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Gonadotropin-releasing hormone-like gene in the cephalopod, *Sepia pharaonis*: characterization, expression analysis, and localization in the brain

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ABSTRACT

Gonadotropin-releasing hormone (GnRH), a decapeptide secreted by hypothalamic neurons, is a key signalling molecule of the hypothalamic–pituitary–gonadal axis for the control of reproduction in vertebrates. In this study, a full-length cDNA of GnRH-like gene from the cuttlefish *Sepia pharaonis* (denoted as *SpGnRH*, GenBank Accession NO. QPB69198.1) was cloned, which is 432 bp and the open reading frame (ORF) encodes a protein of 90 aa. Multi-sequence alignment revealed high homology of *SpGnRH* with GnRH from other cephalopod species: 99%, 91%, 86% and 71% similarity with *Sepia lycidas*, *Sepiella japonica*, *Uroteuthis edulis* and *Octopus vulgaris*, respectively. Phylogenetic analysis supported the finding that *SpGnRH* was a new member of the GnRH protein family, which was clustered into a group with octopus, squid, and other cuttlefish species. Tissue distribution analysis revealed that *SpGnRH* mRNA was highly expressed in the brain at three developmental stages (stage III, stage IV, and stage V). *In situ* hybridization showed that *SpGnRH* was localized throughout several different functional brain lobes, suggesting *SpGnRH* might be involved in physiological regulations such as reproduction and feeding. This study might provide a useful theoretical basis for studying reproductive regulation, genetic resource conservation, and artificial breeding.

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Introduction

Gonadotropin-releasing hormone (GnRH), a widespread peptide conserved in both vertebrates and invertebrates, performs a central role in sexual development and reproduction (Sharker et al. 2020). GnRH is grouped into five major types: GnRH-I, GnRH-II, GnRH-III, GnRH-IV, and GnRH-V, based on phylogenetic analysis, distribution, function, and evolutionary origin (Gorbman and Sower 2003; Silver et al. 2004; Ngernsoungnern et al. 2008; Zhang et al. 2008). To date, studies have reported the presence of GnRH in many molluscs: *Octopus vulgaris* (Di Cosmo et al. 1998; Di Cristo et al. 2002; Iwakoshi et al. 2002), *Uroteuthis edulis* (Onitsuka et al. 2009), *Sepia officinalis* (Di Cristo et al. 2009), *Aplysia californica* (Zhang et al. 2008), *Crassostrea gigas* (Treen et al. 2012), *Haliotis discus hannai* (Sharker et al. 2020), *H. asinina* (Nuurai et al. 2016), *Charonia tritonis* (Bose et al. 2017), *Lottia gigantea* (Veenstra 2010), *Patella caerulea* (De Lisa et al. 2013) and *Lymnaea stagnalis* (Young et al. 1999).

The function of GnRH has been clarified in several invertebrate species. It can induce ovarian cell proliferation and spawning in Pacific white shrimp (Tinikul et al. 2014). It can control oocyte maturation, ovulation, and

spawning in scleractinian corals (Twan et al. 2006). It is also crucial for the early development of gametogenesis and germ cell proliferation in bivalves (Lubet and Mathieu 1982), and it can also play a role at the initial sexual maturity stages of the clam *Ruditapes philippinarum* (Song et al. 2015). This molecule is also in charge of the steroid hormones produced by the follicle cells of the ovary and spermatozoa in octopus (Di Cosmo et al. 1998, 2001, 2002; De Lisa et al. 2012).

Up to about 50% sustained electrical discharge time, called after discharge (AD), was reduced in the *in vitro* treatment of GnRH II in *A. californica* cell preparation without affecting the release of egg-laying hormone (ELH). As shorter AD will significantly reduce the bio-synthesis of ELH, it is speculated that GnRH has a strong and continuous inhibitory effect on the egg laying capacity of these animals (Di Cosmo et al. 2002; Tsai 2006). In *O. vulgaris*, GnRH can not only buffer the inhibitory effect of FMRFamide on the visual activity hypothesis, but also acts at the peripheral level, which means that GnRH can directly control the contraction of the reproductive tract (Iwakoshi-Ukena et al. 2004). At the same time, oct-GnRH can induce oct-GnRH receptors

to express progesterone, steroidogenesis and 17 β -oestradiol in the ovary and testis of *O. vulgaris*, suggesting that oct-GnRH acts as a multifunctional neurotransmitter, neuromodulator and hormone-like factor, both in the *Octopus* central nervous system (CNS) and peripheral tissues (Kanda et al. 2006; De Lisa et al. 2012). GnRH is also implicated in regulating feeding activity, oesophageal motility (Minakata et al. 2009), and affects visual sense (Di Cristo et al. 2009) and tactile sense (Di Cosmo et al. 2001). As a neuromodulator, it is involved in the autonomic roles of the brain, locomotion, feeding, and memory of the octopus (Iwakoshi-Ukena et al. 2004; Kanda et al. 2006). The expression of GnRH in the CNS has been reported in detail for *O. vulgaris* (Iwakoshi et al. 2002), *Loligo bleekeri* (Amano et al. 2008), and *H. discus hannai* (Sharker et al. 2020). All of these findings reinforce the hypothesis that GnRH peptides play an important part in the growth and especially in reproduction of molluscs (Tsai 2006).

Sepia pharaonis, widely distributed in China, is considered a species of major economic interest for commercial aquaculture. Many research groups have paid more attention to the fisheries and food science technology, but there is lack of studies to clarify the mechanisms of growth, development, reproduction, and molecular adaptation of *S. pharaonis*. In this study, we identified and cloned a GnRH-like gene for the first time from the brain of the decapod *S. pharaonis* and examined the expression of precursor mRNA using quantitative real-time PCR (qRT-PCR) and *in situ* hybridization (ISH). The results of this study will be helpful for understanding the physiological function of the GnRH peptide in cephalopods and the correlation with reproduction.

Material and methods

Animals

Females and males of *S. pharaonis* were sampled from an artificial breeding farm (Shacheng, Fuding, Fujian Province, PR China). The cuttlefishes were cultured in filtered seawater at 24°C with a photoperiod of 14 h light: 10 h dark and were fed with shrimps twice per day. Three or six cuttlefish were used for the tissue distribution analysis at every developmental stage: stage III (102.8 ± 6.9 g), stage IV (female 498.4 ± 4.0 g, male 497.7 ± 10.3 g), stage V (female 1003.1 ± 9.2 g, male 1000.5 ± 9.7 g), classified according to the growth time and the appearance of the gonad (Jiang et al. 2007; Luo et al. 2014). All the *in vivo* tests were carried out at the School of Marine Science and Technology of Zhejiang Ocean University (China), which obtained the permission for performing the research protocols and all animal

experiments conducted during the present study from the ethics committee of Zhejiang Ocean University (2,021,031). All experimental procedures were conducted under the oversight and approval of the Academy of Experimental Animal Center of Zhejiang Ocean University and in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Total RNA extraction and the first-strand cDNA synthesis

The cuttlefishes were anesthetized with ice about 2 minutes before sacrifice. Tissue samples of the muscle, heart, gill, stomach, liver, testis, nidamental gland, accessory nidamental gland, ovary, brain, and optic lobe, were dissected separately, then conserved in RNAlater (Sangon Biotech, Shanghai, Co., Ltd, China), and finally stored at -80°C for further use. Total RNA of different tissues was isolated by Trizol reagent (Takara Bio Inc., Otsu, Kyoto, Japan), quantified by nucleic acid protein detector (Nanodrop2000) and verified by gel electrophoresis. The first-strand cDNA synthesis was performed using PowerScript reverse transcriptase (Takara Bio Inc.) at 42°C for 90 min.

Cloning of the full-length cDNA for the SpGnRH gene

To obtain the full-length cDNA, a pair of primers GnRH-F/GnRH-R (Table 1) was designed by comparing the conserved fragments of *Sepiella japonica* (KP982885), *O. vulgaris* (AB037165.1), *U. edulis* (AB447557.1), *M. yessoensis* (AB486004.1), *A. californica* (EU204144.1), and *C. gigas* (HQ712119.1). The mature cuttlefishes were used as the source of the cloning of *SpGnRH* cDNA.

The amplification reaction was performed according to the methods from our laboratory with slight changes (Cao et al. 2016). PCR products containing a single band were constructed to the pUCm-T Vector (Bio Basic, Canada) and conducted using two-way sequencing

Table 1. Primers used in this study.

Primer	Sequence (5'-3')	Position
GnRH-F	CAGACNCAGCACARAAYTA	168–187
GnRH-R	TYTCTATCAAAGCYTTGT	264–282
GnRH-5 outer	TTACCACGGGGTGCCATCC	201–220
GnRH-5 inner	TTGCTAAATGGTAATTCTG	180–199
GnRH-3 outer	CACCTGTGCTATTCTCCCTCTTC	134–161
GnRH-3 inner	TGCATATCCAGGCACAGATTACCA	166–190
RT-actin-F	TGAGAGGGAGATTGTGCGTG	815–834
RT-actin-R	GAACATAGATTCTGGAGCACGG	968–989
RT-GnRH-F	ACTTCTTTACACTCGTCCCT	1–21
RT-GnRH-R	GGGTGCCATCCATGCTAA	193–211
GnRH-probeF	CCGGTCGACATCTCATCAGCTAACATC	47–69
GnRH-probeR	CCGGAATTCCGTGTGATTCCTCGTCAAG	287–307

(Sangon, China). After cloning the core sequence, the first-strand cDNA was used to amplify the two ends of the *SpGnRH* gene based on gene-specific primers (GnRH-5' outer/GnRH-5' inner, GnRH-3' outer/GnRH-3' inner, Table 1) by the RACE (rapid-amplification of cDNA ends) method. For 5'-RACE and 3'-RACE, we conducted two rounds of PCR with the similar reagents and procedures except the template. For the first round PCR, the first-strand cDNA was used as the template, while the PCR product was subjected as the template of the second round PCR. The 5'-RACE and 3'-RACE were handled with the same method.

Sequence and phylogenetic analyses

The BLASTx was used to identify the nucleotide sequence comparison from the National Center for Biotechnology Information database (NCBI). Predict Protein websites were used to deduce the amino acid sequences and the potential coding region. Amino acid sequences were aligned with other species using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). According to previous records, we speculated that the signal sequence was present and affirmed by SignalP v3.0 (<http://www.cbs.dtu.dk/services/SignalP>). The molecular weight (MW) and theoretical isoelectric point (*pI*) of the precursor protein could be calculated by the ExPASy ProtParam. The phylogenetic tree based on the deduced amino acid was constructed by the maximum likelihood method (MEGA 10.0), and the bootstrap test was assessed from 1000 replications to assure the reliability.

Quantitative real-time PCR and tissue distribution analysis

SpGnRH mRNA expression levels of various tissues at different developmental stages were tested on a 7500 Real Time PCR System (Applied Biosystems, UK) with the help of a SYBR Premix Ex TaqTM II Kit (Takara Bio, Inc.). All tissues including heart, brain, optic lobe, gill, testis, ovary, nidamental gland, accessory nidamental gland, liver, muscle, and stomach were conducted in triplicate. The primers (RT-GnRH-F/RT-GnRH-R, Table 1) of qRT-PCR were designed specifically, and β -actin (JN564496.1) was chosen as the reference gene. The qRT-PCR was run at 95°C for 1 min, followed by 40 cycles at 95°C for 10s, then at 60°C for 45s, and the melting curve was used to check accurate amplification of the target gene.

The relative *SpGnRH* mRNA expression was determined based on the cycle threshold (*Ct*), and the $2^{-\Delta\Delta Ct}$ method was used to analyse the expression level (Schmittgen and Livak 2008). The mRNA expression level of the heart was taken as the standard. All data

were normalized with it and are given means \pm S.D. (*n* = 3). Least Significant Difference (LSD) multiple comparison test was performed to determine the significant differences (*p* < 0.05) between means by the Statistical Product and Service Solutions (SPSS 22.0).

In situ hybridization assay of SpGnRH

ISH was performed to localize the *SpGnRH* gene. PCR products of *SpGnRH* with the DIG-labelled sense and antisense probes (Table 1) and plasmid vector pSPT18 were respectively double digested using Acc I enzyme and EcoRI enzyme. Then the recombinant plasmids were dialled with one single enzyme and transcribed to RNA *in vitro* under the action of a DIG RNA Labelling Kit SP6/T7 (Roche Diagnostics, Mannheim, Germany).

Brain tissues of cuttlefish at stage V were fixed with 4% paraformaldehyde (PFA, dissolved in 1× PBS) for 20 h, dehydrated with alcohol of gradient concentrations, transparentized by dimethyl benzene, dipped successively in melted soft paraffin wax and hard paraffin wax, then embedded in hard paraffin wax, and finally sectioned (7 μ m thickness). Paraffin wax sections were dewaxed, rehydrated, fixed twice with 4% PFA, rinsed twice with 1× PBS, and next processed with Glycine solution and peptidase K (1 μ g/ml) in TE buffer (Tris-EDTA buffer). To reduce the background staining, pre-hybridization should be performed. Then the sections were treated with hybridization solution and the DIG Easy Hyb (Roche Molecular Biochemicals, Mannheim, Germany) overnight at 43°C. Then, the sections were treated with PBS buffer and 1% Roche blocking solution. Finally, sections of tissue were incubated with anti-DIG-AP conjugate (Roche Diagnostics, dilution rate was 1:500) and the probes were detected using a chromogenic substrate: nitroblue tetrazolium/4-bromo-4-chloro-3'-indolylphosphate (NBT/BCIP, Promega, Madison, Wisconsin, USA). The sections were washed with 1× PBS, then sealed with clear nail polish, and photographed with a microscope (Olympus CX31, Olympus, Japan).

Results

Cloning and analysis of SpGnRH gene

By using the RACE method, we obtained the full cDNA transcripts of one GnRH-like neuropeptide, *SpGnRH*. The whole length sequence of *SpGnRH* was 432 bp (GenBank Accession No. QPB69198.1), containing a 5'-untranslated region (5'-UTR) of 86 bp, an open reading frame (ORF) of 273 bp encoding 90 amino acids (aa), and a 3'-UTR of 73 bp (Figure 1). The predicted MW was 10.1 kDa and the

1 ACTTCCTTACACTCGCCCTCGAAGAACAAAAGAACACTCAAATCTCCATCATCAG
 61 CTAACATCTACCAGACAGTATTATC**ATG**TCAACCTCCACAGCCTCGCCAGCCTGAGAA
M S T S T A S S S L R
 121 GAATGGCCTTTCACCTGTGCTATTCTCCCCTCTTCTGCATGCATATCCAGGCAC
R M A F F T C A I L P L S F C M H I O A
 181 AGAATTACCATTAGCAATGGATGGCACCCCGTGGTAAACGAAGTGGACTTCAGACA
Q N Y H F S N G W H P G G K R S G L P D
 241 TGCACTGTCAATTAGCACCACAAACAAAGCTTGATCAGAAACTCTTAGACGAGGAA
 M Q C H F R P Q T K A L I E K L L D E E
 301 TCACACGTATAATTACTACATGTACCAATACAGTCATGACATCGCAGACTGCAG**TAAT**
 I T R I I T T C T N T V N D I A D L Q *
 361 TTTTCACAGGTCAAGCAAATTCACTGGATATACAACCTACCAAAAAAAA
 421 AAAAAAAA

Figure 1. Nucleotide sequence and deduced amino acid sequence of *SpGnRH*. The initiation codon (ATG) and termination codon (TAA) are marked with black boxes. The putative signal peptide is marked with a black underline in bold and italics, the predicted mature product is highlighted in red, cleavage sites in green and the glycines used for C-terminus amidation in orange.

theoretical *pI* was 7.73. The deductive *SpGnRH* included a signal peptide of 31 aa and a mature product: one dodecapeptide (pGlu-Asn-Tyr-His-Phe- Ser-Asn-Gly-Trp-His-Pro-Gly). There is a glycine at the C-terminus of the mature peptide, which means post-translationally amidatedlysine-arginine or arginine residues served as an internal proteolytic cleavage site of the mature peptide during post-translational processing at the C-terminus.

Multiple sequence alignment and phylogenetic analysis

The predicted amino acid sequence of *SpGnRH* was compared with other known species from the NCBI database by ClustalW2, and the results showed that *SpGnRH* protein in *S. pharaonis* shares 99%, 91%, 86%, 71%, 37%, 41%, 31% similarity with *S. lycidas*, *S. japonica*,

U. edulis, *O. vulgaris*, *C. gigas*, *M. yessoensis*, and *A. californica*, respectively (Figure 2). To study the evolutionary status of the *SpGnRH*, a phylogenetic tree was reconstructed on the basis of 13 protein sequences by using the Maximum Likelihood method. It showed that the *SpGnRH* in the molluscs, including cephalopods, gastropods, and bivalves, are grouped in a separate branch from the vertebrates, echinoderms, and tunicate (Figure 3). The phylogenetic tree analysis revealed that *SpGnRH* was clustered into the invertebrate branch and formed a cluster with GnRH of *S. lycidas*, which was consistent with the traditional taxonomy (Dong 1988).

Expression of *SpGnRH* mRNA in various tissues

The results of qRT-PCR revealed the mRNA expression levels of the *SpGnRH* gene in various tissues of three

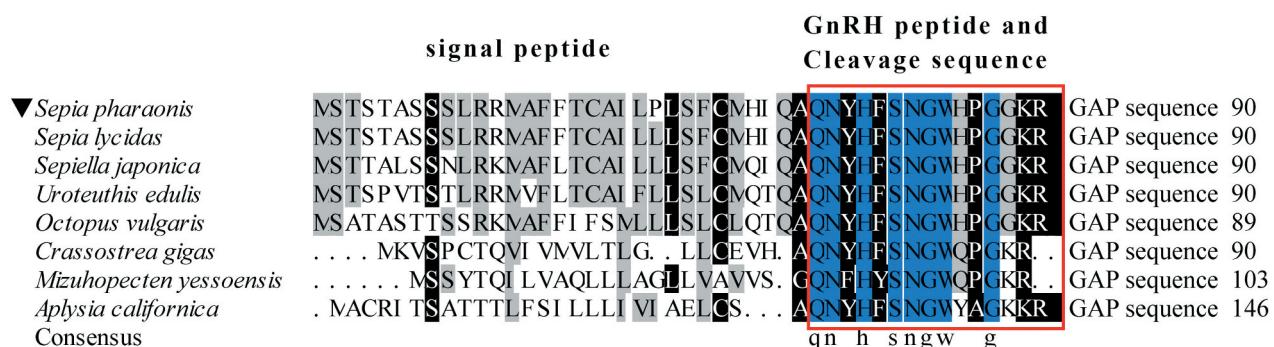


Figure 2. Sequence alignment of GnRH protein precursors of *S. pharaonis* (QPB69198.1), *S. lycidas* (BCG29712.1); *S. japonica* (KP982885); *U. edulis* (AB447557.1); *O. vulgaris* (AB037165.1); *C. gigas* (HQ712119.1); *M. yessoensis* (AB486004.1); *A. californica* (EU204144.1). The shared residues of all sequences are in blue and conservative substitutions are in black.

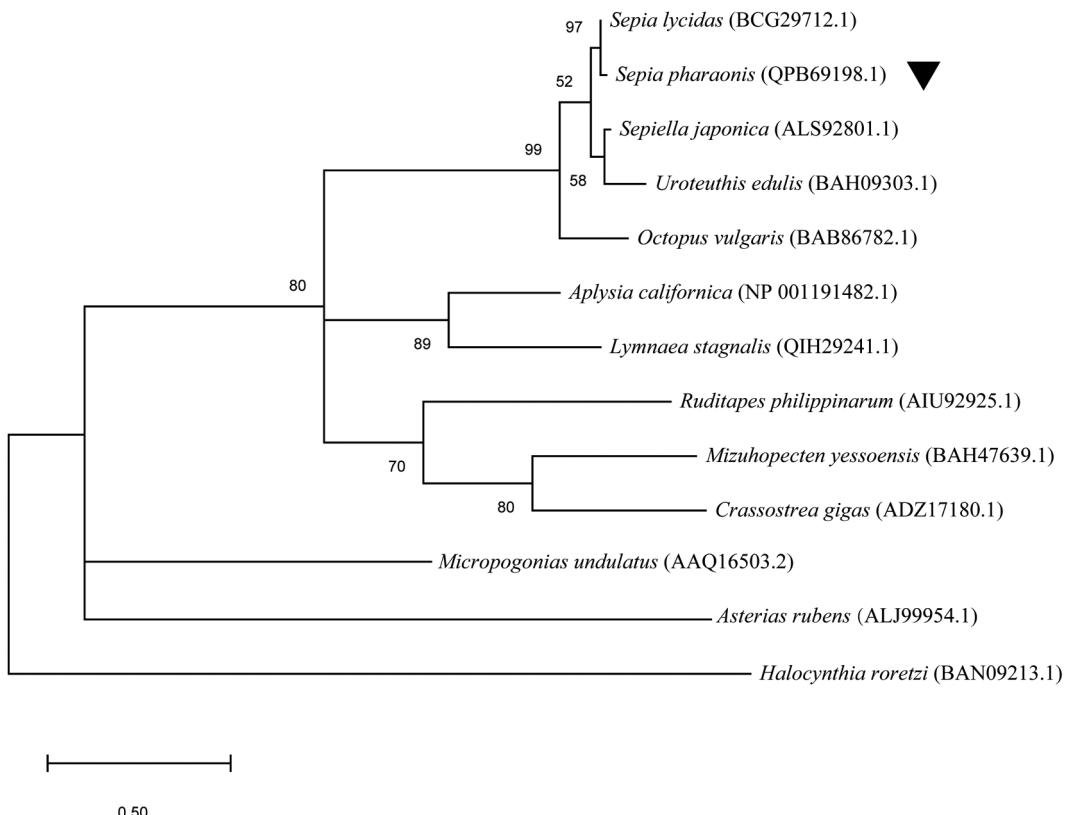


Figure 3. The sequence homology analysis between *SpGnRH* and other identified GnRH was conducted with multiple sequence alignment (ClustalW2) and maximum likelihood phylogenetic analysis (MEGA 10.0). All protein sequences were obtained from GenBank of NCBI, and the GenBank accession numbers are shown in parentheses.

different developmental stages of both sexual cuttlefish (**Figure 4**). The developmental stages of *S. pharaonis* were divided into six stages based on published work (Jiang et al. 2007; Luo et al. 2014). In each case, the expression level of the heart was regarded as the reference value. The data are normalized, and the expression

level of *SpGnRH* in the heart was set to 1. Tissue expression analysis confirmed that *SpGnRH* was widely distributed among tissues and predominantly expressed in the brain at all three developmental stages. At stage III, the relative expression in the brain was more than 120 times that of the heart, and the expression was significantly

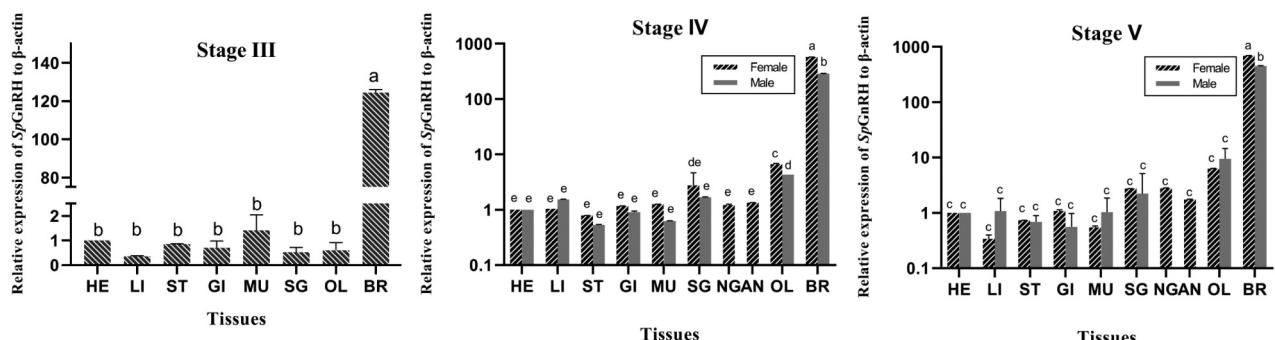


Figure 4. The relative expression variance is shown as a ratio (the amounts of *SpGnRH* mRNA normalized to the corresponding β -actin values) in various tissues of three developmental stages (stage III, stage IV, stage V). Transcript distribution of all tissues is presented relative to that in the heart. Vertical bars represent the mean \pm S.D. ($n = 3$). The analyses were conducted in eight/ten different tissues including the heart (HE), liver (LI), stomach (ST), gill (GI), muscle (MU), sexual gland (SG, including testis and ovary), brain (BR), nidamental gland (NG), accessory nidamental gland (AN), and optic lobe (OL). Least Significant Difference (LSD) multiple comparison test is performed to determine significant differences between means of the Statistical Product and Service Solutions (SPSS). Columns sharing different letters (a-e) show significant difference ($p < 0.05$).

higher in the brain than in other tissues ($p < 0.05$). At stage IV, the expression in the brain of females and males was approximately 570 times and 290 times of the heart, respectively, and the expression in the optic lobes of females and males was 6.7 times and 4.3 times of the heart, respectively. At stage V, the expression in the brain of females and males was approximately 687 times and 451 times of the heart. The mRNA expression of *SpGnRH* in the brain was generally higher in female cuttlefish. ($p < 0.05$).

Localization of *SpGnRH* in the brain

According to previous research, the brain of *S. pharaonis* is divided into three parts: suprakoophgeal mass, subrakoophgeal mass, and optic lobes (Yu et al. 2011). ISH was used to locate the expression of *SpGnRH* in the brain of *S. pharaonis*. As shown in Figure 5, *SpGnRH* gene mRNA-positive hybridization signals concentrated in

some areas of the brain. Figure 5(a) serves as the negative control in which the antisense probe hybridization could not be observed. In some areas of the suprakoophgeal mass, signals of specific hybrid of *SpGnRH* were observed, and all these areas were concentrated on the functional lobe of the medulla. The most strongly positive signals were in subpeduncle lobe (SPL) and the subvertical lobe (SVL) (Figure 5(b)), followed by the posterior basal lobe (PBL) (Figure 5(e)) and anterior basal lobe (ABL) (Figure 5(d)). The signals in the inferior frontal lobe (IFL) (Figure 5(c)) were weakest. In the subboesophagel mass, the effective positive signals of *SpGnRH* mainly existed in the magnocellular lobe (MAG) and the palliovisceral lobe (PVL) (Figure 5(h)) of the posterior subboesophagel masses (PSEM). In the middle subboesophagel mass (MSEM), positive signals could also be observed in the anterior pedal lobe (APL) (Figure 5(f)) and anterior chromatophore lobe (ACL) (Figure 5(g)). In the anterior subboesophagel mass (ASEM), positive

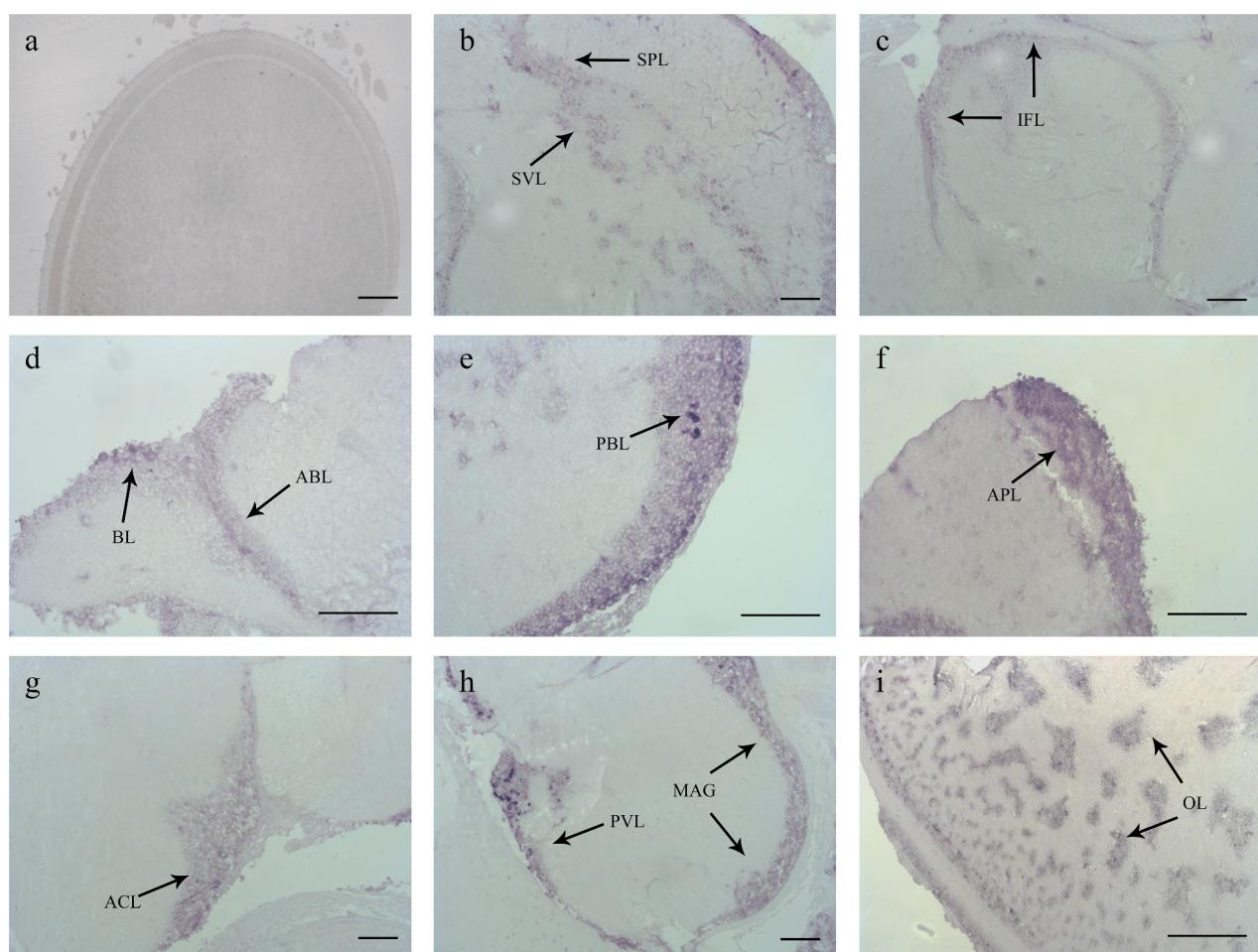


Figure 5. Localization of *SpGnRH* mRNA in brain. (A) A medial sagittal section of the optic lobe stained with the sense GnRH probe. (B) subvertical lobe, SVL; subpeduncle lobe, SPL. (C) inferior frontal lobe (IFL). (D) brachial lobe, BL; anterior basal lobe, ABL. (E) posterior basal lobe, PBL. (F) anterior pedal lobe, APL. (G) anterior chromatophore lobe, ACL. (H) palliovisceral lobe, PVL; magnocellular lobe, MAG. (I) optic lobe, OL. The black arrows indicate positive signals. Scale bars: 200 μ m.

signals could be stained only in the brachial lobe (BL) (**Figure 5(d)**). Similarly, these positive signals of *SpGnRH* antisense oligonucleotide probe hybridization formed in optic lobes (OL) (**Figure 5(i)**), and these signals could just be observed in the medulla. None was observed in the cortex.

Discussion

In this study, we reported the first cloning and characterization of *GnRH* in *S. pharaonis*. Multiple sequence alignment analysis indicated that the deduced amino acid sequence of *SpGnRH* precursor shares 99%, 90%, 86%, 71%, 37%, 41% and 31% homology with *S. lycidas*, *S. japonica*, *U. edulis*, *O. vulgaris*, *C. gigas*, *M. yessoensis* and *A. californica* respectively. This indicated that *SpGnRH* neuropeptide may be highly conserved across the known molluscan members (Nagle 1981).

The results of the phylogenetic tree showed that *SpGnRH* is clustered with other invertebrate species while the vertebrate, echinoderms, and tunicate are clustered into others, respectively. Meanwhile, *S. pharaonis* and *S. lycidas* belong to the same clade with the closest relationship with 99% homology, which is consistent with the traditional classification, indicating that *GnRH* of these types in cephalopods might have originated from a common ancestor. Consequently, this transcript is allocated to the *GnRH-V* group, as previously described for the *GnRHs* of other molluscs (Zhang et al. 2008).

The expression pattern of the *GnRH* transcript of *S. pharaonis* was evaluated by qRT-PCR in several representative tissues. The highest level of expression was detected in the brain (**Figure 5**). In contrast, *GnRH-I* and *GnRH-II* are primarily expressed in the CNS and peripheral tissues in humans and other piscine vertebrates (Nabissi et al. 2000; Metallinou et al. 2007). *SpGnRH* was distributed in the brain of *S. pharaonis* in three developmental stages of females and males. The *GnRH* mRNA was mainly expressed in the CNS of *U. edulis*, whereas *oct-GnRH* mRNA is distributed in CNS and other peripheral organs such as heart, oviduct, and oviductal gland of *O. vulgaris* (Iwakoshi-Ukena et al. 2004). Notably, *ap-GnRH* transcripts were also found in the CNS, ovotestis, and atrial gland (Zhang et al. 2000). However, *GnRH* transcripts were highly and exclusively expressed in the visceral ganglia of the Pacific oyster, *C. gigas* (Bigot et al. 2012). The expression pattern of *GnRH* in molluscs shows that *GnRH* is a multipotent decapeptide that plays a role in many organs such as brain and gonad.

Our results showed that *SpGnRH* is expressed early in life and potentially plays many roles during the development. For further confirmation, the brain of

S. pharaonis would be picked to conduct the ISH assay to detect the distribution of *SpGnRH* mRNA. We found that the mRNA-positive hybridization signals of *SpGnRH* were detected in almost all the functional lobes of the brain, including SPL, SVL, ABL, PBL, IFL, ACL, BL, PVL, MAG, and OL. The distribution of *GnRH* in the CNS of the cephalopods has been reported in detail. It was found that *GnRH* immunoreactive neurons were found in the optic lobes of *O. vulgaris*, that there was an immunoreactive nerve fibre outside the nerve fibre web, and that there are many long axons in the nerve fibres (Di Cosmo et al. 1998). Through immunological staining of c*GnRH-I* in the reproductive tract and gonad of *O. vulgaris*, there was a strong immunoreactive reaction of the c*GnRH-I* in both male (seminal vesicle) and female (oviduct and oviducal gland). While positive signals only can be observed in the nerve fibres around the oviduct and the oviducal gland, and some of the nerve fibres in the central area of the nerve plexus (Di Cosmo et al. 2001, 2002; Di Cristo et al. 2002). RT-PCR and immunolocalization of *oct-GnRH* have been performed in the brain and ovary of *S. officinalis*. The results showed that *oct-GnRH* was expressed in the brain, ovary, eggs and olfactory lobes, especially in the olfactory lobes (Di Cristo et al. 2009). In cephalopods, the olfactory lobe is involved in a variety of physiological and behavioural controls through chemical cues, including growth and reproductive behaviours (Di Cosmo and Polese 2017). Results of immunolocalization showed that immunoreactive neurons were mainly located in the dorsal-lateral basal lobe and nerve fibres (Di Cristo et al. 2009). While compared with *O. vulgaris*, the cortical region of the positive signal is relatively weak.

GnRH analogues and c*GnRH-II* antibody were used for an immunohistochemical study of the brain tissue of *L. bleekeri* and found that most of the immunoreactive nerve fibres was located in the brain and only part of the nerve fibres in the optic gland was stained (Amano et al. 2008). A large number of c*GnRH-II* immunoreactive cell bodies were found to be distributed in the ventral magnocellular lobe and the optic gland, and positive nerve fibres were widely present in the brain. The axons of the bundles were extended from the giant cell leaves to the inside of the brain, and the weak positive signal was also detected in the palliovisceral lobe and olfactory lobes (Amano et al. 2008).

In the preset study, for the first time, a decapeptide *GnRH*-like gene was cloned and characterized from the cuttlefish *S. pharaonis*, which belongs to *oct-GnRH*. qRT-PCR analysis and ISH of the brain indicated the dominant expression profile of *GnRH*-like throughout different developmental stages in both male and female *S. pharaonis*. These findings are expected to provide

some theoretical basis for the diverse functions of GnRH and the reproductive physiology of cephalopods.

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Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the author(s).

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References

- Amano M, Oka Y, Nagai Y, Amiya N, Yamamori K. 2008. Immunohistochemical localization of a GnRH-like peptide in the brain of the cephalopod spear-squid, *Loligo bleekeri*. *Gen Comp Endocrinol.* 156(2):277–284. doi:[10.1016/j.ygcn.2008.01.008](https://doi.org/10.1016/j.ygcn.2008.01.008).
- Bigot L, Zatylny-Gaudin C, Rodet F, Bernay B, Boudry P, Favrel P. 2012. Characterization of GnRH-related peptides from the Pacific oyster *Crassostrea gigas*. *Peptides.* 34(2):303–310. doi:[10.1016/j.peptides.2012.01.017](https://doi.org/10.1016/j.peptides.2012.01.017).
- Bose U, Suwansa-Ard S, Maikaeo L, Motti CA, Hall MR, Cummins SF. 2017. Neuropeptides encoded within a neural transcriptome of the giant triton snail *Charonia tritonis*, a Crown-of-Thorns starfish predator. *Peptides.* 98:3–14. doi:[10.1016/j.peptides.2017.01.004](https://doi.org/10.1016/j.peptides.2017.01.004).
- Cao ZH, Sun LL, Chi CF, Liu -H-H, Zhou L-Q, Lv Z-M, Wu C-W. 2016. Molecular cloning, expression analysis and cellular localization of an LFRFamide gene in the cuttlefish *Sepiella japonica*. *Peptides.* 80:40–47. doi:[10.1016/j.peptides.2015.10.005](https://doi.org/10.1016/j.peptides.2015.10.005)
- De Lisa E, Carella F, De Vico G, Cosmo AD. 2013. The Gonadotropin Releasing Hormone (GnRH)-like molecule in prosobranch *Patella caerulea*: potential biomarker of endocrine-disrupting compounds in marine environments. *Zool Sci.* 30(2):135–140. doi:[10.2108/zsj.30.135](https://doi.org/10.2108/zsj.30.135).
- De Lisa E, Paolucci M, Di Cosmo A. 2012. Conservative nature of oestradiol signalling pathways in the brain lobes of *Octopus vulgaris* involved in reproduction, learning and motor coordination. *J Neuroendocrinol.* 24(2):275–284. doi:[10.1111/j.1365-2826.2011.02240](https://doi.org/10.1111/j.1365-2826.2011.02240).
- Di Cosmo A, Di Cristo C. 1998. Neuropeptidergic control of the optic gland of *Octopus vulgaris*: FMRF-amide and GnRH immunoreactivity. *J Comp Neurol.* 398(1):1–12. doi:[10.1002/\(SICI\)1096-9861\(19980817\)398:13.0.CO;2-5](https://doi.org/10.1002/(SICI)1096-9861(19980817)398:13.0.CO;2-5).
- Di Cosmo A, Di Cristo C, Paolucci M. 2001. Sex steroid hormone fluctuations and morphological changes of the reproductive system of the female of *Octopus vulgaris* throughout the annual cycle. *J Exp Zool.* 289(1):33–47. doi:[10.1002/1097-010X\(20010101/31\)289:13.0.CO;2-A](https://doi.org/10.1002/1097-010X(20010101/31)289:13.0.CO;2-A).
- Di Cosmo A, Di Cristo C, Paolucci M. 2002. A estradiol-17 β receptor in the reproductive system of the female of *Octopus vulgaris*: characterization and immunolocalization. *Mol Reprod Dev.* 61(3):367–375. doi:[10.1002/mrd.10014](https://doi.org/10.1002/mrd.10014).
- Di Cosmo A, Paolucci M, Di Cristo C, Botte V, Ciarcia G. 1998. Progesterone receptor in the reproductive system of the female of *Octopus vulgaris*: characterization and immunolocalization. *Mol Reprod Dev.* 50(4):451–460. doi:[10.1002/\(SICI\)1098-2795\(199808\)50:43.0.CO;2-H](https://doi.org/10.1002/(SICI)1098-2795(199808)50:43.0.CO;2-H).
- Di Cosmo A, Polese G. 2017. Cephalopod olfaction. Oxford research encyclopedia of neuroscience. doi:[10.1093/acrefore/9780190264086.013.185](https://doi.org/10.1093/acrefore/9780190264086.013.185)
- Di Cristo C, De Lisa E, Di Cosmo A. 2009. GnRH in the brain and ovary of *Sepia officinalis*. *Peptides.* 30(3):531–537. doi:[10.1016/j.peptides.2008.07.008](https://doi.org/10.1016/j.peptides.2008.07.008).
- Di Cristo C, Paolucci M, Iglesias J, Sanchez J, Di Cosmo A. 2002. Presence of two neuropeptides in the fusiform ganglion and reproductive ducts of *Octopus vulgaris*: fMRFamide and gonadotropin-releasing hormone (GnRH). *J Exp Zool.* 292(3):267–276. doi:[10.1002/jez.90000](https://doi.org/10.1002/jez.90000).
- Dong, ZZ. 1988. China Fauna Sinica Mollusca Cephalopoda. Beijing: Science press. 119–126.
- Gorbman A, Sower SA. 2003. Evolution of the role of GnRH in animal (Metazoan) biology. *Gen Comp Endocrinol.* 134(3):207–213. doi:[10.1016/j.ygcn.2003.09.018](https://doi.org/10.1016/j.ygcn.2003.09.018).
- Iwakoshi E, Takuwa-Kuroda K, Fujisawa Y, Hisada M, Ukena K, Tsutsui K, Minakata H. 2002. Isolation and characterization of a GnRH-like peptide from *Octopus vulgaris*. *Biochem Biophys Res Comm.* 291(5):1187–1193. doi:[10.1006/bbrc.2002.6594](https://doi.org/10.1006/bbrc.2002.6594).
- Iwakoshi-Ukena E, Ukena K, Takuwa-Kuroda K, Kanda A, Tsutsui K, Minakata H. 2004. Expression and distribution of octopus gonadotropin-releasing hormone in the central nervous system and peripheral organs of the octopus (*Octopus vulgaris*) by *in situ* hybridization and immunohistochemistry. *J Comp Neurol.* 477(3):310–323. doi:[10.1002/cne.20260](https://doi.org/10.1002/cne.20260).
- Jiang XM, Fu FY, Li Z, Feng XD. 2007. Study on the oogenesis and ovarian development of *Sepiella maindroni*. *J Fish China.* 5. 607–617. doi:[10.3321/j.issn:1000-0615.2007.05.007](https://doi.org/10.3321/j.issn:1000-0615.2007.05.007)
- Kanda A, Takahashi T, Satake H, Minakata H. 2006. Molecular and functional characterization of a novel gonadotropin-releasing-hormone receptor isolated from the common octopus (*Octopus vulgaris*). *Biochem J.* 395(1):125–135. doi:[10.1042/BJ20051615](https://doi.org/10.1042/BJ20051615).
- Lubet P, Mathieu M. 1982. The action of internal factors on gametogenesis in pelecypod molluscs. *Malacologia.* 22(1–2):131–136.
- Luo J, Jiang X, Liu MH, Tang F, Peng RB. 2014. Oogenesis and ovarian development in *Sepia lycidas*. *Acta Hydrobiol Sinica.* 38(6):1107–1116. doi:[10.7541/2014.162](https://doi.org/10.7541/2014.162)
- Metallinou C, Asimakopoulos B, Schroer A, Nikolettos N. 2007. Gonadotropin-releasing hormone in the ovary. *Reprodu Sci.* 14(8):737–749. doi:[10.1177/1933719107310707](https://doi.org/10.1177/1933719107310707).
- Minakata H, Shigeno S, Kano N, Haraguchi S, Osugi T, Tsutsui K. 2009. Octopus gonadotrophin-releasing hormone: a multifunctional peptide in the endocrine and nervous systems of the cephalopod. *Neuroendocrinology.* 21(4):322–326. doi:[10.1111/j.1365-2826.2009.01852.x](https://doi.org/10.1111/j.1365-2826.2009.01852.x).
- Nabissi M, Soverchia L, Polzonetti-Magni AM, Habibi HR. 2000. Differential splicing of three gonadotropin-releasing hormone transcripts in the ovary of seabream (*Sparus aurata*). *Biol Reprod.* 62(5):1329–1334. doi:[10.1095/biolreprod62.5.1329](https://doi.org/10.1095/biolreprod62.5.1329).

- Nagle GT. 1981. The molluscan cardioactive neuropeptide FMRFamide: subcellular localization in bivalve ganglia. *J Neurobiol.* 12(6):599–611. doi:[10.1002/neu.480120608](https://doi.org/10.1002/neu.480120608).
- Ngernsoungnern A, Ngernsoungnern P, Weerachatyanukul W, Chavadej J, Sobhon P, Sretarugsa P. 2008. The existence of gonadotropin-releasing hormone (GnRH) immunoreactivity in the ovary and the effects of GnRHs on the ovarian maturation in the black tiger shrimp *Penaeus monodon*. *Aquaculture.* 279 (1–4):197–203. doi:[10.1016/j.aquaculture.2008.04.018](https://doi.org/10.1016/j.aquaculture.2008.04.018).
- Nuurai P, Cummins SF, Botwright NA, Sobhon P. 2016. Characterization of an abalone gonadotropin-releasing hormone and its effect on ovarian cell proliferation. *Aquaculture.* 450:116–122. doi:[10.1016/j.aquaculture.2015.07.008](https://doi.org/10.1016/j.aquaculture.2015.07.008).
- Onitsuka C, Yamaguchi A, Kanamaru H, Oikawa S, Takeda T, Matsuyama M. 2009. Molecular cloning and expression analysis of a GnRH-Like dodecapeptide in the swordtip squid, *Loligo edulis*. *Zool Sci.* 26(3):203–208. doi:[10.1016/j.jmatprotec.2008.01.046](https://doi.org/10.1016/j.jmatprotec.2008.01.046).
- Schmittgen TD, Livak K. 2008. Analyzing real-time PCR data by the comparative C T method. *Nat Protoc.* 3(6):1101. doi:[10.1038/nprot.2008.73](https://doi.org/10.1038/nprot.2008.73).
- Sharker MR, Kim SC, Sumi KR, Sukhan ZP, Sohn YC, Lee WK, Kho KH. 2020. Characterization and expression analysis of a GnRH-like peptide in the Pacific abalone, *Haliotis discus hawaii*. *Agri Gene.* 15:100099. doi:[10.1016/j.agrige.2019.100099](https://doi.org/10.1016/j.agrige.2019.100099).
- Silver MR, Kawauchi H, Nozaki M, Sower SA. 2004. 2–4. Cloning and analysis of the lamprey GnRH-III cDNA from eight species of lamprey representing the three families of Petromyzoniformes. *Gen Comp Endocrinol.* 139(1):85–94. doi:[10.1016/j.ygenc.2004.07.011](https://doi.org/10.1016/j.ygenc.2004.07.011).
- Song Y, Miao J, Cai Y, Pan L. 2015. Molecular cloning, characterization, and expression analysis of a gonadotropin-releasing hormone-like cDNA in the clam, *Ruditapes philippinarum*. *Comp Biochem Physiol B Biochem Mol Biol.* 189:47–54. doi:[10.1016/j.cbpb.2015.07.005](https://doi.org/10.1016/j.cbpb.2015.07.005)
- Tinikul Y, Poljaroen J, Tinikul R, Anuracpreeda P, Chotwiwatthanakun C, Senin N, Poomtong T, Hanna PJ, Sobhon P. 2014. Effects of gonadotropin-releasing hormones and dopamine on ovarian maturation in the Pacific white shrimp, *Litopenaeus vannamei*, and their presence in the ovary during ovarian development. *Aquaculture.* 420–421:79–88. doi:[10.1016/j.aquaculture.2013.10.036](https://doi.org/10.1016/j.aquaculture.2013.10.036).
- Treen N, Itoh N, Miura H, Kikuchi I, Ueda T, Takahashi KG, Ubuka T, Yamamoto K, Sharp PJ, Tsutsui K, et al.. 2012. Mollusc gonadotropin-releasing hormone directly regulates gonadal functions: a primitive endocrine system controlling reproduction. *Gen Comp Endocrinol.* 176(2):167–172. doi:[10.1016/j.ygenc.2012.01.008](https://doi.org/10.1016/j.ygenc.2012.01.008).
- Tsai PS. 2006. Gonadotropin-releasing hormone in invertebrates: structure, function, and evolution. *Gen Comp Endocrinol.* 148 (1):48–53. doi:[10.1016/j.ygenc.2005.09.016](https://doi.org/10.1016/j.ygenc.2005.09.016).
- Twan WH, Hwang JS, Lee YH, Jeng S-R, Yueh W-S, Tung Y-H, Wu H-F, Dufour S, Chang C-F. 2006. The presence and ancestral role of gonadotropin-releasing hormone in the reproduction of scleractinian coral, *Euphyllia ancora*. *Endocrinology.* 147(1):397–406. doi:[10.1210/en.2005-0584](https://doi.org/10.1210/en.2005-0584).
- Veenstra JA. 2010. Neurohormones and neuropeptides encoded by the genome of *Lottia gigantea*, with reference to other mollusks and insects. *Gen Comp Endocrinol.* 167 (1):86–103. doi:[10.1016/j.ygenc.2010.02.010](https://doi.org/10.1016/j.ygenc.2010.02.010).
- Young KG, Chang JP, Goldberg JL. 1999. Gonadotropin-releasing hormone neuronal system of the freshwater snails *Helisoma trivolvis* and *Lymnaea stagnalis*: possible involvement in reproduction. *J Comp Neurol.* 404(4):427–437. doi:[10.1002/\(sici\)1096-9861\(19990222\)404:4<427::aid-cne1>3.0.co;2-r](https://doi.org/10.1002/(sici)1096-9861(19990222)404:4<427::aid-cne1>3.0.co;2-r).
- Yu XX, Wu CW, Chi CF. 2011. Brain microstructure and optic gland ultrastructure in *Sepiella maindroni*. *J Oceanol Limnol.* 42(2):300–304. doi:[10.11693/hyz201102022022](https://doi.org/10.11693/hyz201102022022).
- Zhang L, Tello JA, Zhang W, Tsai P-S. 2008. Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, *Aplysia californica*. *Gen Comp Endocrinol.* 156 (2):201–209. doi:[10.1016/j.ygenc.2007.11.015](https://doi.org/10.1016/j.ygenc.2007.11.015).
- Zhang L, Wayne NL, Sherwood NM, Postigo HR, Tsai P-S. 2000. Biological and immunological characterization of multiple GnRH in an opisthobranch mollusk, *Aplysia californica*. *Gen Comp Endocrinol.* 118(1):77–89. doi:[10.1006/gcen.2000.7457](https://doi.org/10.1006/gcen.2000.7457).