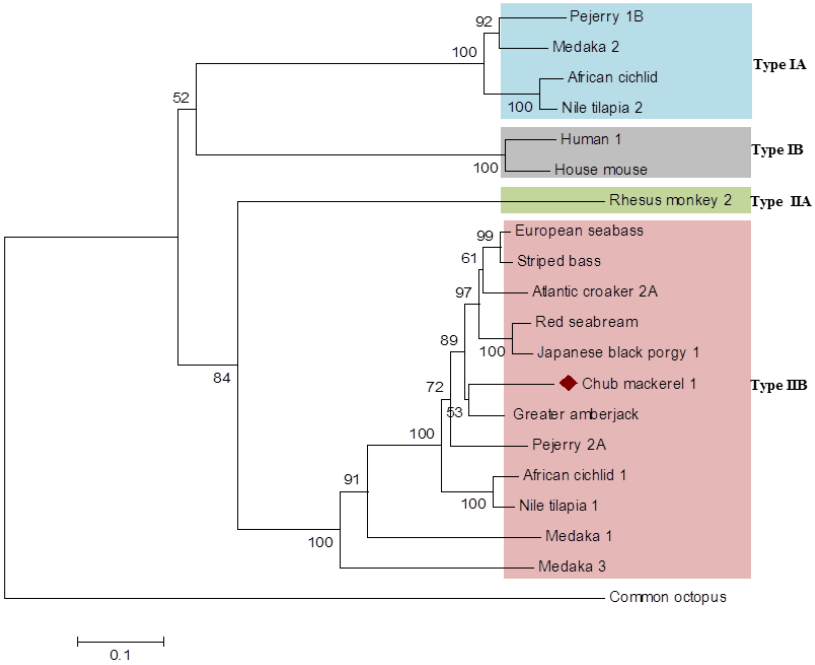


Figure 5. Phylogenetic tree. The phylogenetic tree was generated in MEGA6 using the Neigbour-joining method. The percentages of replicate trees in which the associated taxa clustered together in bootrstrap tests (10000 replicates) are shown next to the branches. The GenBank accession Nos. of GnRHRs are as follows: African cichlid [AAK29745; NP_001273217]; Atlantic croaker [ABB97085]; black porgy [AAV71128]; chub mackerel [ANC48011]; common octopus [Q2V2K5]; European seabass [CAD11992]; greater amberjack [CAB65407]; house mouse [NP_034453]; human [NP_000397]; medaka [NP_001098352; NP_001098392; NP_001098393]; Nile tilapia [BAC77240; NP_001266689]; pejerrey [ABI75336; ABI75337]; red seabream [BAM17648]; Rhesus monkey [NP_001028014] and striped bass [AAF28464]. Octopus GnRHR was used as an outgroup.

medaka 3 (order Beloniformes) showed the lowest identity of 59%. However, when compared with TypeIA GnRHRs, cmGnRHR1 amino acid sequence identity ranges only from 36.1-38.2%, with African cichlid (order Perciformes) as the highest identity (38.2%). Human 1 and house mouse which belong to Type IB GnRHRs both have 30.8% amino acid sequence identity to cmGnRHR1 while Rhesus monkey 2 (a representative of Type IIA GnRHR), has 39.3% amino acid sequence identity to cmGnRHR1.

3.3. Ligand selectivity of chub mackerel pituitary GnRHR

The CRE-Luc was used as a reporter gene for cAMP activation in examining the ligand selectivity for cmGnRHR1. Four GnRH synthetic peptides, cmGnRH1, cmGnRH2, cmGnRH3 and GnRHa stimulated luciferase activity in CHO cells transfected with cmGnRHR1 (Fig. 6). The present receptor showed highest sensitivity for GnRHa with an EC50 value of 2.6313e-010 and lowest for cmGnRH1 (EC50=7.8148e-009).

4. DISCUSSION

Various GnRH receptors have already been identified from different species, but there is currently no well-established classification system for these receptors. In this study, the author adopted the phylogenetic classification consolidated by Hildahl et al. (2011) for its simplicity, in which

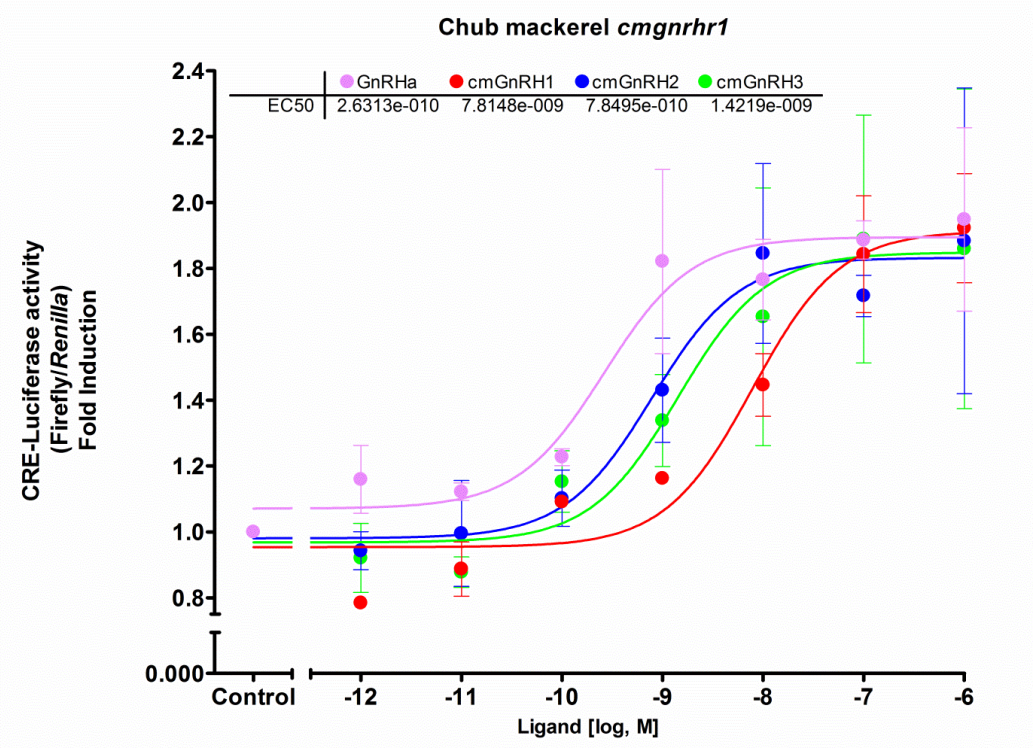


Figure 6. Ligand selectivity of the chub mackerel pituitary GnRH receptor, cmGnRHR1 with CRE-Luc. Transfected cells were treated with GnRHa and chub mackerel GnRH 1, 2, and 3. The data is expressed as the ratio of changes in firefly luciferase activity with graded over the control *Renilla* luciferase activity. Each point was determined in triplicate and is given as a mean ± SEM.

GnRHRs are segregated into two types, Type I (mammalian and non-mammalian receptors) and Type II (fish, tree frog, and all other tetrapod receptors). Phylogenetic analysis using ORF amino acid sequences showed that cmGnRHR1 clustered with Type IIB receptors. Many similar studies have suggested the involvement of this receptor type in fish reproduction and gonadotropic function control, as exhibited by the African cichlid (Flanagan et al., 2007), Atlantic cod (*Gadus morhua*; Hildahl et al., 2011), European eel (*Anguilla anguilla*; Peñaranda et al., 2013), European seabass (Gonzalez-Martinez et al., 2004), and Nile tilapia (Soga et al., 2005). On the other hand, studies have suggested that Type IA receptors of the African cichlid and Nile tilapia play a role in modulating sensory, metabolic, and motor systems (Soga et al., 2005; Chen and Fernald, 2006). To confirm that our isolated GnRH receptor is the functional form involved in the pubertal process in chub mackerel, four experiments were conducted.

The author performed a cAMP response element–luciferase (CRE–Luc) reporter assay on the three deduced GnRH ligands in chub mackerel (cmGnRH1, 2, and 3). The author also performed the assay on a synthetic GnRHa peptide widely used to induce gonadal development in different farmed species (Zohar and Mylonas, 2001). All four synthetic peptides showed potency to cmGnRHR1. All studies of fish carried out in transfected cells have shown that GnRH receptors can be activated by different GnRH

ligands, with a clear preference for GnRH2, followed by GnRH3 and GnRH1 (Lethimonier et al., 2004). In all non-mammalian vertebrate receptors, GnRH2 has a higher binding affinity (relative to GnRH1) regardless of classification, due to the preconfigured β-II' turn conformation (Pfleger et al., 2002). In the same way, the present result indicated potency in the order GnRHa > cmGnRH2 > cmGnRH3 > cmGnRH1. The synthetic GnRHa used in the previous and present experiments has D-Ala^6 substitution for Gly^6, and this substitution is believed to stabilize the β-II' turn conformation that increases binding affinity for the receptor (Monahan et al., 1973).

CHAPTER 2

Regulation of LH by GnRHs and expression variation of *cmgnrhr1* mRNA levels in the pituitary of chub mackerel

1. INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the name given to the members of a family of neuropeptides and the central role of GnRH in the reproductive hierarchy of brain-pituitary-gonad (BPG) axis is undisputed. The GnRH regulation to synthesis and release of pituitary gonadotropins are essential event for pubertal onset and their action is mediated through GnRH receptor (GnRHR) in the pituitary gonadotrophs. The mechanism of the GnRH–GnRHR system has become more complex due to multiple receptor variants present in the pituitary cells at different developmental stages in fish (Hildahl et al., 2011; Parhar et al., 2005). Understanding the expression patterns of GnRHs and GnRHRs at different pubertal stages would improve our knowledge of the functions of GnRH and its receptors during puberty.

In chub mackerel, the key molecular elements in the BPG axis have already been isolated, namely kisspeptins (Selvaraj et al., 2010), kisspeptin receptors (Ohga et al., 2013), GnRHs (Selvaraj et al., 2009), GtHs (Nyuji et al., 2012a, 2012b; Ohga et al., 2012), GtH receptors (Nyuji et al., 2013), and sex steroids (Matsuyama et al., 2005). Two *kiss* genes and two *kissr* genes in the brain of the chub mackerel exhibit sexually dimorphic changes during the seasonal reproductive cycle (Ohga et al., 2013; Selvaraj et al., 2010). Additionally, the recent mRNA analyses of the mackerel brain during puberty showed that for male fish, *kiss2* and *gnrh1* (hypophysiotropic form) levels

increased significantly just prior to the onset of spermatogenesis, whereas for female fish, *kiss1-kissr1*, *kiss2-kissr2*, and *gnrh1* levels increased significantly just before the start of vitellogenesis (Ohga et al., 2015). Furthermore, pituitary *fshβ* and *lhβ* mRNA levels gradually increased at the onset of puberty in parallel with the gonadal development of both sexes in chub mackerel (Nyuji et al., 2014). These results provide useful information about the involvement of the kisspeptin–GnRH–GtH system during the puberty of chub mackerel. However, it is essential to study the functional GnRH receptor in this fish to clearly understand the regulation of puberty in chub mackerel.

2. MATERIALS AND METHODS

2.1. Primary pituitary cell culture

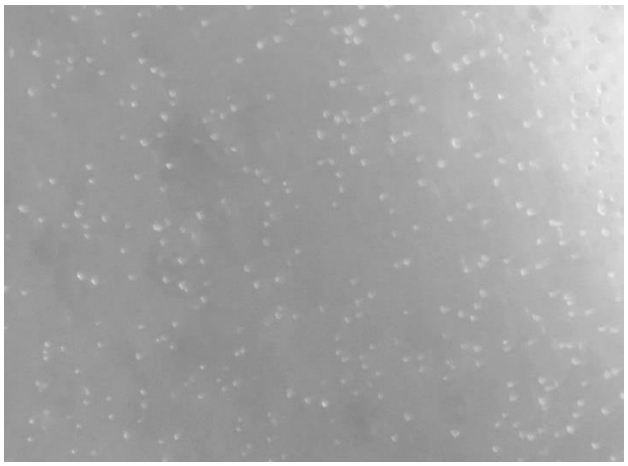
Adult immature female chub mackerel were euthanized at Fishery Research Laboratory of Kyushu University. Briefly, pituitaries from 9 fish were collectively dispersed and placed in ice-cold serum free Leibovitz's L-15 culture medium (Sigma) (pH 7.5) containing 10mM HEPES and 0.02% Gentamycin sulfate (Sigma). Excised pituitaries were washed three times with D-PBS (-) (Dulbecco's phosphate-buffered saline: (100ml: KCl 0.2g, NaCl 8.0g, KH_2PO_4 0.2g, $NaHPO_4 \cdot H_2O$ 2.9g) and 100U penicillin/ml, 100mg streptomycin/ml, 0.25 μg fungizone/ml) and diced into 1 mm^3 fragments with

sterilized scalpel. The fragments were washed again with D-PBS (-) and treated with 0.1% collagenase type I (Sigma) in D-PBS (-) for 3 hours with gentle shaking at 20˚C. Following enzymatic treatment, fragments were mechanically dispersed by gentle pipetting several times with a sterilized Pasteur pipette. Dispersed cells were filtered through 40μm nylon mesh cell strainer and harvested by centrifugation at 200g for 10 min. Collected cells were resuspended in L15 culture medium (L15 with 10% fetal bovine serum (FBS), 100U penicillin/ml, 100μg streptomycin/ml, 0.25μg fungizone/ml: pH 7.4). The number of viable cells was counted using a microscope after trypan blue staining (≥91.2% of viability). Dispersed pituitary cells were cultured (2.5×10^5 cells/ml) on a 96-well cell culture plates at 20˚C in L15 culture medium. Development of primary pituitary cell culture from adult immature female chub mackerel is shown in Fig. 7. After 8 days of culture, cells were exposed for 6 h to 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M of cmGnRH1, cmGnRH2 and cmGnRH3. After peptide treatment, the medium was collected. The samples were stored at -20˚C until analysis.

2.2. Hormonal analysis

Bioactive LH levels in culture medium were estimated using CHO cells transfected by chub mackerel LHR. The activity of LHR was measured

A.) 0-day culture



B.) 8-day culture

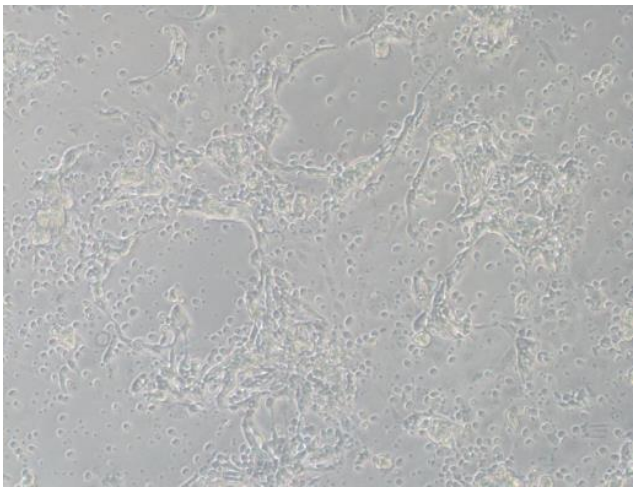


Figure 7. Development of primary pituitary cell culture from adult immature female chub mackerel.

by changes in luciferase activity under the control of a cAMP response element promoter (pCRE). The assay was performed as described previously

(Nyuji et al., 2013). Collected culture media from each tested wells on 6 h treatment was diluted 1/20 with serum free Ham's F-12 medium. After 3 h incubation, luciferase activity was quantified using Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The LH-R expression vector was prepared according to previous report (Nyuji et al., 2013).

2.3. RNA preparation and quantitative real-time PCR

The brain and pituitary samples that were previously used by Nyuji et al. (2014) and Ohga et al. (2015) were also used in the present study for measuring *cmgnrhr1* gene expression. The excised brain samples were stored in RNA later (QIAGEN) for a week at room temperature and kept in -20˚C until use. All the fish that were sacrificed had followed the guidelines in agreement with laws (No. 105) and declaration (No. 6) of the Japanese Government. The brain samples from the immature adult male and female (3 each sex) were sectioned and cut accordingly into different parts namely; pituitary (PIT) olfactory bulb (OB), preoptic area (POA), hypothalamus (HYP), midbrain tegmentum (MB.T). cerebellum (CEB), and medulla oblongata and spinal cord (MO.SC). Pituitary samples were previously histologically divided by Ohga et al. (2015) into five gonadal stages for male fish (Fig. 8) as: (1) Immature (IM); (2) Early spermatogenesis (ES); (3) Mid spermatogenesis (MS); (4) Late spermatogenesis (LS) and (5) Spermation

(SP) and four gonadal stages for female fish (Fig. 9) as: (1) Immature (IM); (2) Early vitellogenesis (EV); (3) Mid vitellogenesis (MV) and (4) Late vitellogenesis (LV). RNA preparation was carried out according to Ohga et al. (2013). The qPCR analysis was performed on an Mx 3000P quantitative PCR system (Stratagene, USA). All transcripts were quantified using a standard curve method and strong linear relationship with a correlation coefficient of R^2 > 0.999 between the fractional cycle number was demonstrated for all standard curves (from 10^4 to 10^8 copies/µl). The ORF sequence of *cmgnrhr1* was subcloned into pGEM-T Easy Vector (Promega). The amplicon length (from the sense to antisense primers) is 115 base pairs (bp) of the ORF receptor sequence. For negative control, cDNA sample was replaced with PCR-grade water. Duplicate reactions were performed for standards, target and reference genes. PCR conditions were as follows: 95˚C (5min); 40 cycles at 95˚C for 10 sec; 60˚C for 30 sec. Melting curve analysis was also included at 1 cycle of 94˚C for 1 min, 55˚C for 30 sec, 95˚C for 30 sec. The reference gene elongation factor 1 alpha (*ef1α)* was measured in each tissue samples for relative quantitation as its expression did not vary between individual samples. Threshold cycle (Ct) values of *ef1α* are shown in Fig. 10 and Fig. 11. Primer

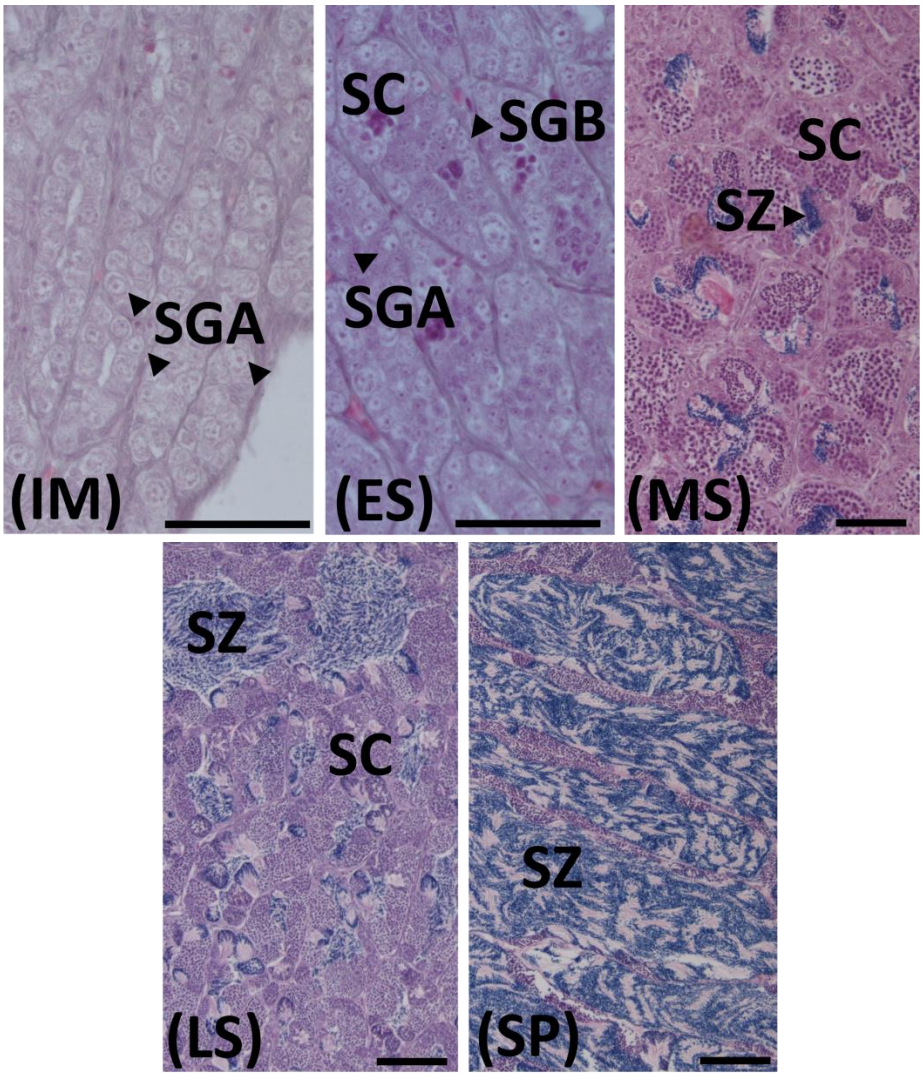


Figure 8. Different testicular stages of chub mackerel sampled during puberty. The thickness of sections cut from gonads is 5µm thick. The abbreviations used are: IM, immature; ES, early spermatogenesis; MS, mid spermatogenesis; LS, late spermatogenesis; SP, spermiation; SGA, typeA spermatogonia; SGB, type B spermatogonia; SC, spermatocytes; and SZ, spermatozoa. Scale bars correspond to 50µm.

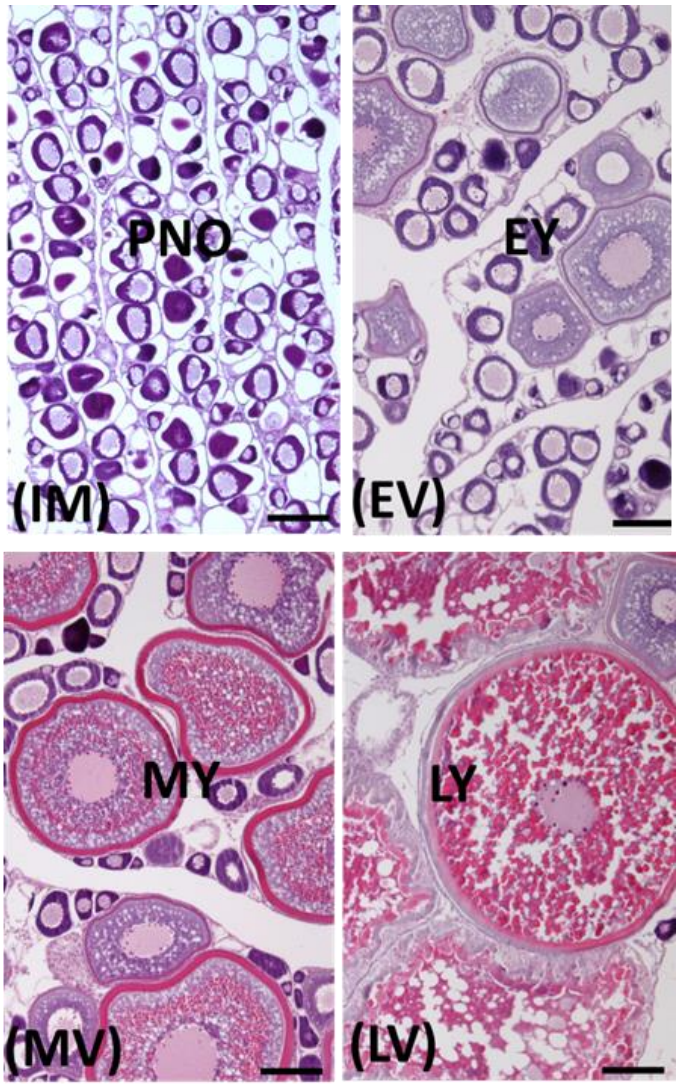


Figure 9. Different ovarian stages of chub mackerel sampled during puberty. The thickness of sections cut from gonads is 5µm thick. The abbreviations used are: IM, immature; EV, early vitellogenesis; MV, mid vitellogenesis; LV, late vitellogenesis; PNO, peri-nucleolar oocyte; EY, early yolk oocyte; MY, mid yolk oocyte; and LY, late yolk oocyte. Scale bars correspond to 100µm.

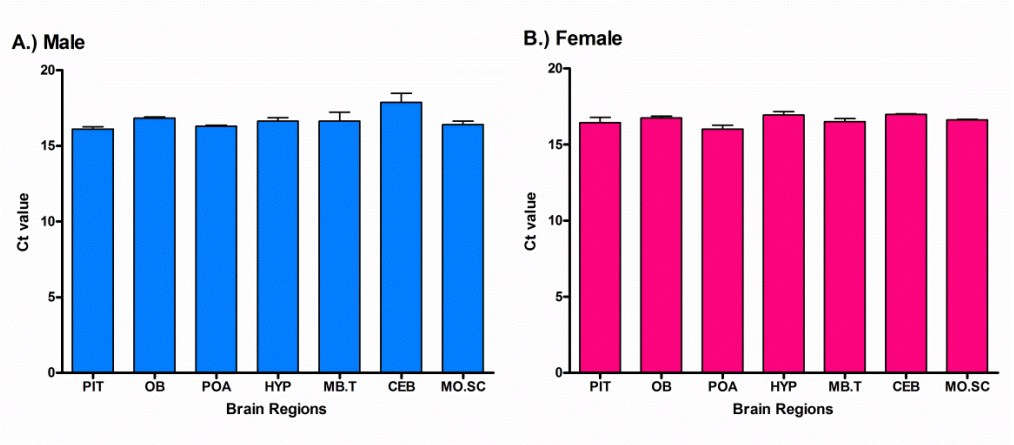


Figure 10. Threshold cycle (Ct) values of *ef1α* gene expressed in A.) male and B.) female chub mackerel from different regions of the brain. Results are expressed as means ± SEM (n=3). Statistically significant changes in gene expression were not observed.

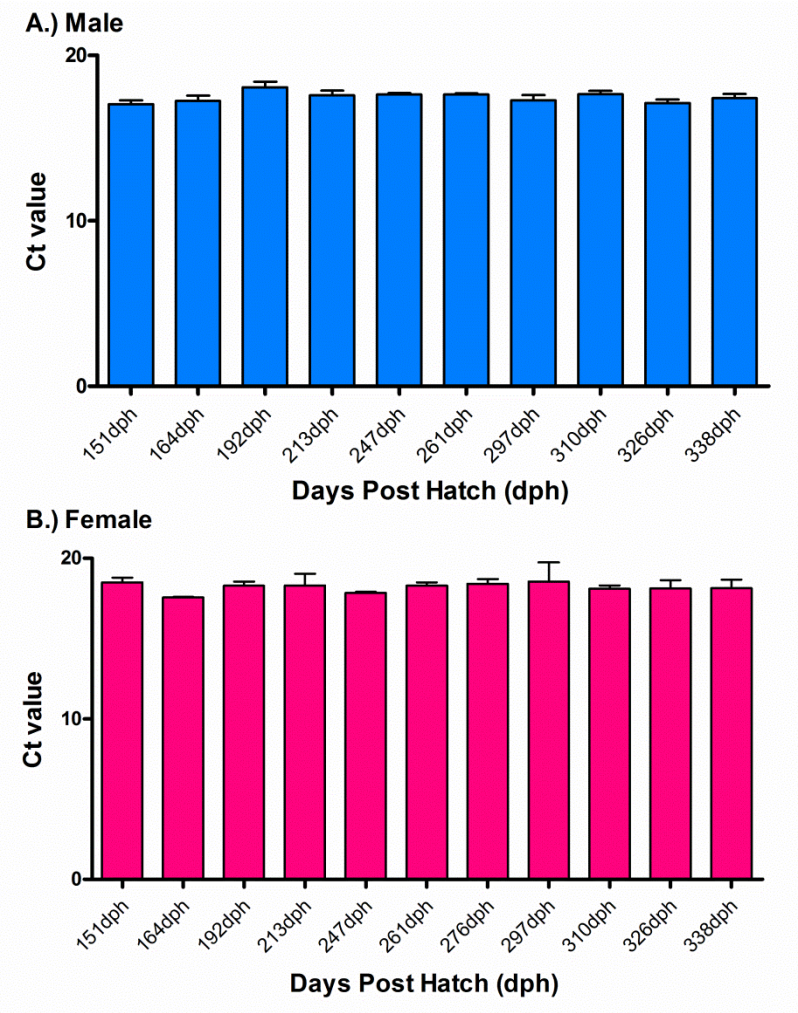


Figure 11. Threshold cycle (Ct) values of *ef1*α gene expressed in A.) male and B.) female chub mackerel from different developmental stages (151-338 dph). Results are expressed as means ± SEM (n=2-7 in male; 2-8 in female). Statistically significant changes in gene expression were not observed.

sets for each gene are listed in Table 2. Melting points for all primers ranged between 60–61˚C. All qPCR assays that were conducted abide on the MIQE (Minimum Information for Publication of qPCR experiments) guidelines (Bustin et al., 2009).

2.4. Statistical analysis

All the data were expressed as means ± SEM (standard errors of the mean). Statistical analysis was analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test using Prism 4 (Graphpad Software, San Diego, CA). Differences were considered significant at $P<0.05$.

3. RESULTS

3.1. Effects of cmGnRH1, cmGnRH2, and cmGnRH3 on *in vitro* LH release in cultured medium by pituitary cells

At 6 h treatment, cmGnRH1 (Fig. 12A), cmGnRH2 (Fig. 12B) and cmGnRH3 (Fig. 12C) peptides significantly elevated LH secretion in cultured medium by pituitary cells above basal values from lower concentration to higher concentration. Even though no significant change was shown for cmGnRH2 at 10^{-6} M dosage, around 25% increase above basal value was still observed.

Table 2. Primer sequence used in quantitative real-time PCR.

cDNA	GenBank accession		Primer sequence (5'-3')
cmgnrhr1	KX153188	Sense	GCTGTCAGTCCCCCAGATATT
		Antisense	ATATGCCGTTTCATCCCAGTT
ef1α	KP642749	Sense	AGGACGTCTACAAGATTGGCG
		Antisense	TGCATCTCCACAGACTTCACC

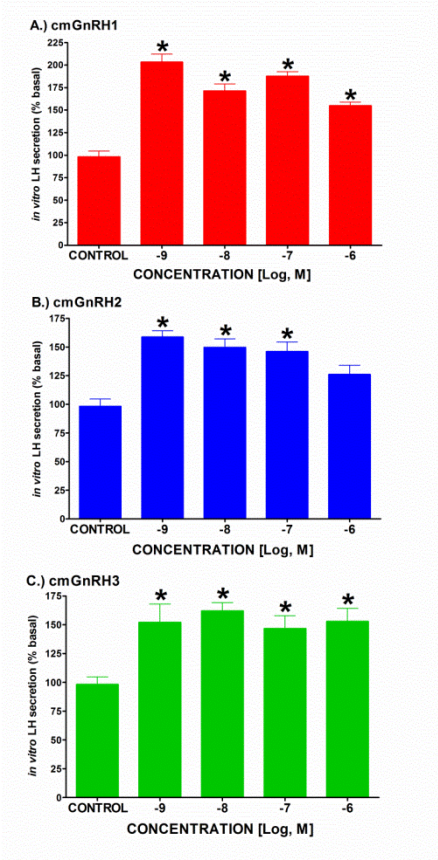


Figure 12. Effects of A.) cmGnRH1, B.) cmGnRH2 and C.) cmGnRH3 peptides on LH secretion in cultured medium from pituitary cells of immature adult female chub mackerel *in vitro*. The data is expressed as the ratio of changes in firefly luciferase activity over the control *Renilla* luciferase activity. The data were analysed using one-way ANOVA; asterisks (*) indicate significant differences ($P<0.05$) between the control. Data are expressed as mean ± SEM (n=4-5).

3.2. Brain tissue distribution of *cmgnrhr1*

Quantitative real-time PCR revealed that *cmgnrhr1* is distributed in different regions of the brain tissues of immature adult male and female chub mackerel. Interestingly, a significantly high level of *cmgnrhr1* mRNA was detected in the pituitary of both sexes in comparison to other parts of the brain (Fig. 13). In the pituitary, male showed a slightly higher expression compared to females, though no significant difference was detected.

3.3. Expression variation of *cmgnrhr1* mRNA levels in the pituitary during puberty

Male fish. The expression of *cmgnrhr1* in the pituitaries of male chub mackerel during puberty is shown in Fig. 14A. The lowest expression of *cmgnrhr1* is detected during the IM stage. An increasing trend of *cmgnrhr1* expression is observed as chub mackerel undergoes testicular development. Furthermore, the highest *cmgnrhr1* expression (that also showed significant increase from the IM stage) is detected at the SP stage.

Female fish. The expression of *cmgnrhr1* in the pituitaries of female chub mackerel during puberty is shown in Fig. 14B. The lowest expression of *cmgnrhr1* is detected during the IM stage. LV stage displayed the highest *cmgnrhr1* expression significantly increased from the IM stage. Furthermore,

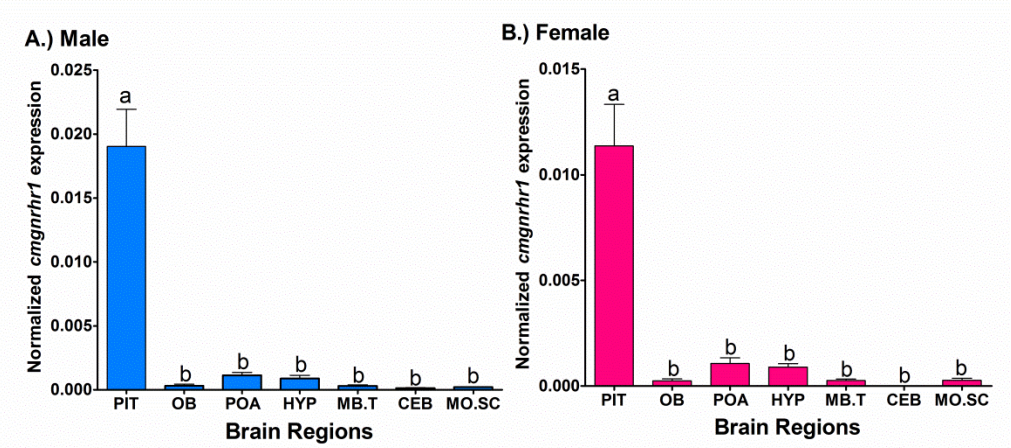


Figure 13. Normalized *cmgnrhr1* mRNA expression in immature adult A.) male and B.) female chub mackerel at different regions of the brain. Quantitative PCR data were normalized to *ef1α* mRNA data. The data were analysed using one-way ANOVA; different letters indicate significant differences ($P<0.05$). Data are expressed as mean±SEM (n=3). The abbreviations used are: PIT, pituitary; OB, olfactory bulb; POA, preoptic area; HYP, hypothalamus; MB.T, midbrain Tegmentum; CEB, cerebellum; and MO.SC, medulla oblongata and spinal cord.

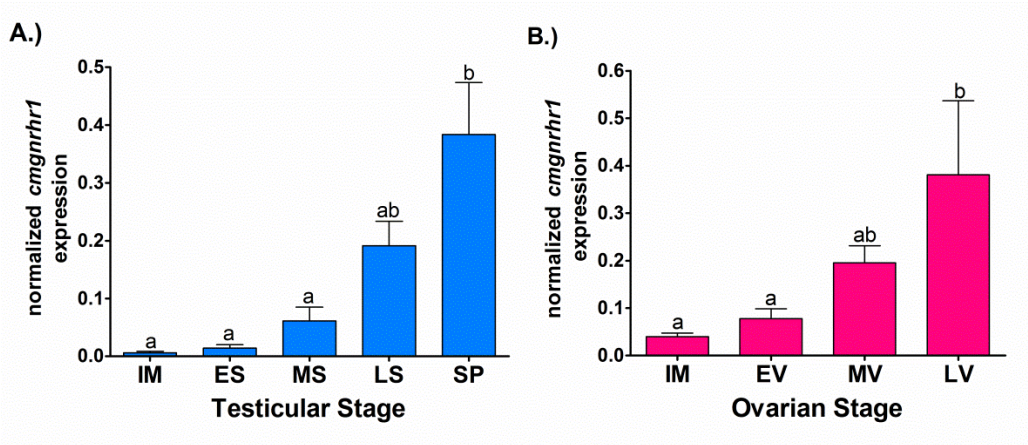


Figure 14. Normalized *cmgnrhr1* mRNA expression in the pituitary of A.) male and B.) female chub mackerel as determined by qPCR in different gonadal stages (151-338 days post hatch (dph)). Quantitative PCR data were normalized to *ef1α* mRNA data. The data were analysed using one-way ANOVA; different letters indicate significant differences ($P<0.05$). Data are expressed as mean±SEM (n= 3-26). The abbreviations used are: IM, immature; ES, early spermatogenesis; MS, mid spermatogenesis; LS, late spermatogenesis; SP, spermiation; EV, early vitellogenesis; MV, mid vitellogenesis; and LV, late vitellogenesis.

an increasing trend of *cmgnrhr1* expression is also observed as it undergoes ovarian development.

4. DISCUSSION

In this study, the author administered cmGnRH1, 2 and 3 peptides *in vitro* to test their effects on LH release. The results clearly indicate that the three deduced GnRH ligands are not only capable of binding to the present receptor but are also effective in releasing LH. Previous *in vivo* studies of chub mackerel have shown that upon administration of a synthetic GnRHa, pituitary *lhβ* but not *fshβ* mRNA levels increased significantly in immature females (Selvaraj et al., 2013). Preliminary experiments showed that cmGnRH1 did not effectively release FSH *in vitro* (Fig.15). Likewise, LH secretion, but not FSH secretion, was stimulated by GnRHa in an *in vitro* experiment using dispersed pituitary cells in mature male red seabream (Okuzawa et al., 2016). The present result is limited to immature adult female chub mackerel. Further studies on both sexes in different gonadal stages are required to understand their reactions in response to the three GnRH ligands.

Additionally, the author also examined the expression of the present receptor in different regions of the chub mackerel brain. Previous study showed that only the GnRH1 (functional form) fibers innervated the pituitary

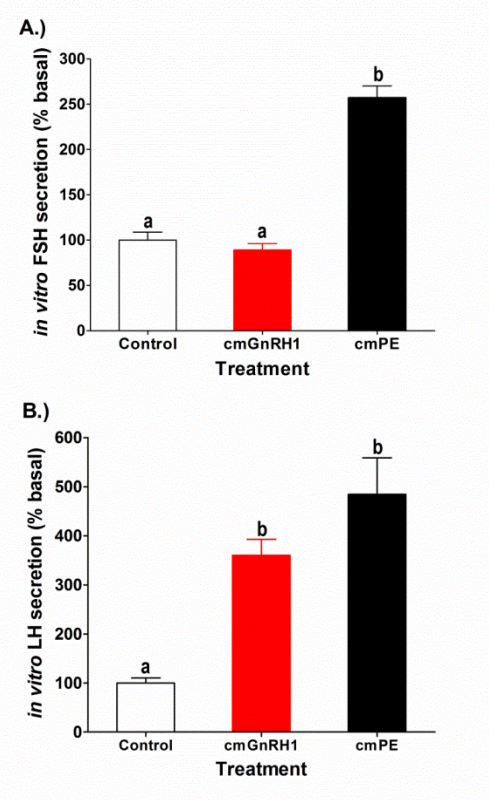


Figure 15. *In vitro* effects of chub mackerel GnRH1 (cmGnRH1) peptide with a dosage of 10^{-6} M on secretion of A.) follicle stimulating hormone (FSH) and B.) luteinizing hormone (LH) in cultured medium from pituitary cells of late spermatogenic male and midvitellogenic female chub mackerel. Chub mackerel pituitary extract (cmPE) was also measured for positive control. The data is expressed as the ratio of changes in firefly luciferase activity over the control *Renilla* luciferase activity. The data were analysed using one-way ANOVA; different letters indicate significant differences ($P<0.05$). Each treatment was determined in quadruplicate and is given as a mean ± SEM.

glands of the chub mackerel (Selvaraj et al., 2009). Interestingly, the present qPCR analysis showed that *cmgnrhr1* is only highly expressed in the pituitary of immature adult chub mackerel, for both sexes. Slightly higher expression was observed in males, although no sexually dimorphic expression of *cmgnrhr1* was seen in the pituitary between immature adult male and female chub mackerel. In previous studies of fish, the African cichlid (Chen and Fernald, 2006), European sea bass (Gonzalez-Martinez et al., 2004), striped bass (Alok et al., 2000), and pejerry (Guilgur et al., 2009) type IIB GnRHRs have been shown to be highly expressed in the pituitary compared with other parts of the brain.

The author also verified gene expression levels of *cmgnrhr1* in the pituitary during the pubertal process. Pubertal onset for teleosts occurs at the beginning of spermatogenesis in males and of vitellegonesis in females (Holland et al., 2000; Okuzawa, 2002). Previous studies of male and female chub mackerel pituitary gene expression showed that functional *gnrh1* expression increased significantly just prior to the start of puberty (Ohga et al., 2015). Notably, upon pubertal onset, *lhβ* started to increase gradually in both sexes of the same species (Nyuji et al., 2014). Interestingly, in the present study, *cmgnrhr1* gene expression gradually increased in the pituitary in both sexes at the onset of puberty, which is consistent with previous results for *lhβ* expression. Taken together, the levels of *lhβ*, *gnrh1*, and *cmgnrhr1* were low

during pre-puberty. In post-puberty, the levels of *lhβ* and *cmgnrhr1* were significantly higher than those of functional *gnrh1*. These results suggest that cmGnRHR1 is a functionally important receptor to the puberty of chub mackerel. In previous studies of fish, Type IIB GnRHR gene expression in the pituitary of European sea bass, striped bass, and pejerry also increased as the fish matured sexually (Alok et al., 2000; Gonzalez-Martinez et al., 2004; Guilgur et al., 2009).

FINAL REMARKS

Studies on the hormones involved in the puberty of fishes are already widely available but largely focused on small model fishes such as zebrafish, medaka, and goldfish. Data from teleost studies on the regulation of puberty can be species-specific, thus, understanding the physiological and endocrine mechanisms in each important fish species are essential. The present study focused on the function of pituitary GnRH receptor in chub mackerel, one of the most commercially utilized fish resources in Japan. Until recently, no information on GnRHRs in this species was available. The aim of this study was to isolate the functional GnRHR involved in the reproduction of chub mackerel.

From one to five GnRHR genes identified in various fish species, still, no well-established classifications for these receptors have been made. Several investigators proposed different nomenclatures for GnRHRs which are composed of two (Flanagan et al., 2007; Lethimonier et al., 2004), three (Levavi-Sivan B and Avitan A., 2005; Millar et al., 2004), or four types (Ikemoto et al., 2004; 2005; Kim et al., 2011) divided into different subtypes. In this paper, the phylogenetic classification consolidated by Hildahl et al. (2011) was adopted, in which GnRHRs are segregated into two types: the

Type I divided by mammalian and non-mammalian receptors, and Type II divided between fish, tree frog an all other tetrapod receptors.

Phylogenetic analysis using the amino acid ORF sequences from the representatives of each subtype showed that the present receptor, *cmgnrhr1*, clustered into TypeIIB receptors. Many similar studies have suggested the involvement of this receptor type in fish reproduction and gonadotropic function control, as exhibited by the African cichlid (Flanagan et al., 2007), Atlantic cod (Hildahl et al., 2011), European eel (Peñaranda et al., 2013), European seabass (Gonzalez-Martinez et al., 2004), and Nile tilapia (Soga et al., 2005). On the other hand, studies have suggested that Type IA receptors of the African cichlid and Nile tilapia play a role in modulating sensory, metabolic, and motor systems (Soga et al., 2005; Chen and Fernald, 2006). Mostly all fish Type I GnRHRs share a conserved AAFIL/SAFIL and DRYRAI/DRHSAI motif in TM3 region (Hildahl et al., 2011). In contrast, nearly all the fish type II GnRHRs has the CAFVT and DRQSAI motif in TM3 (Hildahl et al., 2011). Furthermore, Hildahl et al., (2011) added that in almost all fish, Types I and II have conserved PEY and SHS tri-peptide, respectively, found in the third extra-cellular membrane. For the *cmgnrhr1*, a CAFVT and DRQSAI micro-domain motif in TM3 and SHS tri-peptide are present.

Binding affinity studies in mammals showed that the Type IB GnRHRs have greater potency to GnRH1 ligand, while Type IIA GnRHRs bind to GnRH2 but not GnRH1 (Millar et al., 2004). However, from all the studies on fish carried out in transfected cells such as in African cichlid (Flanagan et al, 2007), African catfish (Bogerd et al., 2002), European seabass (Servili et al., 2010), goldfish (Illing et al., 1999), and medaka (Okubo et al., 2001), GnRH receptors where activated by different GnRH ligands; they also showed higher affinity for GnRH2 with regards to GnRH1. In the same way, the present result indicated potency in the order GnRHa > GnRH2 > GnRH3 > GnRH1. These results are very interesting since in fish GnRHR studies, mostly type IIB receptors is considered as the main hypophysiotropic receptor and said to be involved in the control of gonadotropic function (Chen and Fernald, 2006; Levavi-Sivan et al., 2004; Parhar et al., 2002; 2005; Guilgur et al., 2009; Hildahl et al., 2011). However in GnRHs, GnRH1 is the hypophysiotropic form that plays a major role in reproduction in all vertebrates by stimulating anterior pituitary hormones while GnRH2 could be involved in reproductive behaviour, food intake and neuromodulation in some fishes such as musk shrew (*Suncus marinus*; Temple et al., 2003), goldfish (Volkoff and Peter, 1999; Matsuda et al., 2008) and other teleosts (Oka and Ichikawa, 1990; Oka, 1997). According to Pfleger et al. (2002), GnRH2 (relative to GnRH1) has higher binding affinity to all non-mammalian receptors regardless of their

classification, due to the preconfigured β-II' turn conformation that increases its binding affinity to the receptor. Likewise, the synthetic GnRHa that was used in the present experiment had the D-Ala^6 substitution for Gly^6, believed to stabilize the β-II' turn conformation (Monahan et al., 1973). In line with this, the present binding result serves as a support on previous GnRHa administration studies for effectively inducing spawning in chub mackerel.

The physiological functions of GtHs are already well established in mammalian vertebrates, but information on teleosts on the effects of GnRH on GtH synthesis and secretion are quite limited due to technical difficulties in purifying native GtHs and developing immunoassay systems. In salmonid species, which exhibit synchronous or group-synchronous gonadal development and spawn once per life or year, the FSH plasma levels are high during early phase of spermatogenic males and during early phase of vitellogenic females, whereas levels of LH increases around spermiation in males and final oocyte maturation and ovulation in females (Prat et al., 1996; Schulz et al., 2001; Suzuki et al., 1988; Swanson et al., 2003). In contrast, in non-salmonid species, including multiple spawning Perciform fishes with asynchronous gonadal development, physiological mechanisms of GtHs are quite complex and varies among different species. It is essential to conduct more GtH regulation studies on each species especially on multiple spawners. Thus, one of the aims of the present study is to develop a method on

estimating bioactive levels of GtH in the cultured medium from the pituitary cells of chub mackerel. The results clearly indicate that the three deduced GnRH ligands are not only capable of binding to the present receptor but are also effective in releasing LH. This technique has provided a new and effective way to evaluate LH levels in chub mackerel which would be helpful for future studies. In case of FSH, preliminary GnRH1 administration test did not effectively release FSH *in vitro*. Further tests using different techniques are suggested to study FSH in this species.

The qPCR findings in the present study showed that the present receptor is only significantly expressed in the pituitary. Furthermore, gene expression analysis of *cmgnrhr1* in the pituitary showed a synchronous pattern with the previous results for *fshβ* and *lhβ* expression during pubertal process. Upon combining the previous and present gene expression results (Fig. 16), the levels of *fshβ, lhβ, gnrh1*, and *cmgnrhr1* were low during pre-puberty. On the other hand, in post-puberty, the levels of *fshβ, lhβ* and *cmgnrhr1* were significantly higher than those of functional *gnrh1*. These data suggest that cmGnRHR1 is a functionally important receptor to the puberty of chub mackerel.

Since there was no available information regarding the GnRH receptor in chub mackerel before, these findings have provided baseline information on the missing key component involved in the BPG axis of this fish. Furthermore,

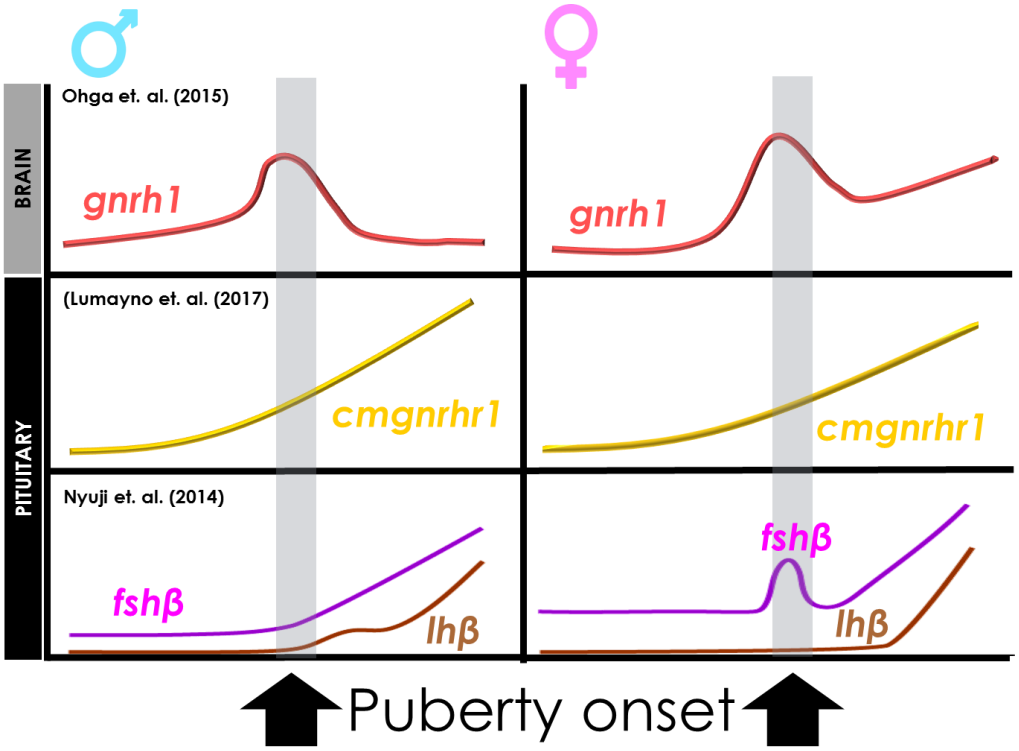


Figure 16. Schematic representation showing changes in *gnrh1* gene levels in the brain; *cmgnrhr1*, *fshβ*, and *lhβ* gene levels in the pituitary of male and female chub mackerel during the puberty.

these findings will be helpful for future studies to better understand the Kiss/KissR-GnRH/GnRHR-GtH/GtHR systems involved in the reproduction of this species. Lastly, the author believes that these findings will be beneficial to the development of aquaculture and fishery management in the future.

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SUMMARY

CHAPTER 1: To understand the missing key component involved in the BPG axis in chub mackerel, this study aimed to identify the GnRH receptor of this fish. At present, one GnRHR was isolated in the pituitary of chub mackerel. The sequence was submitted online with GenBank accession number KX153188. The ORF encodes 1284 bp in length with 428 amino acids and contains seven TM domains. Phylogenetic analysis clustered the cmGnRHR1 amino acid sequence with other teleost type IIB GnRHRs. In most fish studies, this type of receptor is usually involved in the reproduction of the organisms. Reporter gene assay showed that all four tested synthetic peptides bind to the cloned receptor via the PKA/cAMP pathway in the order GnRHa > cmGnRH2 > cmGnRH3 > cmGnRH1.

CHAPTER 2: In order to clarify that the presently isolated GnRH receptor is indeed a functional form involved in the reproduction of chub mackerel, these studies were conducted. In an *in vitro* study, cmGnRH1, cmGnRH2, and cmGnRH3 peptides significantly elevated LH secretion in cultured medium by pituitary cells above basal values from lower concentration to higher concentration. In addition, qPCR revealed that *cmgnrhr1* is distributed in

different regions of the brain tissues of immature adult male and female chub mackerel but only significantly expressed in the pituitary. Interestingly, previous study showed that only the GnRH1 neurons innervate anterior pituitary regions where FSH and LH are localized. Furthermore, receptor gene expression in the pituitary showed an increasing trend in the developing gonadal stages of both sexes during the pubertal process; this process was synchronous with previous studies of *fshβ* and *lhβ* gene expression in chub mackerel. These results suggest that the presently cloned receptor is likely involved in the regulation of pubertal onset in this species.

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ALL THE GLORY AND HONOR BELONGS TO OUR LORD AND SAVIOR, JESUS CHRIST.

Amen.