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MOLECULAR CLONING OF GONADOTROPIN HORMONES I (GtH-I) AND II (GtH-II) GENES IN THE HARD-LIPPED BARB (*Osteochillus hasseltii*) AND THE EFFECT OF PHOTOPERIODS ON THE GENES EXPRESSION

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MOLECULAR CLONING OF GONADOTROPIN HORMONES I (GtH-I) AND II (GtH-II) GENES IN THE HARD-LIPPED BARB (*Osteochillus hasseltii*) AND THE EFFECT OF PHOTOPERIODS ON THE GENES EXPRESSION**

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Running title: Cloning and expression of GtH genes in hard-lipped barb

ABSTRACT

Photoperiods is one of the factors that influence regulation on endocrine gland in producing hormones necessary for gonadal growth and development, gametogenesis and reproductive cycles in fish reproduction. This study clones the gonadotropin hormones I (GtH-I) and II (GtH-II) genes in hard-lipped bard, and investigates the effect of photoperiod on the genes expression. Experiment was designed using three treatments; 14L: 10D (control), 8L: 16D (short photoperiods), and 18L: 6D (long photoperiods). Four aquariums with nine fish/tank were used, serving as replicates. Fish were kept under this photoperiods treatment for eight weeks. Pituitary activities were observed by GtH genes expression measurement. The length of cDNA GtH-I was 222 bp, and the cDNA GtH-II was 354 bp. The GtH-I precursors encoded by cDNA consisted of 67 amino acids, including mature peptide. The level of GtH gene expression was significantly increased as longer photoperiods given ($P<0.05$). The results indicated that hard-lipped barb reproductive performance is affected by photoperiods treatment.

Keywords: Gonadotrhrophin GtH-I, GtH-II, photoperiods, hard lipped barb, cDNA, amino acid

INTRODUCTION

Fish reproduction is controlled by internal and external factors (Prayogo et al., 2016b). Photoperiod exerts its role on reproduction through the brain that integrates and conveys input from external and internal cues to the pituitary via melatonin (Prayogo et al., 2012.). Melatonin downregulating the synthesis and secretion of gonadotrophins releasing hormones (GnRH) gene (Shin at al., 2014). GnRH induced gonadotrophins (GtHs) secretion and that regulate the two main activities of the gonads, i.e. hormone and gamete production (Shin et al., 2014). Estradiol and progesterone are ovarian hormones that play an essential role in maintaining and promoting gamete reproduction (Prayogo et al., 2016a). In salmonids, GtH-I plasma level is elevated during spermatogenesis and vitellogenesis but declined during spermiation in males, and final maturation

and ovulation in females (Minniti et al., 2009). In comparison, GtH-II level is low during the early stages and increased in the later stages of reproduction spermiation in males, and final maturation and ovulation in females (Miranda et al., 2008). Both GTHs are equipotent in stimulating estradiol-17B(E_2) production, but GTH-II is more potent than GTH-I in stimulating maturation-inducing steroid, DHP (17α,20-dihydroxy-pregnen-3-one) (Versa et al., 2013).

Photoperiod is one the important cues for the spawn time in fishes such as carp, *cyprinus carpio* (Shin et al., 2014), Atlantic cod, *Gadus morhua* (Skjæraasen et al., 2006), and Chinook salmon, *Oncorhynchus tshawytscha* (Xiong and Hew., 1991). The effects of photoperiods on the synthesis of GTHs have long been recognized and are considered to be a part of the positive feedback mechanisms operating in fish (Prayogo, et al., 2012). These effects may be exerted indirectly, through modulation of the neuroendocrine factors regulating GtH release or directly at the level of GtH gene transcription. Some studies have shown the expression GtH gene in fish (Martins et al., 2015).

The majority of studies were conducted on temperate-zone fishes in which photoperiod strictly differ between seasons. Studies on the influence of photoperiod on tropical fishes are still limited. Hard-lipped barb,*Osteochilus hasselti*, in tropic condition with longer photoperiods 18L:6D were significantly decreased melatonin level and increased gene expression cGnRH-II (Prayogo et al., 2012). However in that study, it is still not clear whether long photoperiods will increasing gene expression GtH altered steroidogenesis activity in hard lipped barb ovarium.

Hard-lipped bard is an indigenous species and economically fish in Indonesia. This species was synchronous batch spawner fish (Prayogo et al., 2018). Hard-lipped barb is capable to spawn in 60 days after the previous spawning under good environmental. Hard-lipped barb has been adapted to a photoperiod of 12L:12D to 14L:10D. In this study, the isolation and identification of GtH-I cDNAs, and GtH-II cDNAs in the hard-lipped barb are reported for the first time. This study also examined the effect of different photoperiods on GtH gene expression of the hard-lipped barb.

MATERIALS AND METHODS

Treatment and sampling of fish

Total of 144 female hard lipped barb that were sexually matures (ages in 6 month) with the average weight of ±100 g was prepared. The fish were maintained in Laboratory of Fisheries and Marine Science, Jenderal Soedirman University, Indonesia. Every mature hard-lipped barb was induced by ovaprim with 0.5 ml/kg body weight doses to initiate spawning. Day zero was determined by the day of fish spawned. The fish were divided into four groups, each consisted of 50 L water aquariums, with nine fish in each aquarium.

Three photoperiod treatments which were; 6L: 18D, 14L: 10D and 18L: 6D was tested toward GtH gene expression. Blacklight proof polybag was used to covered aquariums. A 25-watt (Phillips) bulb was used as a source of light, regulated by 24 hours automatic timers placed on the top of each aquarium. The fish was maintained for eight weeks Samples of fish were collected every two weeks. Pituitary from nine fish of each group were collected, and the samples were snap-freezed on liquid nitrogen for GtH expression study. Real-Time PCR was used to evaluate GtH genes expression by applying primers derived from GtH genes.

RNA isolation and DNAse treatment

For total RNA isolation we used Sepasol R-RNA super-1 reagent (Takara, Otsu, Japan) to extract from the brain, (Prayogo et al., 2018). Denatured agarose gel, stained with ethidium bromide was used to verify RNA integrity. The RNA samples were treated with DNAse free RNAse (Takara). The quality and concentrations of total RNA were determined by agarose gel electrophoresis and optical density reading at 260 nm and 280 nm, and the RNA was aliquoted in batches and frozen at -70°C.

RT-PCR

cDNA synthesis kit (Takara, Otsu, Japan) were used to reserve transcript total mRNA samples (1 μL) using 6mers (sequence pd (N)6, 50μM) primers and prime script R-tase with manufacture instruction.

cDNA amplification

GtH-I and GtH-II primers were designed from cyprinids like *Cyprinus carpio* and *Carrasius auratus* cDNA. Multalin was used to align sequence to identify the conserved region in open reading frame (ORF) region. The primer to amplify the GtH-I and GtH-II gene were designed using primer 3 software (Table 1).

Thermal cycler (Robocycler, Stratagene,United statde) was used to carrying 35 cycles of PCR for hard-lipped barb GtH-I and GtH-II. Thermal cycler was used according to the following cycle, 95°C for 2 min, 35 cycles to 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, followed by a 5 min extension at 72°C. Post-amplification, the PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide electrophoretically.

Table 1. The primers used to amplify the GtH genes and their PCR product

No	Name/Primer Code	DNA sequence (primer)	Tm	PCR product

1.	Forward G1 Real Time (F2)	GCGCTTCGTTGTTATGGTGA	62,81	201 bp
2.	Reverse G1 Real Time (R2)	CATTGCAGCCGAGTGTCTG	62,83	
3.	Forward GIIReal Time (F3)	AGCTCTTATCTTCCACCCTGT	61,23	150 bp
4.	Reverse GII Real Time (F3)	AAGACTTGTGATAGT AGC AGG	62,34	
5.	Forward Actin (FA)	GAGCTATGAGCTCCCTGA CGG	58,3	53 bp
6.	Reverse Actin (RA)	AAACGCTCATTGCCAATG GT	55,6	

Cloning and sequencing of PCR products

Amplified PCR products separated by agarose gel electrophoresis and DNA gel extraction procedure was used to purify the incised gels. The desired DNA fragments from mRNA GtH-I, and mRNA GtH-II were subcloned into T vector (10 ng) (Takara) and ligated with T4 ligase. The plasmid was transfected into *E. coli* and spread into LB medium. The positive recombinant colonies screened using ampicillin. Positive colonies were purifying with mini scale plasmid preparation for sequencing. Fragments DNA sequences were determined using the Big Dye version 3.1 sequencing method with specific primers (Table 1). The data were automatically collected on the ABI PRISM 3100 Genetic Analyzer (PE Applied Bio-systems).

Sequence analysis

GtH-I and GtH-II gene checked by searching cDNA sequences using BLASTN searches (http://www.ncbi.nlm.nih.gov/ BLAST/) performed with default setting, non-redundant GenBank database nucleotide sequences.

Phylogenetic analysis

Hard lipped barb cDNA GtH-I, and GtH-II genes was compared to cDNA GtH-I, and GtH-II sequences from ten fish species, retrieved from NCBI GenBank. Fishes relationship in GtH genes was generated with CLUSTAL W, Treeview version 1.5.2 was used to generated scoring method percent and the unrooted tree.

Quantitative Real-Time analysis

The primers were designed based on GtH-I (KT947119) and GtH-II (KT762151) using the Primer 3.0 software. Hard lipped barb actin was used as endogenous control, amplified by the following primers-actin forward 5-GAGCTATGAGCTCCCTGACGG- 3, actin reverse 5-AAACGCTCATTGCCAATGGT-3- and were used to normalize variations in RNA. After

optimization, PCR reactions were performed in a 10 µL volume containing 2 µL cDNA, 5 µL SYBR mix (Applied Biosystem, Massachusetts,USA), 0.3 µL forward primer, 0.3 µL reverse primer, and 2.4 µL of double destilled water (DDW) using the following condition: 95°C for 45 s, (45 cycles of 95°C for 15 s and 60°C for 1 min), then 95°C for 15 s, 60°C for 15 s, and 95°C for 15s. $\Delta\Delta C_t$ method was used to calculate relative fold change of gene expression. Hard-lipped barb elongation actin gene is a stable reference gene, was used to normalize the C_t values of the target genes. Normalized qPCR data were LOG transformed prior to statistical testing.

Data analysis

SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA) was used to analyze the Real-Time data. Variance one-way analysis followed by Tukey's post hoc test was used to test for significant differences in the data ($p \leq 0.05$). The values are expressed as the means ± standard error (SE).

RESULTS AND DISCUSSION

Identification of GtH genes in hard-lipped barb

Hard-lipped barb GtH-I and GtH-II genes were successfully amplified from the total RNA. The agarose gel electrophoresis of the cDNA GtH-I showed a specific band, approximately 203 bp (KT947119). The corresponding cDNA sequences were called GtH-I. The cDNA sequences were checked with BLAST and we found there wasn't 100% identity with another gene GtH-I. The nucleotide sequence identity of GtH-I cDNAs was 98% with carp (*Cyprinus carpio* M37379.1), 97 % with goldfish (*Carrasius auratus*, AY800266.1), 95% with minnow (*Gobiocypris rarus*, JF340640.1), and 94% with grass carp (*Ctenopharyngodon idella*, EU095936.1).

Hard lipped barb GtH-II genes of were successfully amplified from the cDNA. The agarose gel electrophoresis of the cDNA also showed a specific band, approximately 353 bp for GtH-II, which was called GtH-II cDNA (GenBank accession no. KT762151). The corresponding cDNA sequences were called GtH-II. The cDNA sequences were checked with BLAST and the identity was not 100% identical with another genes of GtH-II. The nucleotide sequence identity of GtH-II cDNAs was 96% with carp C*cyprinus carpio* AY189961.1), 95% with goldfish (*Carrasius auratus*, U30386.1), 94% with roach (*Rutilus rutilus*, U60668.1), and 94% with grass carp (*Ctenopharyngodon idella*, EU981284.1).

Gene structure GtH-I and GtH-II genes

GtH-I genes share the same basic structure as other cyprinids. Translation of GtH-I genes in

hard-lipped barb showed GtH-I had 67 amino acid and identified as mature peptide (Figure1). GtH-II genes also share the same basic structure as other cyprinids. Translation of GtH-II genes in hard-lipped barb showed GtH-II hads 1 signal peptide and 117 mature peptide (amino acid no 2-118) (figure.2).

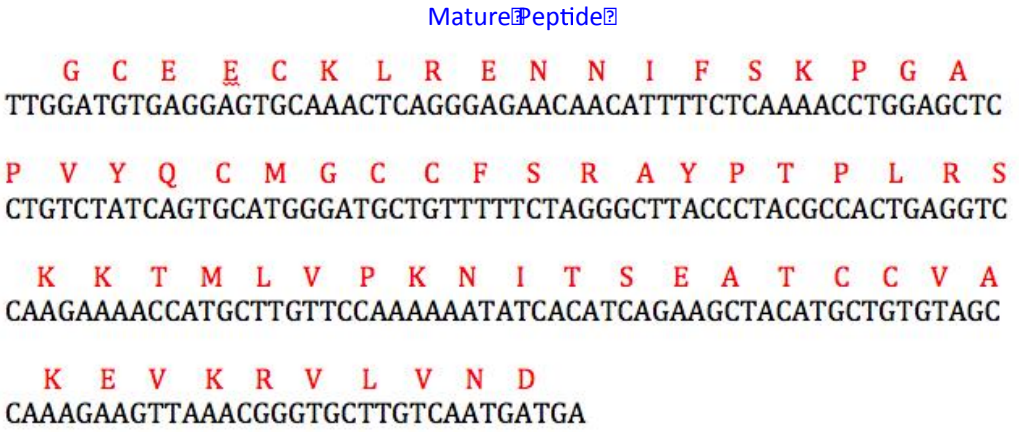

Figure 1 The nucleotide sequences of GtH-I in hard-lipped barb

Signal Peptide Mature Peptide

S S Y L P P C E P V N E T V A V E K E
AGCTCTTATCTTCCACCCTGTGAGCCAGTTAATGAGACTGTTGCTGTGGAAAAAGAG

G C P K C L V F Q T T I C S G H C L T
GGCTGTCCAAAATGTCTGGTGTTTCAGACTACCATCTGCAGTGGTCACTGCCTGACA
K
AAG

Mature Peptide

E P V Y K S P F S T V Y Q H V C T Y R
GAACCTGTATACAAGAGCCCATTTTCCACTGTCTACCAACACGTGTGCACTTACCGA

D V R Y E T V H L P D C P P G V D P H
GACGTGCGCTACGAAACTGTCCACTTGCCAGACTGTCCGCCAGGAGTGGACCCCCAT

I T Y P V A L S C D C S L C T M D T S

ATCACCTACCCTGTGGCTCTCAGCTGCGACTGCAGCCTCTGCACCATGGATACATCT

D C T I E S L Q P D F C M S K R E D F

GACTGTACAATTGAAAGCCTGCAGCCTGATTTTTGCATGTCTAAGAGAGAGGATTTC

L L Y Stop

CTTTTATACTAACCCCTTCTGACCACAAGACTACTATTCTGTGTTTAGCACATCAAAC

CAAAGTGTACACAAAA

Figure 2 The nucleotide sequences of GtH-II in hard-lipped barb. (Black color: Signal peptide; Yellow color: Mature peptide).

Hard-lipped barb GtH-I and GtH-II cDNA had a high similarity in the coding sequences with another teleost which can be seen as the distance at the phylogenetic trees (Figure 3-6). The greatest differences within the preprohormone were within the gene-associated peptide (GAP) coding sequences. The striking contrast between conservation of the GtH coding sequence and lack there of in GAP coding sequence is the evidence of differential selective pressure within the gene. This is evident in cases where the identity and similarity of GtH and GAP coding sequences have been compared for cDNA of different GtH genes within a species.

Phylogenetic analyses

The GtH-I and GtH-II genes were analyzed using phylogenetic analyses. Genetic distances (measured as substitutions/site) showed moderate low values, and the topology was well supported by strong bootstrap values. As expected, GtH-I and GtH-II genes in hard-lipped barb was included within a sub-cluster of the carp (*Cyprinus carpio)* and goldfish *(Carrasius auratus*) with high bootstrap values (Figure 3).

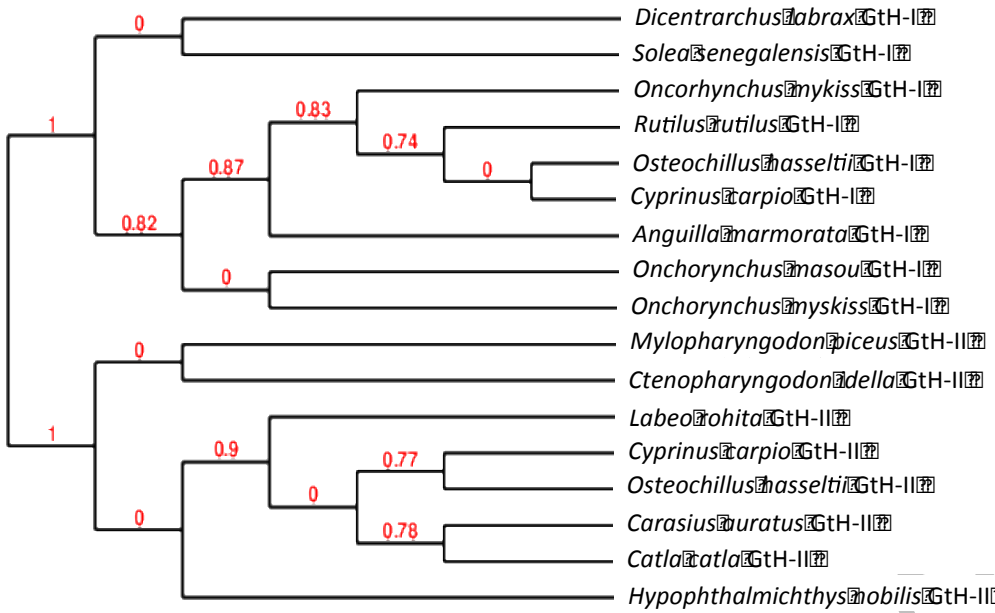

Figure 3 Phylogenetic relationship of precursors derived from known nucleotide encoding gonadotropin hormone (GtH-I and GtH-II). The relationship was generated with CLUSTAL W and the unrooted tree was generated using Treeview version 1.5.2. The scale bar represents the estimated evolutionary distance as 0.1 amino acid substitutions per site.

Expression of GtH mRNA under photoperiods manipulation

In hard-lipped barb females, relative GtH-I mRNA expression levels in eight weeks were 0.08-4.29 (Figure. 4). The highest GtH-I mRNA expression (4.29) was observed long photoperiods (LP) group in eight weeks significantly different ($P<0.05$) compared from short photoperiods (SP) and control (C). mRNA expression for 18L:6D group increased significantly ($P<0.05$) with post spawning periods compared to short photoperiods (SP) and control (C). The GtH-I mRNA expression for other treatment photoperiods in second weeks and fourth weeks had non-significantly different ($P>0.05$), but in six weeks and eight weeks of LP had higher gene GtH-I than short photoperiods (SP) and control ($P<0.05$). For the relative GtH-II mRNA expression level in eight weeks was 0.049-1.938 (Figure. 5). The highest GtH-II mRNA expression (1.938) was observed LP group in eight weeks was significantly different ($P<0.05$) compared to short photoperiods (SP) and control (C). mRNA expression for 18L:6D (LP) group increased with post spawning periods ($P<0.05$). The GtH-II mRNA expression for other treatment photoperiods in second weeks and fourth weeks had non significantly different ($P>0.05$), but in six weeks and eight weeks LP had higher gene GtH-II than SP and control ($P<0.05$).

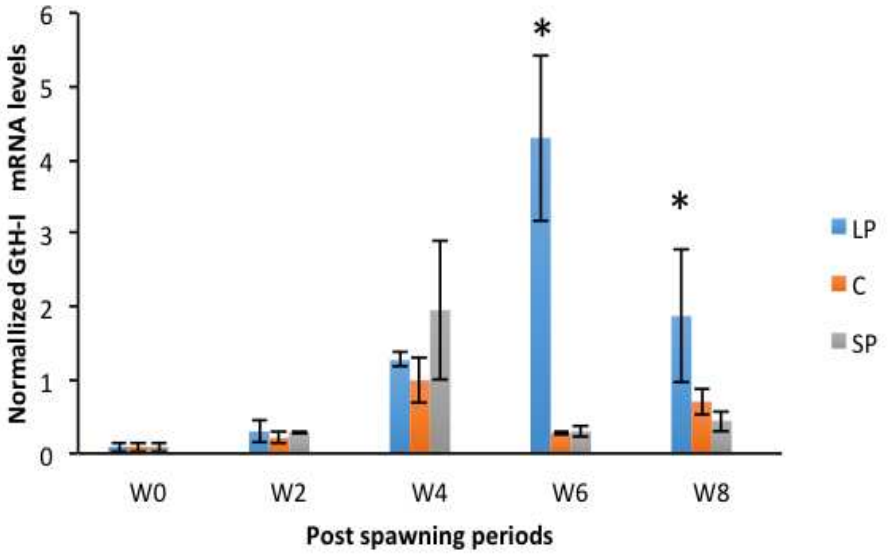

Figure 4 GtH-I gene expression of hardlipped barb kept under different photoperiod for eight weeks. C=14L: 10D (control), SP= 8L: 16D (short photoperiods) and LP=18L: 6D (long photoperiods). (* : Significantly different, ANOVA were analyzed every two weeks)

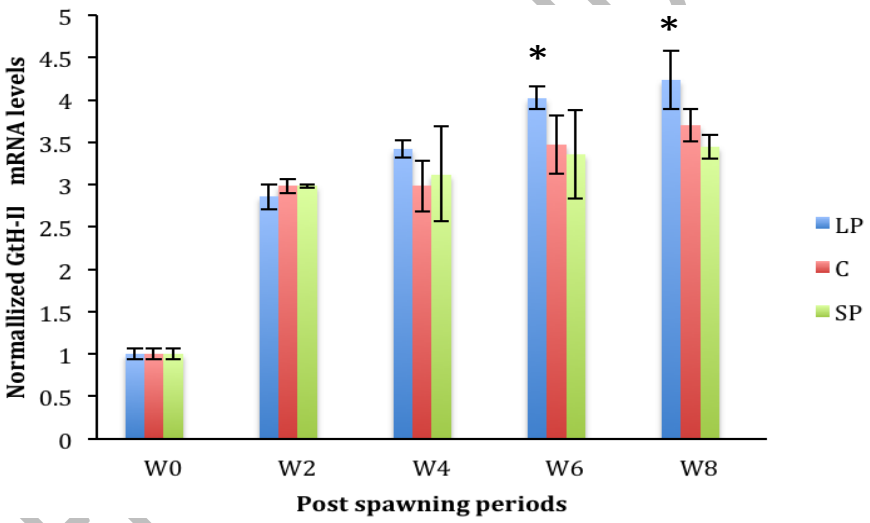

Figure 5 GtH-II gene expression of hardlipped barb kept under different photoperiod for eight weeks. C=14L: 10D (control), SP= 8L: 16D (short photoperiods) and LP=18L:6D (long photoperiods),). (*): Significantly different, ANOVA were analyzed every two weeks)

This study reports for the first time cloning of differing cDNAs for GtH encoding the GtH-I and GtH-II from the pituitary tissues hard-lipped barb. Comparison of the nucleotide sequence identity of GtH-I cDNAs was very similar with another variant GtH-I. BLAST analyses showed 98% similarity with carp (*Cyprinus carpio* M37379.1), 97% with goldfish (*Carrasius auratus*, AY800266.1), 95 % with *Gobiocypris rarus*, (JF340640.1), and 94% with grass carp (*Ctenopharyngodon idella*, EU095936.1) (Figure 6). The same pattern was also reported in GtH-II. The nucleotide sequence identity of GtH-I cDNAs also very similar with another variant GtH-I, from BLAST, where the results showed 98% similarity with carp (*Cyprinus carpio* M37379.1)

and97% with goldfish (*Carrasius auratus*, AY800266.1), The nucleotide sequence identity of GtH-II cDNAs was 96% similar with carp (*Cyprinus carpio* AY189961.1), 95% with goldfish (*Carrasius auratus*, U30386.1), 94% with roach (*Rutilus rutilus*, U60668.1), and 94% with grass carp (*Ctenopharyngodon idella*, EU981284.1) (Figure.7).

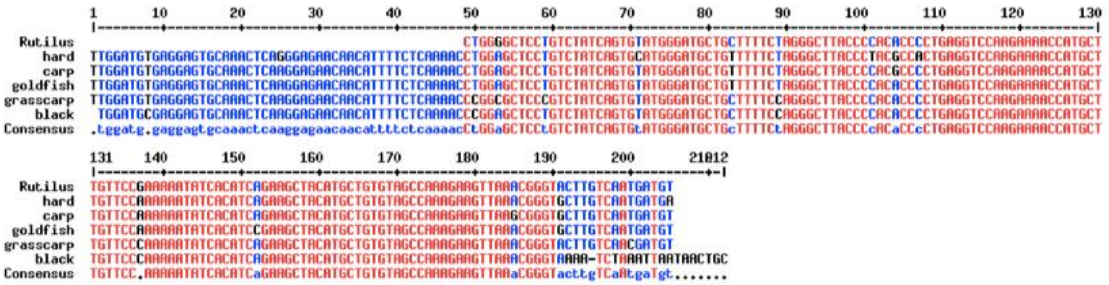

Figure 6 Nucleotides alignment of GtH-I cDNA in hardlipped barb with another teleost. (Red colour: conserved area; blue and black colour: not conserved area)

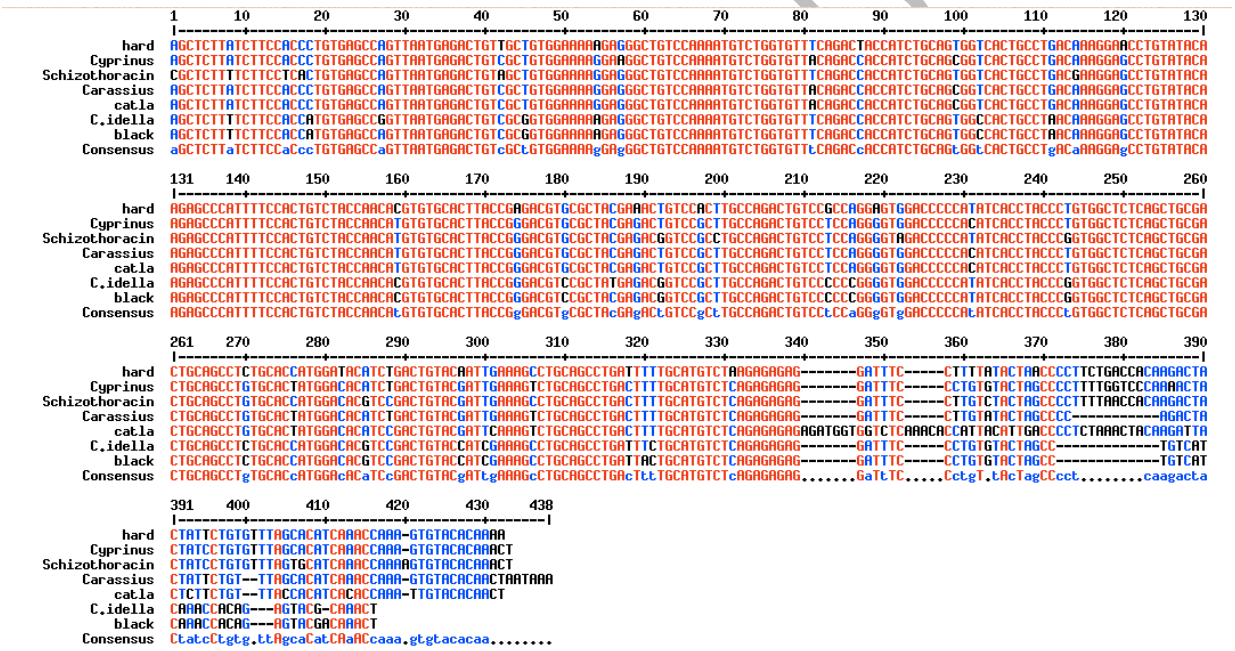

Figure 7 Nucleotides alignment of GtH-II cDNA in hardlipped barb with another teleost. (Red colour: conserved area; blue and black colour: not conserved area)

The partial GtH-I precursor encoded by cDNAs, translated to 67 amino acid residues. The partial GtH-I precursor was composed of mature peptide (Figure. 1). The amino acid sequences of hard-lipped barb GtH-I precursors encoded by cDNA were compared with some of previously GtH-I precursors (Figure. 8), such as the precursors of rohu (*Labeo rohita*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), rainbow trout (*Oncorhyncus mykiss*), and rock carp (*Procypris rabaudi*). The results showed that the amino acid homology of GtH-I precursors within Cyprinoids was 85-97%. However, compared to other teleosts GtH-I

precursors, the amino acid homology of GtH-I precursors in teleosts was only 50-71% (Figure.8). However, from that alignment explained that GtH-I mature peptide between hard lipped barb compared with another teleost like rainbow trout and grass carp had different from amino acid number 41(R) and 43 (N).

The partial GtH-II precursor encoded by cDNAs, translated to 118 amino acid residues. The partial GtH-IIb precursor was composed of a 1 amino acids signal peptide, and 117 mature GtH-II decapeptide. (Figure.2). The amino acid sequences of common carp GtH-II precursors encoded by cDNA were compared with some of the previously identified GtH-II precursors, such as the precursors of roach (*Rutilus rutilus*), goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*). The results showed that the amino acid homology of GtH-II precursors within carp was 97% (Figure. 9). From multi-alignment analyze explained that GtH-II mat-peptide hard lipped-barb had different from amino acid number 27 (F), number 66 (H), number 111 (K) and number 117 (L). Compared with another teleost like carp (*Cyprinus carpio*). This can be assumed that peptide function may be changed due to adaptation of nature selection.

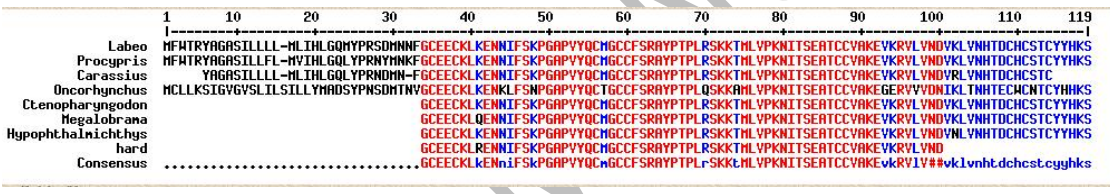

Fig. 8. Amino acids alignment of GtH-I cDNA in hard-lipped barb with other teleost. (Red colour: conserved area; blue and black colour: not conserved area)

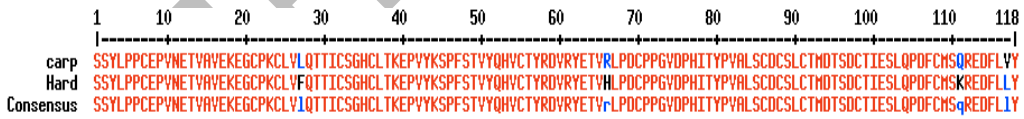

Figure 9 Amino acids alignment of GtH-II cDNA hard-lipped barb with other teleost. (Red colour: conserved area; blue and black colour: not conserved area)

The present study provides new evolutionary information on this GtH gene family in the pituitary. The GtH in hard-lipped barb were grouped together with another teleost in the phylogenetic tree, suggesting a common ancestor for both groups of genes. Phylogenetic analysis shows that GtH can be separated into two major groups. Subgroup I contains GtH-I from sea bass (*Dicentrarchus labrac*) until salmon (Onchorycus *mykiss*), while subgroup II from black carp (*Mylopharingodon piceus*) until bighead carp (*Hypophthalmichtys nobulis*). (Figure.3). For GtH hard-lipped barb are similar to teleost GtH special with goldfish and carp, indicated they also have some same features and functionality. Furthermore GtH-I and GtH-II hard lipped-barb were similar

to goldfish, carp and red crucian carp, which assumed that hard lipped-barb also had two subunits of GtH (alpha and beta).

The mature peptide was a minimal structural requirement for delineated for gonadotropin activity. The GtH-II mature peptide from hard-lipped barb only different 4 amino acid with carp, assumed that they had a same functional. The comparison results of amino acid sequences of GtH-II precursors from different vertebrates showed that the mature GtH-II was very conserved. A mature peptide is a peptide chains that coding sequence for the mature or final peptide or protein product following post-translational modification. So, GtH-II was conserved during the process of evolution. According to reported research, the GtH-II commonly had 12 cysteine residues, which could form six conserved disulfide bridges with three b barrettes. In addition, the glycosylation sites in glycoprotein were important for the synthesis, secretion, ratio metabolism and regulation of hormones (Ulloa et al., 1999). The previous studies showed that GtH-II only had one N-linked glycosylation site (Asn number 11). These glycosylation sites would influence the combination activity between the hormone and its receptor (Yu and Peter., 1992).

Previous studies in teleost showed that extended photoperiod (18L:6D) decreased melatonin and increased gene expression of cGnRH-II, gene expression of sGnRH and estradiol level (Prayogo et al., 2012). This increase was resulted from the melatonin production decrease lead to the increase of GnRH production from the hypothalamus and increases the GtH expression (Miranda et al., 2008; Shin et al., 2014). In this study, changes in gene expression of GtH-I, and GtH-II levels in hard-lipped barb were analyzed to characterize the role of neuropeptides in the control of reproduction under photoperiods manipulation. This study confirms previous results that showing increased in all GtH genes levels during photoperiods manipulation in hard-lipped barb. In addition, we report for the first time, changes in the gene expression levels of GtH genes in correlation with photoperiods manipulation. Although we are aware that mRNA levels do not always match with protein levels and/or the physiological effects of the protein products, the regulation of mRNA levels provides an indication of the activity of a particular peptide neuronal system.

In this study, we showed that GtH-I and GtH-II level increased equivalent with the long photoperiod increased. This proved that photoperiod exerts it role on reproduction through hypotalamus-pituitary-gonad, which integrates and conveys input from external and internal cues to the pituitary organs (Versa et al., 2013; Minniti et al., 2009). Photoperiods regulated melatonin production and melatonin mediated cyclical regulation of GnRH mRNA expression involves the protein kinase C and the extracellular signal-regulated kinase 1 and 2 pathways. Melatonin regulated act through membrane receptors to trigger the protein kinase C pathway and 12-O-tetradecanoyl phorbol-13-acetate (TPA), a modulator of this pathway, has been shown to suppress

GnRH gene expression through the promoter (Qingbo et al., 2005; Prayogo et al., 2012). GnRH binds to GnRH receptor and active G protein-mediated phosphorylation to protein kinase C and synthesis Gonadotrophin (GtH-I and GtH-II). GtH-II secreted into the blood vessel, to the receptor in theca cell activated G protein and adenylate cyclase to phosphorylation cAMP and activation staR protein. staR protein regulated cholesterol (Martins et al., 2015). On the contrary, short photoperiod stimulated melatonin production and suppressed the hypothalamus-pituitary-gonad axis. Therefore, increasing photoperiod should, in theory, increase the reproductive activities (Prayogo et al., 2012).

CONCLUSION

In summary, this study reported for the first time the cDNA sequence of GtH genes variants in a hard-lipped barb. The phylogenetic results presented in this work support the idea that all GtH genes share the same basic structure. Moreover, this mean that GtH-I and GtH-II in hard-lipped barb very conserve, and assumed to havesame function with another teleost. Photoperiod affected regulation of gene expression of GtH-I and GtH-II in the hard-lipped barb. The more extended photoperiod increased gene expression of GtH-I and GtH-II, via HPG axis.

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