
**UNIVERSITI PUTRA MALAYSIA**

***MOLECULAR CHARACTERIZATION AND RECOMBINANT GONADOTROPIN SUBUNIT DEVELOPMENT FOR IMPROVING REPRODUCTIVE PERFORMANCE IN FEMALE Hemibagrus nemurus VALENCIENNES***

**ZARIRAH BINTI MOHAMED ZULPERI**

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# MOLECULAR CHARACTERIZATION AND RECOMBINANT GONADOTROPIN SUBUNIT DEVELOPMENT FOR IMPROVING REPRODUCTIVE PERFORMANCE IN FEMALE *Hemibagrus nemurus* VALENCIENNES

By

**ZARIRAH BINTI MOHAMED ZULPERI**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

**October 2016**

*Assalamualaikum…*

*Peace be upon you…*

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

# MOLECULAR CHARACTERIZATION AND RECOMBINANT GONADOTROPIN SUBUNIT DEVELOPMENT FOR IMPROVING REPRODUCTIVE PERFORMANCE IN FEMALE *Hemibagrus nemurus* VALENCIENNES

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**October 2016**

**Chairman : Ina Salwany Md Yasin, PhD**
**Faculty : Agriculture**

*Hemibagrus nemurus*, locally known as 'ikan baung', is a valuable cultured catfish in Malaysia, but the production is limited due to problems associated with its reproduction. Most hatcheries encountered problems in induced spawning due to the fact that the fish do not expose to their natural spawning habitat, especially the failure of female brood stock to undergo final oocyte maturation (FOM), preventing its ability to ovulate and spawn. Gonadotropin (GtH), comprised of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), play an important role in gametogenesis and sexual maturation in mammals as well as in fish. This research was conducted to study the molecular characterization and to monitor changes in the mRNA expression of three GtH subunits, $\alpha$, FSH$\beta$, and LH$\beta$, during the reproductive cycle of the female *H. nemurus*. Next, a single-chain recombinant LH (rLH) of *H. nemurus* was expressed using *E. coli* expression system. The bioactivity of the recombinant hormone was assessed for their potency in stimulating the production of 17$\beta$-estradiol (E2), ovarian development and mRNA expression level of GtH subunits in experimental fish. Molecular characterization using PCR amplifications revealed that the cDNA of $\alpha$, FSH$\beta$ and LH$\beta$ in *H. nemurus* were 656, 728 and 602 bp, and encoded for 116, 134 and 140 amino acids, respectively. Multiple sequence alignment showed all the three subunits shared the highest homology with Siluriformes and Anguilliformes, and least similar to higher vertebrates. Phylogenetic analysis of $\alpha$ and FSH$\beta$ grouped fishes and higher vertebrates together, while in LH$\beta$, the tree was divided in two distinct groups, the first group contained all fishes together, and the second group only contained higher vertebrates. The abundance of transcripts encoding the GtH subunits, $\alpha$, FSH$\beta$ and LH$\beta$ subunits in pituitaries of female *H. nemurus* was tracked throughout the reproductive cycle using quantitative real-time PCR. Data were collected from August 2013 until October 2015, with fish aged between 1 to 27 month-old. The mRNA expression of GtH subunits increased together

with ovarian development throughout the reproductive cycle. The FSHβ mRNA level increased during ovarian development and reached its peak in mid-vitellogenesis and reduced during ovulation and spawning, whereas the LHβ mRNA level significantly increased (P<0.05) in late vitellogenesis and reached its peak during ovulation and spawning period. In α subunit, the mRNA level corresponded to FSHβ and LHβ, which showed its highest expression during ovulation. This suggest that FSH could be involved in vitellogenesis, while LHβ subunit raised at the end of reproductive cycle, determined the involvement of LH in ovulation and final oocyte maturation. A single chain recombinant LH (rLH) from *H.* nemurus consisted of LHβα fusion proteins was constructed and derived through gene-synthesis. The rLH construct was successfully cloned into pET-32 Ek/LIC and expressed in *E. coli* BL21 (DE3) cells. A specific band at 45.4 kDa was detected which corresponded to the molecular size of fusion rLH resolved by SDS PAGE. Two monoclonal antibodies, His-Tag and S-tag, were used to confirm the same expressed bands in Western immunoblotting. In order to investigate the bioactivity of the rLH hormone, fish trial was performed to assess the effect of exogenous rLH on the plasma level of E2, their mRNA level of GtH subunits and oocyte development in the gonad of female *H. nemurus*. Immature female *H. nemurus* were divided into four different treatments of ten fish/group, and each group received a single injection of: Group 1= 1X PBS (sham control), Group 2= 0.5 ml/kg Ovaprim (positive control), Group 3= rLH 50 μg/kg, and Group 4= rLH 150 μg/kg, respectively. Study parameters including the oocyte development through observation on gonad histology, the E2 plasma levels using enzyme-linked immunosorbent assay (ELISA) and the mRNA transcript level of α, FSHβ and LHβ subunits using real-time PCR. Based on the histological observation of the gonad, fish received treatment with rLH 50 μg/kg, rLH 150 μg/kg, as well as Ovaprim were able to induce the maturation of oocyte after 48 hr p.i. The E2 plasma level in fish treated with rLH 50 μg/kg raised after 6 hr p.i., significantly increased (p<0.05) after 12 hr p.i., and the level was sustained up to 24 hr p.i. The mRNA expression levels of α, FSHβ and LHβ also showed significant increment (p<0.05) in fish treated with rLH 50 μg/kg after 48 hr p.i. Fish treated with Ovaprim showed significant increased (p<0.05) of E2 plasma level after 6 hr p.i., but the level was reduced after 12 hr p.i. There was no significant increased (p>0.05) of mRNA subunits in fish treated with Ovaprim after 48 hr p.i., as compared to sham control fish. Meanwhile, treatment with rLH 150 μg/kg showed a weak stimulatory effect on the E2 production and mRNA transcript level of GtH subunits. The results revealed that treatment with rLH at 50 μg/kg is the best dosage to induce spawning and oocyte maturation in maturing female *H. nemurus*. These findings enhanced the understanding of GtH subunit in *H. nemurus*, and the temporal profile helped to estimate the correct time for hormonal therapies to induce ovulation and spawning in female *H. nemurus* reared in captivity. The production of recombinant GtH from *H. nemurus* would offer the establishment of a potentially efficient method to induce the gonadal development in this species and possibly in other catfish species.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENCIRIAN MOLEKUL DAN PENGHASILAN REKOMBINAN SUBUNIT GONADOTROPIN UNTUK PENINGKATAN PRESTASI DALAM SISTEM PEMBIAKAN BETINA *Hemibagrus nemurus* VALENCIENNES**

Oleh

**ZARIRAH BINTI MOHAMED ZULPERI**

**Oktober 2016**

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*Hemibagrus nemurus*, dikenali sebagai ikan baung, adalah spesis ikan keli yang dikultur dan sangat berharga di Malaysia, namun pengeluaran hasil ikan berkenaan terbatas akibat permasalahan dalam sistem pembiakan. Kebanyakan hatcheri menghadapi masalah dalam pembiakan aruhan disebabkan ikan tersebut tidak terdedah dengan pembiakan semulajadi mereka, terutama kegagalan induk betina menjalani tempoh kematangan telur seterusnya menghalang proses ovulasi and pembiakan. Gonadotropin (GtH), yang terdiri daripada hormon perangsang folikel (FSH) dan hormon pengluteinan (LH), memainkan peranan penting dalam gametogenesis dan kematangan seks pada mammalia, termasuk kumpulan ikan. Penyelidikan ini dijalankan untuk memahami ciri-ciri molekul dan memantau perubahan dalam pengekspresan mRNA untuk ketiga-tiga subunit GtH, $\alpha$, FSH$\beta$ dan LH$\beta$, dalam kitaran sistem pembiakan betina *H. nemurus*. Kemudian, satu rekombinan rantaian tunggal LH (rLH) *H. nemurus* telah dihasilkan di dalam sistem *E. coli*. Keaktifan hormon rekombinan (rLH) akan diuji sama ada ia berpotensi dalam merangsang pengeluaran hormon 17$\beta$-estradiol (E2), perkembangan ovari dan pengekspresan mRNA subunit GtH dalam ikan yang dikaji. Melalui analisis molekul menggunakan kaedah PCR menunjukkan cDNA $\alpha$, FSH$\beta$ dan LH$\beta$ dalam *H. nemurus* ialah 656, 728 dan 602 bp, bersamaan 116, 134 dan 140 asid amino. Ketiga-tiga subunit tersebut mempunyai homologi tertinggi dengan kumpulan Siluriformes dan Anguillaformes, manakala paling rendah dengan vertebrata kelas tinggi. Analisis filogenetik mengkelaskan $\alpha$ dan FSH$\beta$ bersama-sama dengan kumpulan vertebra kelas tinggi, manakala bagi LH$\beta$, terdapat dua kelompok yang berbeza, kumpulan satu hanya mengandungi semua kumpulan ikan, dan kumpulan kedua hanya mengkelaskan vertebra kelas tinggi. Aras transkrip subunit GtH, $\alpha$, FSH$\beta$ dan LH$\beta$, dalam pituitari betina *H. nemurus* dipantau sepanjang kitaran pembiakan menggunakan PCR masa nyata. Data dikumpul antara Ogos 2013 dan Oktober 2015, daripada usia ikan 1 hingga 27 bulan. Analisis menunjukkan aras mRNA subunit GtH berkadaran

terus dengan perkembangan ovari di sepanjang tempoh pembiakan. Aras subunit FSHβ mRNA meningkat semasa perkembangan ovari dan mencapai tahap optimum di pertengahan vitellogenesis, dan berkurangan semasa ovulasi, manakala aras subunit LHβ bertambah dengan signifikan ($p<0.05$) pada akhir vitellogenesis dan mencapai tahap optimum semasa proses ovulasi. Bagi subunit α, aras mRNA meningkat berkadaran terus dengan subunit FSHβ dan LHβ, dan mencapai tahap optimum semasa proses ovulasi. Melalui hasil eksperimen, FSH terlibat semasa vitellogenesis, manakala pengekpresan subunit LHβ pada akhir kitaran pembiakan menunjukkan hormon tersebut terlibat dalam ovulasi dan kematangan seks. Seterusnya, satu rantaian rekombinan LH (rLH) daripada *H. nemurus* yang mengandungi susunan struktur LHβα, dihasilkan melalui proses gen sintesis. Struktur protein rLH telah berjaya diklon ke dalam vektor, pET-32 Ek/LIC dan diekspres di dalam sel *E. coli* BL21 (DE3). Melalui SDS PAGE, satu jalur yang bersaiz 45.4 kDa telah dikenalpasti, bersamaan dengan anggaran saiz gabungan protein rLH tersebut. Dua antibodi monoklon, His.Tag dan S.Tag digunakan untuk pengesahan saiz protein rLH melalui Western immunoblot. Untuk mengkaji bioaktiviti hormon rLH, keaktifan hormon rLH tersebut diuji melalui keupayaan rLH untuk menaikkan aras E2 dalam plasma, aras mRNA subunit GtH dan perkembangan ovari dalam sistem pembiakan betina *H. nemurus*. Ikan betina *H. nemurus* yang pra-matang dibahagi kepada empat kumpulan rawatan berbeza dengan sepuluh ikan/kumpulan, dan setiap kumpulan menerima satu suntikan rawatan: kumpulan 1= 1X PBS (kawalan sham), kumpulan 2= 0.5 ml/kg Ovaprim (kawalan positif), kumpulan 3= rLH 50 μg/kg, dan kumpulan 4= rLH 150 μg/kg. Parameter kajian termasuk perkembangan telur melalui pemerhatian histologi gonad, aras E2 dalam plasma melalui esei immunoserap terungkai enzim (ELISA) dan aras mRNA transkrip subunit α, FSHβ dan LHβ melalui PCR masa nyata. Berdasarkan pemerhatian histologi gonad, kumpulan ikan yang menerima suntikan rLH 50 μg/kg, rLH 150 μg/kg, serta Ovaprim, telah menunjukkan perkembangan dan kematangan dalam telur selepas 48 jam rawatan. Aras E2 dalam plasma bagi kumpulan ikan dalam rawatan rLH 50 μg/kg telah menunjukkan kenaikan selepas 6 jam rawatan, dan bertambah dengan signifikan ($p<0.05$) selepas 12 jam rawatan, dan berterusan sehingga 24 jam rawatan. Analisis subunit GtH juga menunjukkan kumpulan ikan yang menerima rawatan rLH 50 μg/kg telah menaikkan ($p<0.05$) aras mRNA α, FSHβ dan LHβ selepas 48 jam rawatan. Kumpulan ikan yang menerima rawatan Ovaprim menunjukkan kenaikan ($p<0.05$) aras E2 dalam plasma selepas 6 jam rawatan, namun, aras tersebut telah berkurangan selepas 12 jam rawatan. Analisis subunit mRNA GtH bagi kumpulan ikan yang menerima rawatan Ovaprim pula tidak menunjukkan perubahan yang signifikan ($p>0.05$) selepas 48 jam rawatan, apabila dibandingkan dengan kumpulan kawalan sham. Manakala kumpulan ikan yang menerima rawatan rLH 150 μg/kg tidak menunjukkan kenaikan yang signifikan dalam aras E2 plasma dan mRNA subunit GtH. Keputusan eksperimen menunjukkan rawatan rLH pada 50 μg/kg adalah dos yang terbaik untuk merangsang pembiakan dan kematangan telur pada ikan betina *H. nemurus* yang sedang matang. Hasil kajian ini akan membantu bagi meningkatkan tahap pemahaman GtH hormon dalam spesis *H. nemurus*, manakala profil bagi subunit GtH di sepanjang kitaran pembiakan

akan membantu dalam menganggar ketepatan masa untuk menjalankan rawatan terapi hormon bagi merangsang ovulasi dan pembiakan dalam ikan betina spesis tersebut. Penghasilan hormon rekombinan LH daripada *H. nemurus* berpotensi untuk menyediakan kaedah yang lebih cekap untuk merangsang pembiakan dalam spesis ini dan berkemungkinan pada spesis ikan keli yang lain.

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"Verily with hardship, comes ease."

[Al-Insyirah, 94:5]

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Thank you very much!

I certify that a Thesis Examination Committee has met on 6 October 2016 to conduct the final examination of Zarirah binti Mohamed Zulperi on her thesis entitled "Molecular Characterization and Recombinant Gonadotropin Subunit Development for Improving Reproductive Performance in Female *Hemibagrus nemurus* Valenciennes" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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| | |
|---|---|
| % | percent |
| °C | degree Celsius |
| 11-KT | 11-ketotestosterone |
| 17α,20β-DHP | 17α,20β-dihydroxy-4-pregnen-3-one |
| 20β-S | 17α,20β,21β-trihydroxy-4-pregnen-3-one |
| A | absorbance |
| AChe | estradiol-acetylcholinesterase |
| AFLP | amplified fragment length polymorphism |
| ANOVA | analysis of variance |
| $B_0$ | maximum binding |
| BLASTn | basic local alignment search tool for nucleotide |
| Blk | blank |
| bp | base-pairs |
| BPG | brain-pituitary-axis |
| BSA | bovine serum albumin |
| BW | body weight |
| C-terminal | carboxy-terminus |
| CCD | computer-controlled cooled |
| cDNA | complementary deoxyribonucleic acid |
| CG | chorionic gonadotropin |
| CHO | Chinese hamster ovary |
| *Col* | coliconogenic |
| Ct | cycle threshold |
| Da | dalton |
| DAB | 3,3'-diaminobenzidine |
| dATP | deoxyadenosine triphosphate |
| $dH_20$ | distilled water |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| dph | days post hatching |
| ds | double stranded |
| dtn | decitonne |
| DTT | dithiothreitol |
| *E. coli* | *Escherichia coli* |
| E2 | 17β-estradiol |
| EDTA | ethylene-diamine-tetraacetic acid (disodium salt) |
| EIA | enzyme immunoassay |
| Ek | enterokinase |
| ELISA | enzyme-linked immunosorbent assay |
| FOM | final oocyte maturation |
| FRET | fluorescence resonance energy transfer |
| FSH | follicle-stimulating-hormone |
| g | gram |
| GnRH | gonadotropin-releasing hormone |
| GnRHa | antagonist gonadotropin-releasing hormone |
| GSI | gonadosomatic index |

| | |
|---|---|
| GtH | gonadotropin |
| GVBD | germinal vesicle breakdown |
| *H. nemurus* | *Hemibagrus nemurus* |
| hCG | human chorionic gonadotropin |
| hr | hour/s |
| HRP | horseradish peroxidase-conjugated |
| IgG | immunoglobulin G |
| IPTG | isopropyl β-galactose |
| kb | kilo base-pairs |
| kDa | kilo Dalton |
| l or L | liter |
| LB | Luria-Bertani |
| LH | luteinizing hormone |
| LIC | ligation independent cloning |
| MBP | maltose binding protein |
| MEGA | Molecular Evolutionary Genetics Analysis |
| mg | miligram |
| $MgCl_2$ | magnesium chloride |
| min | minute/s |
| MIS | maturation inducing steroid |
| ml | mililiter |
| ml | mililiter |
| mM | milimolar |
| mRNA | messenger ribonucleic acid |
| N-terminus | amine-terminus |
| NCBI | National Center for Biotechnology Information |
| NCS | N-linked glycosylation sequences |
| ng | nanogram |
| nm | nanometer |
| NSB | non-specific binding |
| NTC | no template control |
| NusA | N-utilizing substance-A |
| OD | oocyte diameter |
| ORF | open reading frame |
| p.i. | post injection |
| PBS | phosphate buffered saline |
| PBST | phosphate buffered saline with Tween 20 |
| PCR | polymerase chain reaction |
| pET-32/LIC-rLH | Recombinant plasmid (pET-32LIC+rLH gene) |
| pH | puissance hydrogen (hydrogen-ion concentration) |
| *R* | resistance |
| r.p.m. | rotations per minute |
| RACE | rapid amplification of cDNA ends |
| RAPD | random amplified polymorphism DNA |
| RIA | radioimmunoassay |
| rLH | recombinant LH |
| RM | Ringgit Malaysia |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |

| | |
|---|---|
| RT | reverse-transcription |
| RT-PCR | reverse-transcription polymerase chain reaction |
| SDS | sodium dodecyl sulfate |
| SDS PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sec | second/s |
| SEM | standard error mean |
| SOC | super optimal broth |
| std | standard |
| T | testosterone |
| TA | total activity |
| TBE | tris-boric EDTA |
| TBS | tris buffer saline |
| Tm | melting curve |
| Tris | tris (hydroxymethyl) aminomethane |
| trx | thioredoxin |
| TSH | thyroid-stimulating hormone |
| u | unit |
| UPM | universal primer mix |
| USD | United States Dollar |
| UTR | untranslated region |
| UV | ultra-violet |
| V | voltan/volt |
| v/v | volume per volume |
| Vtg | vitellogenin |
| w/v | weight per volume |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |
| μg | microgram |
| μl | microliter |
| μM | micromolar |
| μm | micrometer |
| μm | micrometer |

| Amino Acid | Single Letter | Three Letter Symbol |
|---|---|---|
| Alanine | A | Ala |
| Asparagine or aspartic acid | B | Asx |
| Cysteine | C | Cys |
| Aspartic acid | D | Asp |
| Glutamic acid | E | Glu |
| Phenylalanine | F | Phe |
| Glycine | G | Gly |
| Histidine | H | His |
| Isoleucine | I | Ile |
| Lysine | K | Lys |
| Leucine | L | Leu |
| Methionine | M | Met |
| Asparagine | N | Asn |
| Proline | P | Pro |
| Glutamine | Q | Gln |
| Arginine | R | Arg |
| Serine | S | Ser |
| Threonine | T | Thr |
| Valine | V | Val |
| Tryptophan | W | Trp |
| Tyrosine | Y | Tyr |
| Glutamine or glutamic acid | Z | Glx |

# CHAPTER 1

# INTRODUCTION

Aquaculture is growing and expanding in most of the regions in the world, as human population consumes the aquatic food for their daily nutrition, which provides a highly digestible proteins, essential vitamins and minerals in a simple accessible form. According to Food and Agriculture Organization of the United Nations (FAO, 2014), to sustain the current level per capita consumption, global aquaculture will need to achieve 80 million tons production by 2050. Latest report of fisheries sector in Malaysia generated RM 12,765.3 million in 2014, showing an increment of 11.32% compared to 2013 (Department of Fisheries Malaysia, 2014). Aquaculture production, which comprised of freshwater and brackish water production had contributed about 25.6% to the national economy, an increase of 35.02% compared to 2013.

The *Hemibagrus nemurus* (*H. nemurus*) or known as “ikan baung” is a popular catfish species preferred by local people in Malaysia, due to its good taste and nutritional value. Its non-bony flesh comprises of low cholesterol, high protein and rich in omega 3-polyunsaturated fatty acid (Mesomya et al., 2002). *Hemibagrus nemurus* is an indigenous species, and distributed in most of the Southeast Asian countries including Malaysia, Vietnam, Indonesia and Thailand (Rainboth, 1996). As one of the important catfish species in Malaysia, this fish has been commercially cultured intensively in floating cages or semi-intensively in ponds and pens (Kamarudin et al., 2011; Abidin et al., 2006; Khan et al, 1990). The annual national production of *H. nemurus* has gradually increased over the years, although it remains less than 6% of the total freshwater aquaculture production (Department of Fisheries Malaysia, 2014). The insufficient seed supply coupled with low quality of brood stocks have limited its production. The species is difficult to spawn artificially, due to problems associated with its induced spawning (Muchlisin et al., 2004). The maturity between male and female brood stock is difficult to be synchronized (Muchlisin et al., 2004). In addition, the female brood stock usually experiences reproductive failure of the pituitary to release gonadotropins, which is important in gonadal maturation in fish (Zohar and Mylonas, 2001).

The brain-pituitary-gonadal (BPG) axis plays a crucial role to control sexual development and reproductive system in most vertebrates including fish. This process starts when the gonadotropin-releasing hormone (GnRH) produced in brain, governs the release of the gonadotropins (GtHs) hormones in anterior pituitary into the blood circulation. Via the blood, those hormones reach into gonad and bind to their receptors, and stimulate the production of sex steroid, which are required for spermatogenesis and oogenesis (Amano, 2010; Weltzien et al., 2004; Holland et al., 2001). The

gonadotropins (GtHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are the main hormones in the regulation of gametogenesis and sexual maturation in fish. Both hormones are heterodimeric proteins comprise of a common $\alpha$ subunit and bound non-covalently to a specific $\beta$ subunit hormone (Swanson et al., 2003; Pierce and Parson, 1981). The duality of gonadotropic hormones in teleost fish has now been confirmed in various species mostly salmonid and few non-salmonid fish species. A distinct pattern on the mRNA expression of $\alpha$, FSH$\beta$ and LH$\beta$ subunits was detected in single spawning fish. The FSH is involved in the initiation of gametogenesis, vitellogenesis and spermatogenesis, while LH is involved in the final maturation process; ovulation, oocyte maturation, milt production and spermiation (Levavi-Sivan et al., 2010; Kobayashi et al., 2006; Yaron et al., 2003). In multiple spawning fish, the expression of FSH$\beta$ and LH$\beta$ mRNA levels are high during sexual behavior and oocyte maturation in females (Yoshiura et al., 1997; Jackson et al., 1999), but this is not a common pattern observed in fish such as red seabream (Gen et al., 2003).

In captive brood stock, the females usually exhibit severe reproductive problems than the males. Most fish reared in captivity showed reproductive dysfunction due to the failure of the pituitary to produce maturational gonadotropin, LH hormone (Zohar and Mylonas, 2001, Peter et al., 1993). Thus, the production of recombinant GtHs using various expression systems are being establish to improve method of inducing gonadal development in fish (Kobayashi et al., 2006). To date, a few fish recombinant GtH have been produced in several type of bioreactors, for example baculovirus in silkworm larvae (Ko et al., 2007; Kobayashi et al., 2006), yeast (Chen et al., 2012; Kasuto and Levavi-Sivan, 2005) and bacterial cell (Kim et al., 2012).

Since *H. nemurus* is one of the important cultured catfish in Malaysia, few efforts have been made to improve its breeding and spawning performance. Thalathiah et al. (1992) reported the successful artificial spawning of the species through hormonal induction using heteroplastic pituitary extract in combination with human chorionic gonadotropin (hCG). Latest result by Adebiyi et al. (2013b) revealed a treatment with 50 $\mu$g/kg BW (body weight) of synthetic agonists of GnRH (GnRHa) could increase steroid production in the plasma of *H. nemurus*, thus showing that GnRHa can be used as an inducing agent for reproduction control of this species. Other studies have established the reproduction, nutrition, genetics and morphology of *H. nemurus*, but up to this point, none of them focus on manipulating FSH and LH hormones.

The commercial culture of *H. nemurus* in Malaysia faced with problems associated in induced spawning that lead to its low production to fulfill the market demand. The current spawning method using the commercial synthetic reproductive hormones such as Ovaprim and Ovatide are successfully to induce spawning and ovulation in the species. However,

several disadvantages of using the hormones were the short response of the hormones tested in *in vivo* study (Adebiyi et al., 2013d), the hormones especially Ovaprim was too concentrated that is not practical for farmers, and sometimes two injections were needed to induce spawning and ovulation in the fish (Zohar and Mylonas, 2001) which is time-consuming. Therefore, this study aimed to perform a thorough study of GtH subunits, $\alpha$, FSH$\beta$ and LH$\beta$, in female *H. nemurus*, understand its reproductive cycle and produce a recombinant LH (rLH) in bacterial *E. coli* cells for an effective approach of hormonal manipulation and gonadal development in this species. The long-term goal is to produce hormonal treatment with a simple method and cost effective that can be used by the industry to improve the reproduction of cultured catfishes for sustainable aquaculture production. This study covered the following objectives:

1) to determine the molecular characteristic of GtH subunits, $\alpha$, FSH$\beta$ and LH$\beta$, from *H. nemurus*
2) to assess the expression levels of α, FSHβ and LHβ subunits of female *H. nemurus* at different stages of reproductive cycle
3) to develop and produce the recombinant LH (rLH) from *H. nemurus*
4) to determine the effect of exogenous recombinant LH on the plasma level of 17$\beta$-estradiol (E2) in female *H. nemurus*

Hypothesis:

$H_0$: Treatment with rLH at a specific dose in maturing female *H. nemurus* could not induce oocyte maturation, stimulating the production of sex steroid and up-regulate the mRNA transcript of GtH subunit genes including $\alpha$, FSH$\beta$ and LH$\beta$, resulting ovulation and spawning in the fish.

$H_A$: Treatment with rLH at a specific dose in maturing female *H. nemurus* could induce oocyte maturation, stimulating the production of sex steroid and up-regulate the mRNA transcript of GtH subunit genes including $\alpha$, FSH$\beta$ and LH$\beta$, resulting ovulation and spawning in the fish.

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## LIST OF PUBLICATIONS

### Journals

1. **Zulperi, Z.,** Ina-Salwany, M.Y., Christianus, A., Yusoff, F.M. and Harmin, S.A. (2017). Ovarian development and changes of the expression of pituitary gonadotropin subunits during reproductive cycle of female Malaysian river catfish, *Hemibagrus nemurus* (Valenciennes, 1840) in captivity*. Journal of Applied Ichthyology.* 00: 1-8. doi: 10.1111/jai.13267

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

3. **Zulperi, Z.M.**, Omar, A.R. and Arshad, S.S. (2009). Sequence and phylogenetic analysis of S1, S2, M, and N genes of infectious bronchitis virus isolates from Malaysia. *Virus Genes*. 38(3): 383-391.

4. Nehlah, R., Ina-Salwany, M.Y. and **Zulperi, Z.** (2016). Antigenicity analysis and molecular characterization of two outer membrane proteins of *Vibrio alginolyticus* strain VA2 as vaccine candidates in tiger grouper culture. *Journal of Biological Sciences*. 16(1): 1-11.

5. Ina-Salwany, M.Y., Hishammuddin, H., **Zulperi, Z.**, Salema, M., Karim, M. and Natrah, F.M.I. (2015). Elucidating the probiotic potential of Malaysia *Paenibacillus pabuli* against *Vibrio alginolyticus* in artemia culture. *Asian Journal of Agricultural Research*. 9 (5): 223-236.

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7. Zmora, N., Stubblefield, J., **Zulperi, Z.**, Klenke, U. and Zohar, Y. (2011). Kisspeptin-photoperiod/gonadal steroid relationships in the brain of two perciforms, the striped and hybrid basses. *Indian Journal of Science and Technology*. 4(S8): 10-11.

### Manuscript Submitted

1. Ina-Salwany, M.Y., **Zulperi, Z.**, Christianus, A., Yusoff, F.M. and Harmin, S.A. Recombinant luteinizing hormone development to improve the reproductive performance in female Malaysia catfish, *Hemibagrus nemurus*.

**Proceedings**

1. **Zulperi, Z**., Ina-Salwany, M.Y., Christianus, A. and Yusoff, F.M. (2016). Expression of gonadotropin subunit genes in Malaysian river catfish, *Hemibagrus nemurus* (Valenciennes 1840) in captivity. 7th International Agriculture Congress 2016. 4-6 October 2016. Hotel Bangi-Putrajaya, Malaysia. p. 736.

2. **Zulperi, Z**., Ina-Salwany, M.Y., Christianus, A., Yusoff, F.M. and Harmin, S.A. (2014). Molecular characterization and production of recombinant protein of LHβ subunit to induce spawning in *Hemibagrus nemurus*. The 3rd Thailand-Malaysia Graduate Forum in Life Sciences, Food Science and Agriculture 2014. 16-20 December 2014. Kasetsart University, Bangkok, Thailand. p. 38.

3. **Zulperi, Z**., Ina-Salwany, M.Y., Christianus, A., Yusoff, F.M. and Harmin, S.A. (2014). Molecular cloning and phylogenetic analysis of gonadotropin gene subunits of Asian redtail catfish, *Hemibagrus nemurus*. World Aquaculture Adelaide 2014. 7-11 June 2014. Adelaide Convention Centre, Adelaide, Australia. p. 481.

**GenBank**

1. **Zulperi, Z**., Ina-Salwany, M.Y., Christianus, A., Harmin, S.A. and Yusoff, F.M. (2014). *Hemibagrus nemurus* glycoprotein hormone alpha subunit mRNA, complete cds. Accession no: **KF934189**.

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