Turk. J. Fish.& Aquat. Sci. 19(8), 689-697
http://doi.org/10.4194/1303-2712-v19_8_07

RESEARCH PAPER

Recombinant Luteinizing Hormone Development to Improve the Reproductive Performance of Female Malaysia Catfish, *Hemibagrus nemurus* (Valenciennes, 1840)

Md Yasin Ina-Salwany[1,2], Zarirah Zulperi[1,*], Annie Christianus[1,2], Fatimah Md Yusoff[1,2]

[1] Universiti Putra Malaysia, Faculty of Agriculture, Department of Aquaculture, 43400 UPM Serdang, Selangor, Malaysia
[2] Universiti Putra Malaysia, Institute of Bioscience, Laboratory of Marine Biotechnology, 43400 UPM Serdang, Selangor, Malaysia

Article History
Received 20 April 2018
Accepted 06 August 2018
First Online 07 August 2018

Corresponding Author
Tel.: +60.38947 4919
E-mail: zarirah@upm.edu.my

Keywords
Gonadotropin
Single-chain recombinant protein
17β-estradiol

Abstract

Gonadotropins (GTHs), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) are the major regulators of gonadal development in fish. This study reported the production of recombinant LH (rLH) in *Hemibagrus nemurus*. A single-chain rLH from *H. nemurus* was cloned into pET-32 and expressed in *Escherichia coli*. A specific band at 45.4 kDa was detected; this band corresponded to the molecular size of fusion rLH. Immature female *H. nemurus* were divided into four different treatment groups. Each group received a single injection of either 1X PBS, 0.5 ml/kg Ovaprim, rLH50 µg/kg, or rLH150 µg/kg. Gonad histology revealed that the fish treated with rLH50 µg/kg, rLH150 µg/kg, and Ovaprim could induce oocyte maturation at 48h post injection (p.i.). The 17β-estradiol (E2) plasma level in fish treated with rLH50 µg/kg significantly increased after 12h p.i., and the level was sustained up to 24h p.i. The mRNA expression levels of GTH subunits also significantly increased in fish treated with rLH50 µg/kg after 48h p.i. Fish treated with Ovaprim showed significantly increased E2 plasma levels after 6h p.i., but this level decreased after 12h p.i. No significant increase was found among mRNA subunits in fish treated with Ovaprim after 48h p.i. Treatment with rLH150 µg/kg had a weak stimulatory effect on the E2 production and the mRNA transcript level of GTH subunits.

Introduction

The Malaysian river catfish (*Hemibagrus nemurus*), locally known as "*Ikan Baung*," is an essential freshwater fish species that is cultured in most Asia-Pacific countries including Malaysia (Adebiyi *et al*., 2011; Mesomya *et al*., 2002). However, the annual production of the species is considered low, less than 6% (from 2012-2014) of the production of freshwater fish in Malaysia. In 2014, the production of *H. nemurus* was 1,648 tonnes, down 2.05% compared to 2013 (Department of Fisheries Malaysia, 2014). The decreased production was ascribed to hatchery problems of the species in terms of induced spawning, the synchronized maturation of male and female brood stocks, and the unavailability of high-quality brood stocks (Muchlisin *et al*., 2004).

The gonadotropins (GTHs) hormones; follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are formed in the anterior pituitary glands and play crucial roles in gonadal maturation and the reproductive system in fish (Nyuji *et al*., 2016; Guzmán *et al*., 2009). FSH and LH belong to the glycoprotein hormone family; each member is a heterodimer composed of the α and β subunits (Chaube *et al*., 2015; Mazón *et al*., 2015; Pierce, 1988). Both subunits are non-covalently bound into gonadotropic cells to form a biologically-active dimeric hormone. Studies showed FSH is involved in the initiation of gametogenesis, whereas LH is involved in the final maturation processes (Levavi-Sivan *et al*., 2010;

Published by Central Fisheries Research Institute (SUMAE) Trabzon, Turkey in cooperation with Japan International Cooperation Agency (JICA), Japan

Kobayashi *et al*., 2006). Recombinant fish GTHs, either recombinant FSH or LH, had been developed in various bioreactors, such as transgenic fish (Morita *et al*., 2004), baculovirus–silkworm larvae (Ko *et al*., 2007; Kobayashi *et al*., 2006), yeast (Chen *et al*., 2012) and bacteria (*Escherichia coli*) (Kim *et al*., 2012). Although the production of recombinant GTHs is relatively recent, where most trials were limited to *in vitro* and *in vivo* studies (Levavi-Sivan *et al*., 2010), positive results from previous studies suggest that recombinant GTHs are practical for the induction of gonadal development in the aquaculture industry. In the current study, we successfully produced a single-chain recombinant LH (rLH) from *H. nemurus* in *E. coli* BL21 (DE3) cells for the effective hormonal manipulation and gonadal development in this species. The aims of the study were to determine the effect of exogenous rLH hormones at the steroid level, the mRNA expression of GTH subunits and the oocyte development in immature female *H. nemurus*.

Materials and Methods

Constructs of A Single-Chain LH (rLH)

A single-chain recombinant LH (rLH) was derived by gene synthesis (GenScript, Piscataway, NJ) based on the open reading frame (ORF) of previously cloned *H. nemurus* cDNAs for the GTH subunits (Zulperi *et al*., 2015; Genbank accession numbers for the α and LHβ subunits are KF934189 and KF934190). The designs for the tethered single-chain LH$\beta\alpha$ construct encoding 747 bp (249 amino acids) was comprised of a mature β subunits encoding LHβ (25 amino acids of signal peptide sequences and 115 amino acids without stop codon) and a mature α subunit (93 amino acids including stop codon). To improve its bioactivity, a 48 bp fragment of synthetic DNA encoding Ser-Gly-Ser-Asn-Ala-Thr-Gly-Ser-Gly-Ser-Asn-Ala-Thr-Ser-Gly-Ser (NCS) was inserted between the β and α chains.

Production of rLH and Western Blot Analysis

Primers was specifically designed from the single-chain rLH sequence to clone with Ek/LIC vector (Table 1). The PCR mix was prepared in a 50 µl final volume of 50-100 ng DNA template, 10X *Pfu* Buffer with $MgSO_4$, 40 mM of each dNTP, 0.02 mM of each primer, and 2.5 U *Pfu* DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). Amplification was performed with an initial denaturation of 3 min at 95 °C, following 35 cycles of denaturation at 95°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1:30 min. The final elongation step was extended to 7 min at 72°C. Subsequently, T4 DNA polymerase (Novagen, Germany) treatment was performed on the purified PCR products to create a compatible overhang on the inserts before ligation to the Ek/LIC vector. The treatment was conducted in a final volume of 20 µl, which contained 50–100 ng of the purified PCR product, 10X of T4 DNA Polymerase buffer, 25 mM dATP, 100 mM DTT, and 2.5 U of T4 DNA Polymerase. The treated insert was annealed to the vector by adding 1 µl of the pET-32 Ek/LIC vector (Novagen) and 25 mM EDTA followed by incubation at 22°C for 10 min. The positively cloned rLH and pET-32 Ek/LIC vector was transformed into the *E. coli* BL21 (DE3) host strain (Novagen).

Pilot expression was performed following methods described by Kim *et al*. (2012) with several modifications. Briefly, bacterial culture containing rLH insert was grown on LB agar at 37°C overnight. Subsequently, 4.5 ml of the starter culture was added into 150 ml of LB media with 50 µg/ml ampicillin. The culture was incubated at 37°C with shaking at 200 rpm until the optical density at 600 nm (OD_{600}) was approximately 0.5 to 1.0. Later, the culture was induced by adding 1 mM isopropyl β-galactose (IPTG) and incubated at 37°C with agitation at 200 rpm. The induced culture was taken at different incubation periods (8 and 10 h) to determine the correct time course for the expression of rLH protein cells. The culture was centrifuged to harvest the cells at 10,000×*g* at 4°C and dissolved with BugBuster® Protein Extraction Reagent (Novagen) to release a soluble protein. To concentrate and semi-purified rLH protein solution, the extracted protein produced in previous step was carefully transferred into Amicon® Ultra-15 (EMD Millipore, Germany) spin column, and was centrifuged at 4,000 xg for 15 min at 4°C. Later, the flow-through was discarded as it contained unnecessary protein less than 10 kDa, while our desired rLH protein, was collected from the spin column and transferred into clean tubes, stored at -20°C until further used in fish trial.

Table 1. Gene-specific primers and vector-specific primers for PCR amplification and sequencing analysis

Gene of Interest	Primers	Primer Sequences, (5'-3')	Application
rLH, (LH$\beta\alpha$ sequences)	LIC-LH$\beta\alpha$F LIC-LH$\beta\alpha$R	GAC GAC GAC AAG <u>ATG</u> AGC GTT CCG GCT GAG GAG AAG CCC GGT <u>TTA</u> GAA TTT ATG	Gene specific primers for PCR amplification of rLH, LH$\beta\alpha$ construc sequences
Universal primers	T7 promoter T7 Terminator	TAA TAC GAC TCA CTA TAG GG GCTAGTTATTGCTCAGCG G	Sequencing analysis on the verification of the inserted rLH gene within recombinant *E. coli* expressic expression vector, pET-32/LIC-rLH

Note: The start (ATG) and stop (TAA) are underlined; LIC sequences are colored red

To confirm rLH gene expression, protein samples were subjected to SDS-PAGE and Western blot analysis. SDS-PAGE was performed with 10% polyacrylamide gels. The unstained gel contained the soluble protein of recombinant *E. coli*, was then electrotransferred onto a 0.45 µm nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was incubated in the blocking buffer of phosphate-buffered saline (PBS)/Tween 20 (PBST) (Calbiochem, Merck Millipore, Germany) with 1% bovine serum albumin (BSA) (Calbiochem, Merck Millipore) for 1.5 h with gentle shaking at 37°C. Subsequently, the membrane was incubated in 1:1000 dilution of the His.Tag (Novagen) or S.Tag (Novagen) monoclonal antibody and incubated overnight at 4°C. After washing, the membrane was incubated again in a 1:1500 dilution of HRP-conjugated goat anti-mouse IgG (Novagen) with mild shaking for 2.5 h at 37 °C. Finally, colorimetric detection was performed with 3,3´-diaminobenzidine (DAB; Merck Millipore, Germany), with DAB buffer as the substrate, for 4–5 min to develop the brownish color on the membrane, which indicated the presence of recombinant proteins.

Experimental Fish and Treatment Procedure

Fifteen month-old females of *H. nemurus* (average body weight (BW) = 362.88±16.06 g; gonadosomatic index (GSI)=10.06%±1.35%) were collected from a commercial farm in Cheroh Aquatic Sendirian Berhad, Pahang, Malaysia (total number of fish, *n*=40). The samples were transported to the Aquaculture Research Station, Universiti Putra Malaysia, and later transferred to 2 ton circular fiberglass tanks (round tank maximum capacity at 2300 liters with diameter at 2.1 meter) under a natural photoperiod and acclimatized for 7 days. The fish were randomly assigned into four tanks; each tank contained 10 fish. Feeding was performed once daily in the evening with commercial fish pellets. Two days before the experimental trials, all the fish were fasted to relax their digestive system before proceeding with the treatments.

To further evaluate the bioactivity of the rLH protein hormone, each group of fish (10 fish/group) received a single injection via the intramuscular dorsal route with four different treatments: Group 1, sham control with 1X PBS; Group 2, positive control with 0.5 ml/kg Ovaprim (Syndel Laboratories Ltd, Canada); Group 3, with rLH 50 µg/kg; Group 4, with rLH 150 µg/kg. Fish were anesthetized with clove oil (40 mg/L–60 mg/L dissolved in ethanol), which was added into the water before handling the fish. The body weight of each fish was measured to determine the volume of hormones or PBS to be injected into each fish. Blood samples of approximately 1 ml were collected from each fish at 0, 6, 12, 18, 24, and 48 h post injection (p.i.). Blood was collected from the caudal vasculature with a heparinized syringe and needle, transferred into 1.5 ml microcentrifuge tubes and centrifuged at 2000×*g* for 15 min at 4°C. Plasma samples were transferred to clean tubes and stored at -80°C until further used. Five gonads and pituitaries were collected at 0 and 48 h p.i. The pituitaries were immediately stored at -80°C, and gonads were excised and fixed in Bouin's solution for histological observation.

Gonad Histology

Samples of ovarian tissues were fixed in Bouin's solution for 24 h. After washing with ethanol, each of the ovarian tissues was placed in histological cassettes, stored into 70% ethanol, and processed using the routine histological methods (Shi *et al*., 2010). The dehydrated tissues were embedded in paraffin and sectioned into 5-7 µm. Cut sections were fixed on glass slides and stained with hematoxylin and eosin for histological studies. After staining, the slides were examined using light microscope (Leica DFC 295, Leica Microsystems, Queenstown, Singapore).

Estradiol ELISA Analysis

Plasma 17β-estradiol (E2) levels were analyzed by enzyme-linked immunosorbent assay (ELISA) with an estradiol ELISA (EIA) kit, (Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions. To perform the assay, 50 µl of estradiol EIA standards with

Table 2. Primers for the detection of α, FSHβ and LHβ of *H. nemurus* by quantitative real-time PCR assay. All primers (except 18S rRNA) were designed from the conserved region in each subunit genes

cDNA		Primer sequence (5'-3')	Position	Amplicon (bp)
α	Sense	CCTGTGTATCAGTGCATGGG[a]	196-216	180
	Antisense	GCAGTGGCAGTCTGTGTGAT[a]	356-376	
FSHβ	Sense	GCCGTACATCCAGAACACCT[a]	235-255	120
	Antisense	CACTCACAGCTCAGAGCCAC[a]	335-355	
LHβ	Sense	GATGTCCGCTATGAAACGGT[a]	275-295	151
	Antisense	TACAGAAATCCGGCTTCAGG[a]	406-426	
18S	Sense	TGGTTAATTCCGATAACGAACGA[b]	1325-1347	93
	Antisense	CGCCACTTGTCCCTCTAAGAA[b]	1397-1418	

[a] Primers previously applied for real-time GTH subunits detection of *H. nemurus* (Zulperi et al., 2017)
[b] Primers previously applied for internal control of channel catfish (*Ictalurus punctatus*) (Kumar et al., 2000)

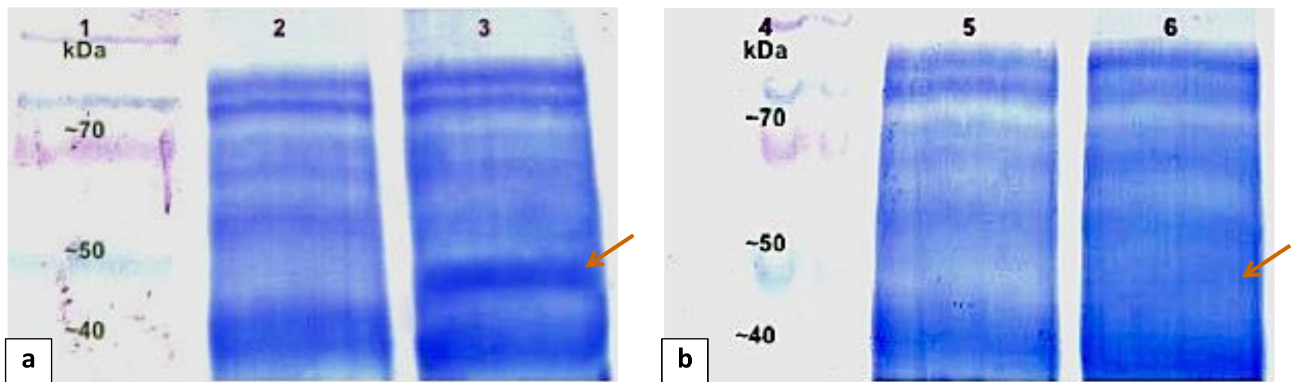

Figure 1. SDS-PAGE analysis of the soluble fusion protein of recombinant rLH after pilot expression in *E. coli* BL21 (DE3) at a) 8 h, and b) 10 h, of the induction–incubation period at 37°C. Lanes 1 and 4: Protein Marker (Spectra™ Multicolor Broad Range Protein Ladder, Thermo Fisher Scientific); Lanes 2 and 5: *E. coli BL21* (DE3) host without insert at 8 and 10 h of induction at 37°C; Lanes 3 and 6: soluble cell protein of *E. coli* BL21 (DE3) expression pET-32/LIC-rLH at 8 and 10 h of induction at 37°C. Note: The arrows indicate the absence of expressed protein at approximately 45.4 kDa.

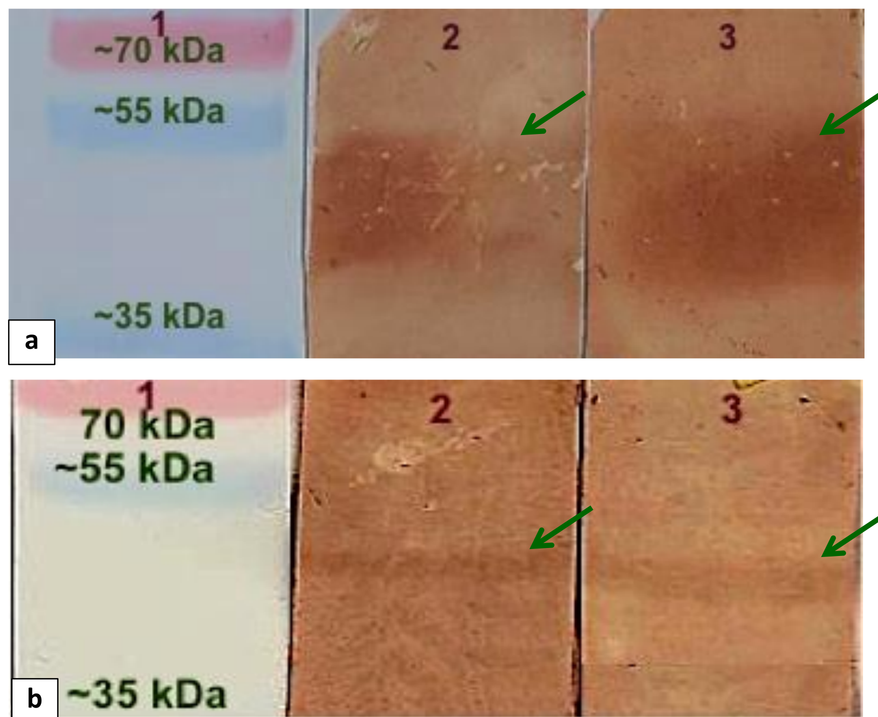

Figure 2. Western immunoblot analysis with (a) the S.tag monoclonal antibody and (b) the His.Tag monoclonal antibody of the soluble fusion protein of the recombinant rLH protein under optimized induction for 8 h at 37°C in *E. coli* BL21 (DE3). Lane 1: Protein Marker (PageRuler™ Plus Pre-stained Protein Ladder); Lane 2 and 3: The soluble cell protein of *E. coli* BL21 (DE3) expression pET-32/LIC-rLH under optimized induction for 8 h at 37°C in *E. coli* BL21 (DE3). Note: The arrows indicate the absence of expressed protein at approximately 45.4 kDa.

different concentrations was added to the standard wells, while for samples, 50 µl from each purified sample was added to the sample wells. Each standards and samples was run in triplicate. Subsequently, 50 µl of the Estradiol AChE Tracer and the estradiol EIA Antiserum was added to each wells, followed by incubation for 1 h at room temperature on an orbital shaker. After washing, 200 µl of Ellman's Reagent was added to each well; the mixture was left for 70 min at room temperature on an orbital shaker to allow development of the assay. The plate was placed on a Multiskan™ FC Microplate Photometer (Thermo Scientific), and the measurements were performed at wavelengths between 405 nm and 420 nm.

Quantitative Real-Time PCR and Statistical Analysis

Primers of each subunit genes from previous study (Zulperi *et al*., 2017) were used to perform the real-time PCR amplification (Table 2). Total RNA was extracted from pituitaries using Trizol Reagent® (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA with

the Quantitect® Reverse Transcription kit (Qiagen, Germany). Standard curves were conducted for each subunit genes with tenfold serial dilution of cDNA template which ranged from 1000 ng to 0.01 ng. The real-time PCR was performed with CFX96™ Real-Time System (Bio-Rad Laboratories, Hercules, CA) in a 20 µl reaction mixture containing 1 µl of cDNA, 10 µM of each forward and reverse primer, 10 µl of the 2X SensiFAST® No-ROX Mix, and nuclease-free water. The real-time PCR profile was: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 59°C for 30 s, and 72°C for 10 s. The reaction mix was incubated at 77°C for 5 s to remove contaminants before the fluorescence reading was obtained. After the amplification process, melting curve analysis was performed to confirm the assay specificity by increasing the incubation temperature from 65°C to 95°C; a reading was obtained every 0.5°C, with a hold period of 5 s at each increment. All amplification reactions, including the samples, standards, and the internal control 18S rRNA (Kumar *et al*., 2000), were run in triplicate. All data were expressed as means ± standard error of the mean (SEM). Statistical analysis was performed with one-way ANOVA, followed by Tukey post hoc tests using the IBM® SPSS® Statistics software, version 21. Differences were considered significant at $P<0.05$.

Results

Confirmation of rLH by Western Blot Analysis

The presence of the protein insert was confirmed by Western blot analysis with the S.tag and His.Tag monoclonal antibodies. Western blots revealed the presence of a ~45.4 kDa single band with soluble proteins from *E. coli* BL21 (DE3) cells containing the recombinant protein insert, rLH (Figure 1 and 2). In the negative controls of *E. coli* BL21 (DE3) cells without the insert, bands with estimated size of the recombinant protein insert were not detected.

Gonad Histology

Females of *H. nemurus* with developing ovaries were selected to assay the function of the rLH protein. Gonad histology was examined at 0 and 48 h p.i. among fish in all four treatments. At 0 h p.i., ovaries from all treatments were composed of cortical alveolar and vitellogenic oocytes, thereby showing that the immature *H. nemurus* were still developing their ovaries at the secondary growth phase (Figure. 3 a-d). After 48 h p.i., no differences in ovarian development was observed in sham control, with a GSI of 11.94%±0.50%. Most of the oocytes were retained at the secondary growth (Figure 3e). Treatment with Ovaprim (Figure 3f) and rLH 50 µg/kg (Figure 3g) increased the mean GSI by 13.42%±0.64% and 12.55%±1.48%, respectively. Ovarian growth development was observed in the gonads; most of the oocytes had entered the maturation phase and were composed of the mature and germinal vesicle migration oocyte stages. Meanwhile, treatment with rLH 150 µg/kg (Figure 3h) had slightly increased the GSI value by 12.01%±0.97%.

Effect of rLH on E2 plasma levels

Plasma levels of E2 receiving rLH with different doses were measured from six different post-injection hours, at 0, 6, 12, 18, 24 and 48 h p.i. (Figure 4). For E2 level analysis, statistical significance was accepted only when the tested groups showed a significant difference relative to the control group, 1X PBS buffer at the same sampling time point. Treatment with rLH 50 µg/kg and 0.5 ml/kg Ovaprim increased circulating E2 concentration after 6 and 12 h p.i. Treatment with rLH 50 µg/kg increased E2 level by 1.8-fold at 6 h p.i., and showed significant increased up to 1.95-fold after 12 h p.i. Meanwhile, treatment with Ovaprim showed the highest significant increased of E2 level by 2.3 to 3-fold at 6 and 12 h p.i. but reduced after 18 h p.i. Only treatment with rLH at 50 µg/kg showed a high E2 level at significant difference compared to other three treatment groups at 18 and 24 h p.i. Treatment with rLH at 150 µg/kg yielded no significant increased when compared to the 1X PBS control fish at the same time point, though we could observe a slight increased of E2 level after 24 h p.i.

Effect of rLH on mRNA Expression of the α, FSHβ, and LHβ Subunit Genes

Pituitary expression level of α, FSHβ and LHβ subunits were measured following treatments with rLH at different dose, 50 µg/kg and 150 µg/kg, and compared to sham control (1X PBS) and positive control (0.5 ml/kg Ovaprim), after 48 h p.i. as all fish were sacrificed (Figure 5). All data were normalized to the amount of 18S RNA in each sample. From the plotted graph, it was observed that treatment with rLH at 50 µg/kg significantly increased α, FSHβ and LHβ when compared between sham control and rLH 150 µg/kg. Treatment with rLH at 150 µg/kg, slightly increased the expression of all the three subunits level but were not significant compared to those treatments. For positive control treatment, Ovaprim at 0.5 ml/kg increased the mRNA expression of α, FSHβ and LHβ subunits, but only LHβ showed significant increment at 48 h p.i. when compared to sham control.

Discussion

This study aimed to produce recombinant luteinizing hormone (rLH) from *H. nemurus* in a bacterial expression system. Most recombinant GTH studies reported the success of heterodimeric recombinant GTH for inducing steroidogenesis and ovarian development

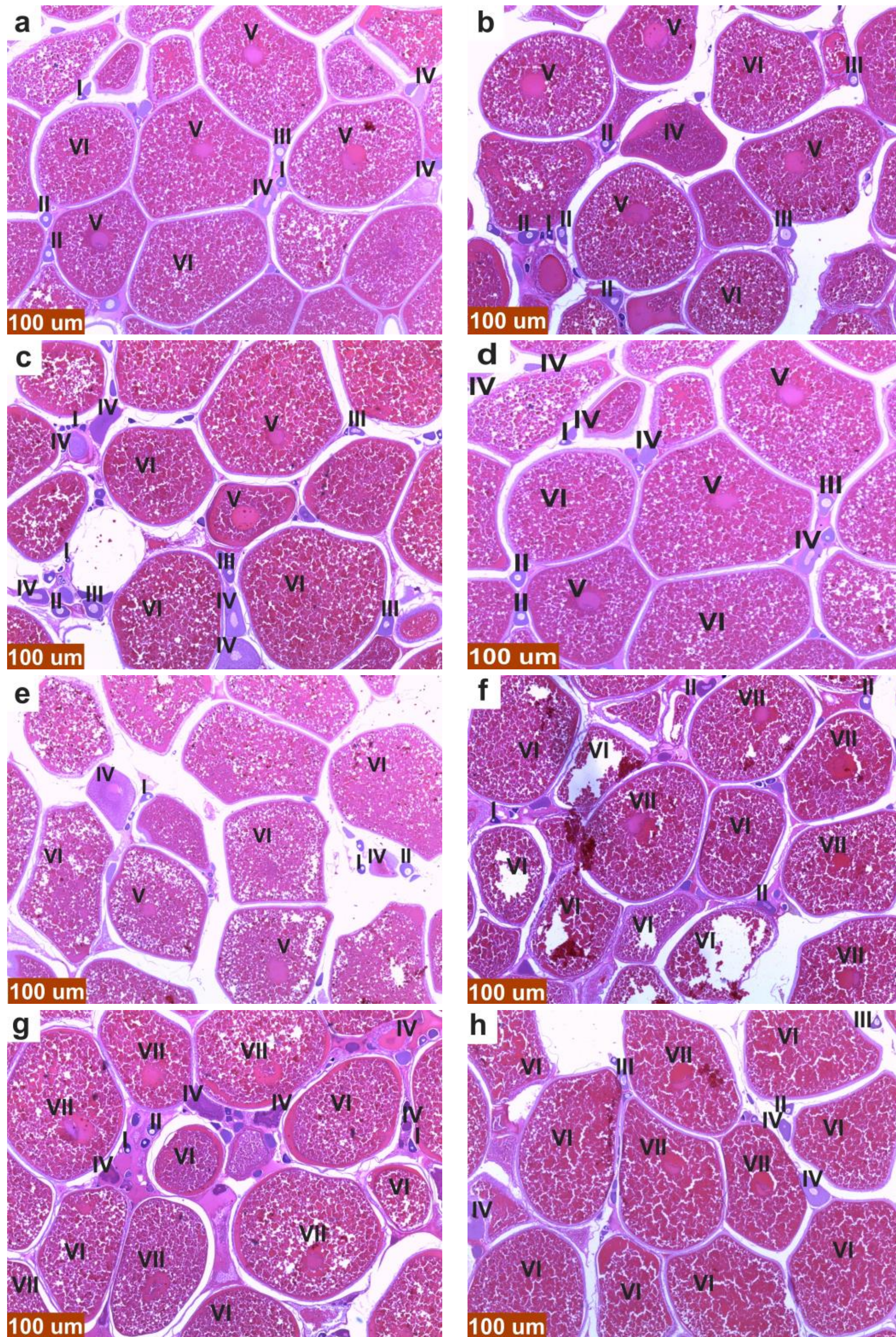

Figure 3. Gonad histology of *H. nemurus* at 0 h p.i. for the (a) sham control (1X PBS), (b) positive control (Ovaprim), (c) rLH 50 µg/kg, and (d) rLH 150 µg/kg groups, and at 48 h p.i. for the (e) sham control, (f) Ovaprim, (g) rLH 50 µg/kg, and (h) rLH 150 µg/kg groups. I, chromatin nucleolar oocyte; II, early perinucleolar oocyte; III, late perinucleolar oocyte; IV, cortical alveolar oocyte; V, vitellogenic oocyte; VI, mature oocyte; VII, germinal vesicle migration oocyte stages.

in fish (Chen *et al*., 2012; Ko *et al*., 2007). In the current study, a single-chain molecule was constructed, where the carboxyl terminus of the LHβ subunit was fused to the amino terminus of the mature α subunit. N-glycosylated (NCS) protein consisted of 16 amino acids was added between the β and α chains. Previous studies reported that the addition of the NCS site can extend the response of recombinant GTH in the reproductive system (Kim *et al*., 2012; Klein *et al*., 2003). The cloned product, which is referred to as the fusion rLH protein, was transformed into the expression host, *E. coli* BL21 (DE3). A similar prokaryotic system was applied to produce rLH in cinnamon clownfish; this protein was expressed in *E. coli* BL21 (DE3) pLysS cells (Kim *et al*.,

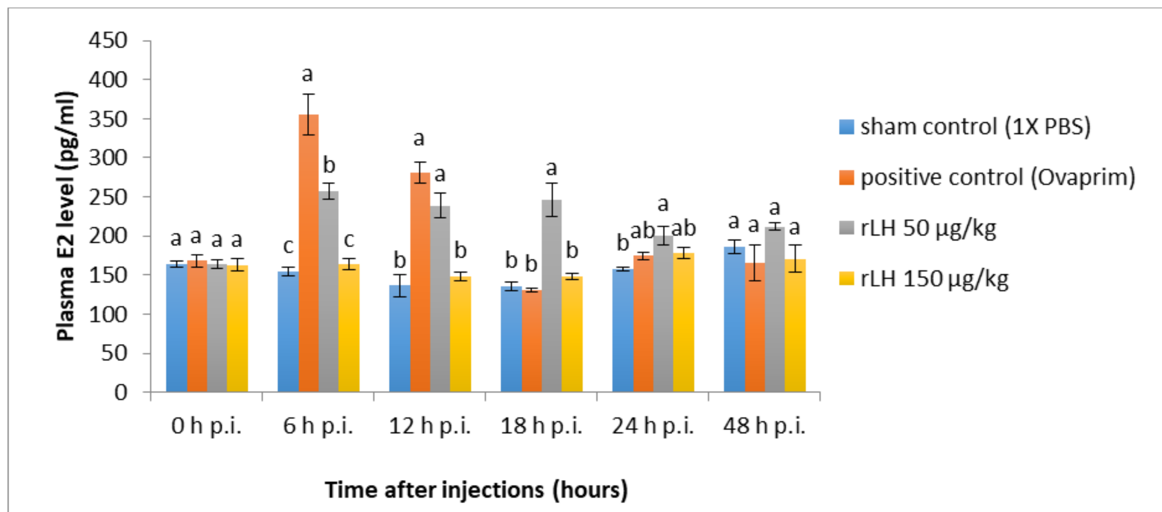

Figure 4. Plasma 17β-estradiol (E2) levels during treatment with sham control (1X PBS), positive control (Ovaprim), rLH 50 µg/kg, and rLH 150 µg/kg, at different sampling point, 0, 6, 12, 18, 24 and 48 h p.i. Plasma E2 level were measured by Estradiol ELISA (EIA) assay. Different letters refer to statistically significant differences between treatment groups, where $p<0.05$. All values are means±SEM, with n =5.

2012). Profiles of SDS-PAGE analysis revealed that the expected band size of rLH was 45.4 kDa, thereby demonstrating that the LHβ and α subunit genes were heterodimerized. Although no data are available to specify the type or degree of glycosylation of rLH proteins, the structural features of recombinant proteins were compatible with the heterodimerization of α and β subunits, which naturally exist in the fish system to activate GTHs, based on the detected band size.

To assess the bioactivity of rLH proteins and their ability to induce E2 secretion and ovarian development, as well as the mRNA expression of α, FSHβ, and LHβ subunits, a short-term fish trial with immature (15 month-old) female *H. nemurus* was performed. The stage classification of ovary and oocyte development of *H. nemurus* during the reproductive cycle was described previously (Adebiyi *et al.*, 2011). The oocyte developmental stages were described as follows: stage I, chromatin nucleolar oocyte; stage II, early perinucleolar oocyte; stage III, late perinucleolar oocyte; stage IV, cortical alveolar oocyte; stage V, vitellogenic oocyte; stage VI, mature oocyte; stage VII, germinal vesicle migration oocyte. Ovaries in the primary growth phase showed immature oocytes in the chromatin nucleolar, early perinucleolar, and late perinucleolar stages of oocyte development (Stages I, II, and III). In the secondary growth phase, oocyte development was at the cortical alveolar and vitellogenic phases (Stages IV and V). During the maturation phase, mature ovaries presented oocytes with mature and germinal vesicle migration, as well as germinal vesicle breakdown (GVBD) (Stages VI and VII). Based on the gonad histology obtained at 0 h p.i., the ovarian development of all these fish was at their vitellogenic phase and ready to enter the maturation phase of oocyte development. Four different treatments were performed in this study; each fish received a single injection of either the sham control (1X PBS), positive control (Ovaprim), rLH at 50 µg/kg, or rLH at 150 µg/kg. A single injection with rLH at concentrations of 50 and 150 µg/ml, as well as the Ovaprim treatment, increased the mean GSI and oocyte maturation as compared with those of the control fish at 48 h p.i. GVBD was observed in most of the oocytes, thereby indicating that ovarian development had reached the maturation phase. These data are consistent with previous reports on goldfish rLH (Kobayashi *et al.*, 2006) and cinnamon clownfish rLH (Kim *et al.*, 2012), which showed that rLH can induce ovulation in both species. The ELISA results showed that treatment with rLH at 50 µg/kg gradually increased the plasma E2 level at 6 h p.i. and significantly increased these levels at 12 h p.i up to 24 h p.i., thereby eliciting a signal that is 1.8 times to 1.95 times higher than the E2 basal level. The E2 secretion decreased at 48 h p.i., but its level remained higher than those of the three other treatments. Therefore, we proved that the addition of highly conserved NCS sites in the rLH construct enabled them to extend the activity up to 48 h p.i. This result agrees with recombinant GTH studies on cinnamon clownfish (Kim *et al.*, 2012) and rainbow trout (Ko *et al.*, 2007). The use of NCS sites was first reported in mammals; a recombinant human FSH added with the NCS site extended the half-life in the bloodstream by 7.3 h, a value twofold higher than that of the native recombinant FSH (Klein *et al.*, 2003). The effects of rLH injection on the mRNA expression levels of α, FSHβ, and LHβ were observed at 48 h p.i. by quantitative real-time PCR. Treatment with rLH 50 µg/kg significantly upregulated the mRNA transcript of α and LHβ subunits, and a slight but significant increment occurred in the FSHβ subunit. The results revealed the ability of rLH to activate both FSHβ and LHβ subunits, as reported in a study on African catfish rLH, which can regulate both FSHβ and LHβ mRNA transcripts, as well as FSH and LH receptors (Vischer *et al.*, 2003). This result is expected

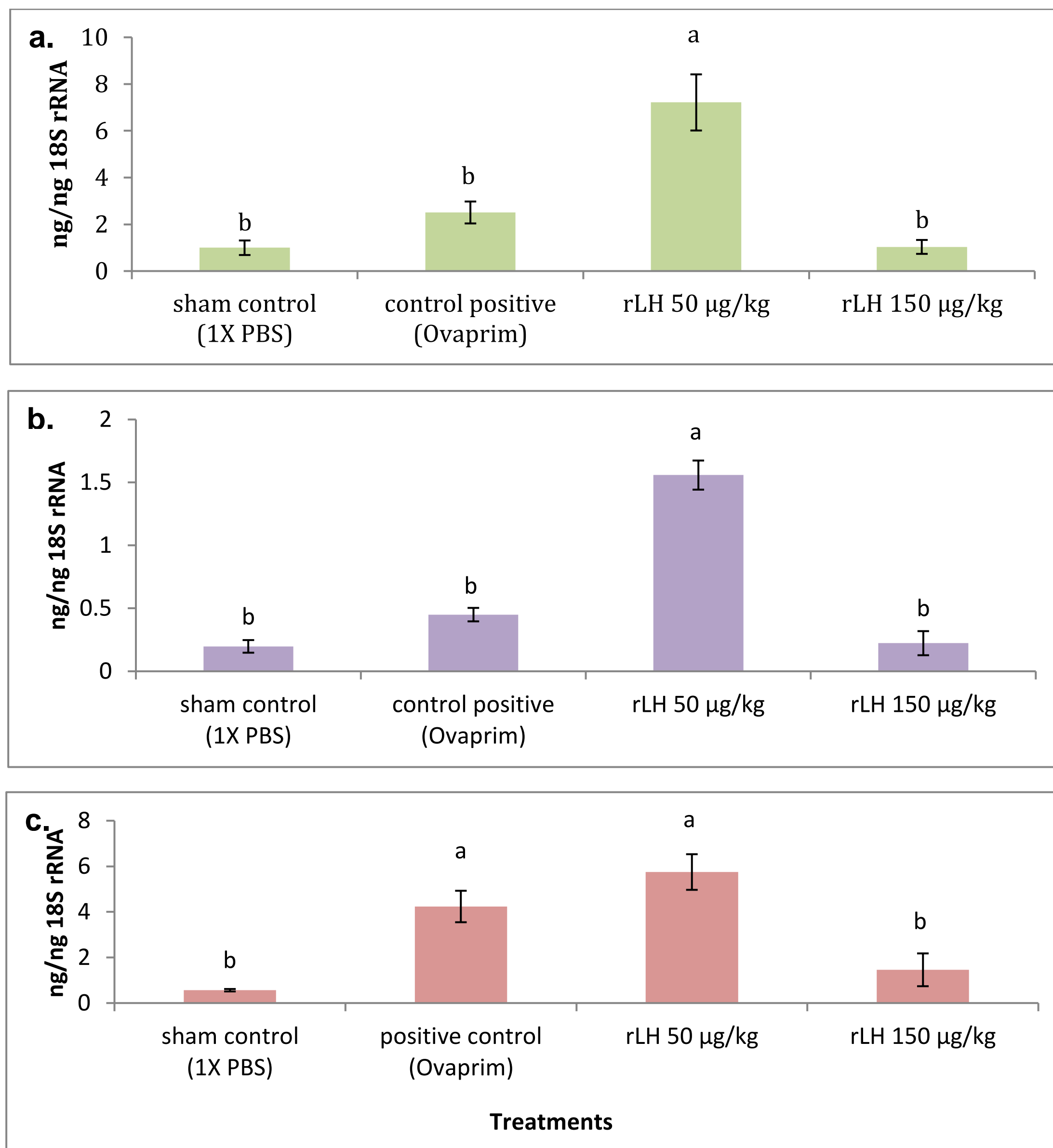

Figure 5. Effect of rLH injection to mRNA expression level of a) α subunit, b) FSHβ subunit, and c) LHβ subunit, after 48 h p.i. mRNA expression level measured using real-time PCR. Different letters refer to statistically significant differences, $p<0.05$. All values are means±SEM, with *n* =5.

because of the phylogenetic lineage of FSHβ and LHβ that diverged from a common ancestral gene; thus, both of them may be expressed as FSH- and LH-type of GTHs (Vischer *et al*., 2003; Quérat *et al*., 2000). Furthermore, this study found that treatment with rLH at high dosage (150 µg/kg) showed a weak stimulatory effect on the E2 production and mRNA transcript level of GTH subunits. This finding suggested the importance of determining the accurate dosage of the rLH hormone to be applied for its optimal performance to induce steroid production and upregulate the mRNA transcript level in female *H. nemurus*.

This study demonstrated that a single injection of rLH with a specific dose of 50 µg/kg in female *H. nemurus* can induce oocyte maturation and ovulation, increase the mean GSI of gonads, and upregulate the mRNA expression levels of the α, FSHβ, and LHβ transcripts, as well as to stimulate steroidogenesis at E2 levels in plasma. The rLH produced in this study presents a high potential in aquaculture as an alternative hormone to induce gonadal development in cultivated fish. The finding will enhance the understanding about hormonal control of reproduction in female *H. nemurus*, as well as other catfish species in Malaysia.

Acknowledgements

This work was supported by the Fundamental Research Grant Scheme, Ministry of Education Malaysia (Grant No. 5524086); and the Geran Putra-Inisiatif Putra Siswazah, Universiti Putra Malaysia (Grant No. 9390700). We would like to thank students and staff from the Department of Aquaculture, Universiti Putra Malaysia for their kind assistance.

References

Adebiyi, F.A., Siraj, S.S., Harmin, S.A. & Christianus, A. (2011). Ovarian development of a river catfish *Hemibagrus nemurus* (Valenciennes, 1840) in captivity. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*. *315*(9), 536-543. https://doi.org/10.1002/jez.702

Chaube, R., Joy, K.P. & Acharjee, A. (2015). Catfish Gonadotrophins: Cellular Origin, Structural Properties and Physiology. *Journal of Neuroendocrinology*. *27*(6), 536-543. https://doi.org/10.1111/jne.12286

Chen, J., Zhang, Y., Tang, Z., Mao, J., Kuang, Z., Qin, C. and Li, W. (2012). Production of recombinant orange-spotted grouper (*Epinephelus coioides*) follicle-stimulating hormone (FSH) in single-chain form and dimer form by *Pichia pastoris* and their biological activities. *General and Comparative Endocrinology*. *178*(2), 237-249. https://doi.org/10.1016/j.ygcen.2012.05.009

Department of Fisheries Malaysia. 2014. Annual Fisheries Statistic 2014 Vol. 1. Ministry of Agriculture and Agro-Based Industry Malaysia, Putrajaya.

Guzmán, J.M., Bayarri, M.J., Ramos, J., Zohar, Y., Sarasquete, C. & Mañanós, E.L. (2009). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) gene expression during larval development in Senegalese sole (*Solea senegalensis*). *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*. *154*(1), 37-43. https://doi.org/10.1016/j.cbpa.2009.04.623

Kim, N.N., Habibi, H.R., Lee, J. & Choi, C.Y. (2012). Effects of recombinant gonadotropin hormones on the expression of vitellogenin, gonadotropin subunits and gonadotropin receptors in cinnamon clownfish, *Amphiprion melanopus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. *162*(4), 73-80. https://doi.org/10.1016/j.cbpb.2012.04.006

Klein, J., Lobel, L., Pollak, S, Lustbader, B., Ogden, R.T., Sauer, M.V. & Lustbader, J.W. (2003). Development and characterization of a long-acting recombinant hFSH agonist. *Human Reproduction*. 18(1), 50-56. https://doi.org/10.1093/humrep/deg024

Ko, H., Park, W., Kim, D.J., Kobayashi, M. & Sohn, Y.C. (2007). Biological activities of recombinant Manchurian trout FSH and LH: their receptor specificity, steroidogenic and vitellogenic potencies. *Journal of Molecular Endocrinology*. 38(1), 99-111. doi: 10.1677/jme.1.02163

Kobayashi, M., Morita, T., Ikeguchi, K., Yoshizaki, G., Suzuki, T. & Watabe, S. (2006). *In vivo* biological activity of recombinant goldfish gonadotropins produced by baculovirus in silkworm larvae. *Aquaculture*. *256*(1), 433-442. https://doi.org/10.1016/j.aquaculture.2006.01.016

Kumar, R.S., Ijiri, S. & Trant, J.M. (2000). Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a reproductive cycle. *Biology of Reproduction*. *63*(6), 1676-1682. https://doi.org/10.1095/biolreprod63.6.1676

Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A. & Lareyre, J.J. (2010). Perspectives on fish gonadotropins and their receptors. *General and Comparative Endocrinology*. *165*(3), 412-437. https://doi.org/10.1016/j.ygcen.2009.07.019

Mazón, M.J., Molés, G., Rocha, A., Crespo, B., Lan-Chow-Wing, O., Espigares, F., Muñoz, I., Felip, A., Carrillo, M., Zanuy, S. & Gómez, A. (2015). Gonadotropins in European sea bass: endocrine roles and biotechnological applications. *General and Comparative Endocrinology*. *221*, 31-41. https://doi.org/10.1016/j.ygcen.2015.05.002

Mesomya, W., Cuptapun, Y., Jittanoonta, P., Hengsawadi, D., Boonvisut, S., Huttayanon, P. & Sriwatana, W. (2002). Nutritional evaluations of green catfish *Mystus nemurus*. *The Kasetsart Journal (Natural Sciences)*. *36*, 69-74.

Morita, T., Yoshizaki, G., Kobayashi, M., Watabe, S. & Takeuchi, T. (2004). Fish eggs as bioreactors: the production of bioactive luteinizing hormone in transgenic trout embryos. *Transgenic Research*. *13*(6), 551-557. https://doi.org/10.1007/s11248-004-2824-5

Muchlisin, Z.A., Hashim, R. & Chong, A.S.C. (2004). Preliminary study on the cryopreservation of tropical bagrid catfish (*Mystus nemurus*) spermatozoa; the effect of extender and cryoprotectant on the motility after short-term storage. *Theriogenology*. *62*(1), 25-34. https://doi.org/10.1016/j.theriogenology.2003.05.006

Nyuji, M., Kazeto, Y., Izumida, D., Tani, K., Suzuki, H., Hamada, K., Mekuchi, M., Gen, K., Soyano, K. & Okuzawa, K. (2016). Greater amberjack Fsh, Lh, and their receptors: plasma and mRNA profiles during ovarian development. *General and Comparative Endocrinology*. *225*, 224-234. https://doi.org/10.1016/j.ygcen.2015.10.008

Pierce, J.G. (1988). Gonadotropins: chemistry and biosynthesis. In E. Knobil, J, Neill (Eds). The Physiology of Reproduction (pp. 1335-1348). New York, Raven Press.

Quérat, B., Sellouk, A. & Salmon, C. (2000). Phylogenetic analysis of the vertebrate glycoprotein hormone family including new sequences of sturgeon (*Acipenser baeri*) β subunits of the two gonadotropins and the thyroid-stimulating hormone. *Biology of Reproduction*. *63*(1), 222-228. https://doi.org/10.1095/biolreprod63.1.222

Shi, Y., Zhang, Y., Li, S., Liu, Q., Lu, D., Liu, M., Meng, Z., Cheng, C.H.K. & Lin, H. (2010). Molecular identification of the Kiss2/Kiss1ra system and its potential function during 17alpha-methyltestosterone-induced sex reversal in the orange-spotted grouper, Epinephelus coioides. *Biology of Reproduction*, *83*(1), 63-74. https://doi.org/10.1095/biolreprod.109.080044

Vischer, H.F., Granneman, J.C., Linskens, M.H., Schulz, R.W. & Bogerd, J. (2003). Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. *Journal of Molecular Endocrinology*. *31*(1), 133-140. doi: 10.1677/jme.0.0310133

Zulperi, Z., Christianus, A., Yusoff, F. M., Harmin, S. A., & Ina-Salwany, M. Y. (2017). Ovarian development and changes in the expression of pituitary gonadotropin subunits during the reproductive cycle of female Malaysian river catfish, Hemibagrus nemurus (Valenciennes, 1840), in captivity. *Journal of Applied Ichthyology*, *33*(3), 429-436. https://doi.org/10.1111/jai.13267

Zulperi, Z., Ina-Salwany, M.Y., Christianus, A., Yusoff, F.M. & Harmin, S.A. 2015. Molecular characterization of *Hemibagrus nemurus* gonadotropin subunits: cDNA cloning and phylogenetic analysis. *Research in Biotechnology, 6*(1), 26-46.