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EFFECTS OF PHOTOPERIODS ON THE CLONED GTH GENES IN HARD-LIPPED BARB (*Osteochillus hasseltii*)

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ABSTRACT

Photoperiod is one factor that regulates the endocrine gland's production of hormones necessary for gonadal growth and development, gametogenesis and reproductive cycles in fish reproduction. However, studies on the influence of photoperiod on tropical fishes are still limited. Hence, this study investigates the effects of photoperiod on the hard-lipped barb, particularly, the genetic expression of its cloned gonadotropin hormones I (GtH-I) and II (GtH-II) genes. Experiment was designed using three treatments; 14L:10D (control), 8L:16D (short photoperiods), and 18L:6D (long photoperiods). Four aquariums with nine fishes/tank were used, serving as replicates. The fishes were kept under these different photoperiod lengths for eight weeks. Pituitary activities were observed by measuring the GtH genetic expression. 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 significantly increased as longer photoperiods were administered. The results indicated that photoperiod lengths affect the hard-lipped barb reproductive performance.

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

INTRODUCTION

Fish reproduction is controlled by both internal and external factors, one of which is the photoperiod that regulates reproduction through the brain that integrates and conveys input from external and internal cues to the pituitary gland via the melatonin (Prayogo *et al.* 2012; Prayogo *et al.* 2016b). Melatonin downregulates the synthesis and secretion of gonadotrophin releasing hormones (GnRH) gene (Shin *et al.* 2014). GnRH induces the gonadotrophins (GtHs) secretion and regulates 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; Sukardi *et al.* 2018). 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 but increases in the later stages of reproduction or 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) (Veras *et al.* 2013).

Photoperiod is one important cue for the spawning time of fishes such as carp (*Cyprinus carpio*) (Shin *et al.* 2014), Atlantic cod (*Gadus morhua*) (Skjæraasen *et al.* 2006), and Chinook

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salmon (*Oncorhynchus tshawytscha*) (Xiong & 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 fishes (Prayogo *et al.* 2012). These effects may be exerted indirectly, through the modulation of the neuroendocrine factors regulating the GtH release or directly at the level of GtH gene transcription. Some studies have shown the GtH gene expression in fish (Martins *et al.* 2015).

The majority of studies were conducted on temperate-zone fishes in which photoperiod strictly differ between seasons. However, little information is available on the influence of photoperiod on tropical fishes. Hard-lipped barb, *Osteochilus hasselti*, growing in tropical conditions with longer photoperiods of 18L:6D has experienced significantly decreased melatonin level and increased cGnRH-II gene expression (Prayogo *et al.* 2012). However, it is still unclear whether long photoperiods will increase the gene expression of GtH-altered steroidogenesis activity in hard lipped barb ovarium.

Hard-lipped barb is an economically important indigenous fish species in Indonesia. This species is synchronous with batch spawner fish (Prayogo *et al.* 2018). Under good environmental connditions, it is capable of spawning again in 60 days after the previous spawning. Hard-lipped barb is adapted to a photoperiod of 12L:12D to 14L:10D.

This study documents for the first time, the isolation and identification of GtH-I cDNAs, and GtH-II cDNAs in the hard-lipped barb and the influence of differing photoperiods on the GtH gene expression.

MATERIALS AND METHODS

Treatment and Sampling of Fishes

A total of 144 six-month old and sexually mature female hard lipped barb with an average weight of ±100 g were maintained at the Laboratory of Fisheries and Marine Science, Universitas Jenderal Soedirman, Indonesia. Each of the mature barb was induced with ovaprim applied at 0.5 mL/kg body weight doses to initiate the spawning process. Day zero was identified as the day the fishes were spawned. The fishes were divided into four groups with nine fishes in each of the 50 L water aquarium.

Three photoperiod treatments, namely; 6L:18D, 14L:10D and 18L:6D were tested toward GtH gene expression. Black-light-proof polybag was used to cover the aquariums. A 25-watt (Phillips) bulb was used as a source of light, regulated by 24-hour automatic timers, and placed at the top of each aquarium. The fishes were maintained for eight weeks and sample fishes were collected every two weeks. The pituitary gland from nine fishes of each group were collected, and the samples were snap-freezed on liquid nitrogen for determination of GtH expression. The real-time PCR was used to evaluate the GtH genes expression by applying primers derived from GtH genes.

RNA Isolation and DNAse Treatment

For total RNA isolation, the Sepasol R-RNA super-1 reagent (Takara, Otsu, Japan) was used to extract total RNA from the brain, (Prayogo *et al.* 2018). Denatured agarose gel, stained with ethidium bromide was used to verify the RNA integrity. The RNA samples were then 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

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

cDNA Amplification

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

The thermal cycler (Robocycler, Stratagene, United statde) was used to carry 35 cycles of PCR for hard-lipped barb GtH-I and GtH-II, according to the following cycles, 95°C for 2

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 GII Real Time (F3)	AGCTCTTATCTTCCACCCTGT	61.23	150 bp
4.	Reverse GII Real Time (F3)	AAGACTTGTGATAGTAGC AGG	62.34	
5.	Forward Actin (FA)	GAGCTATGAGCTCCCTGA CGG	58.3	53 bp
6.	Reverse Actin (RA)	AAACGCTCATTGCCAATG GT	55.6	

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. At post-amplification, the PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide electrophoretically.

Cloning and Sequencing of PCR Products

The amplified PCR products were separated by agarose gel electrophoresis and the 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 were screened using ampicillin. Positive colonies were purified with mini scale plasmid preparation for sequencing. Fragments of DNA sequences were determined using the Big Dye version 3.1 sequencing method with specific primers (Table 1). The data were automatically collected via the ABI PRISM 3100 Genetic Analyzer (PE Applied Bio-systems).

Sequence Analysis

The GtH-I and GtH-II genes were checked by searching cDNA sequences using BLASTN searches (Syngai *et al.* 2013) 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 used to generate the 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 the 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 distilled 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 the relative fold changes of the gene expression. Hard-lipped barb elongation actin gene, 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

The SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA) was used to analyze the real-time data. The one-way ANOVA followed by Tukey's post hoc test were used to test for significant differences in the means ($P \leq 0.05$).

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 were not 100% identical with the other gene GtH-I. The nucleotide sequence identity of GtH-I cDNAs was 98% identical 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).

The GtH-II genes of hard lipped barb 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 *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

The GtH-I genes share the same basic structure as other cyprinids. Translation of these genes showed that GtH-I has 67 amino acids that were identified as mature peptide (Fig. 1). GtH-II genes also share the same basic structure as other cyprinids. The translation of these genes showed that GtH-II had 1 signal peptide and 117 mature peptides (amino acid no 2-118) (Fig. 2).

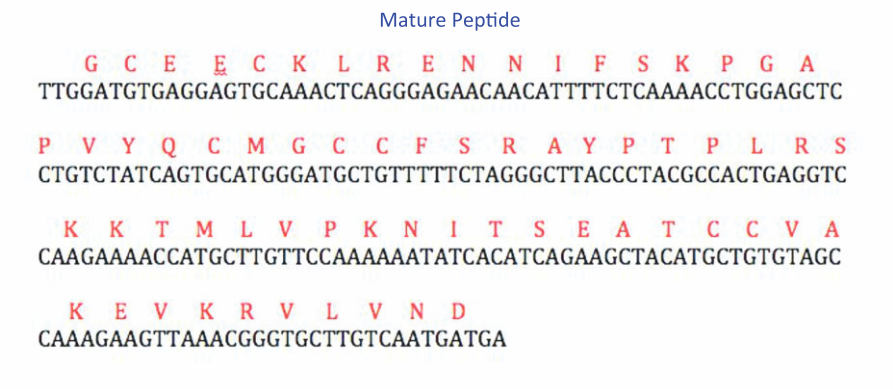

Figure 1 The nucleotide sequences of GtH-I in hard-lipped barb

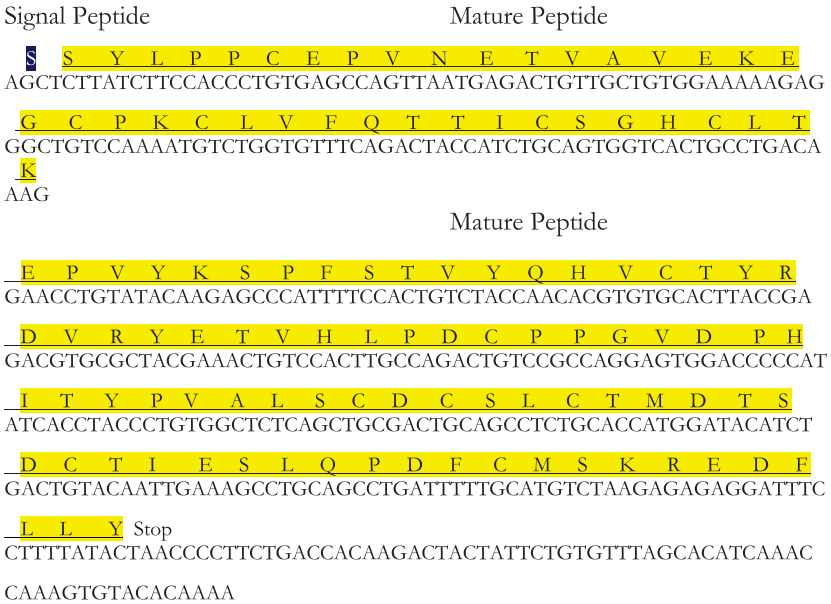

Figure 2 The nucleotide sequences of GtH-II in hard-lipped barb (black color: signal peptide; yellow color: mature peptide)

Hard-lipped barb GtH-I and GtH-II cDNA had a high similarity in the coding sequences of another teleost which can be seen as the distance at the phylogenetic trees (Fig. 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 the lack thereof 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 with cDNAs of different GtH genes within a species.

Phylogenetic Analysis

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 were included within a sub-cluster of the carp (*Cyprinus carpio)* and goldfish *(Carrasius auratus*) with high bootstrap values (Fig. 3).

Expression of GtH mRNA under Photoperiods Manipulation

In eight weeks, the female hard-lipped barbs, recorded a relative GtH-I mRNA expression levels of 0.08-4.29 (Fig. 4). The highest GtH-I mRNA expression (4.29) observed at long photoperiods (LP) group significantly differ ($P<0.05$) from those of the short photoperiods (SP) and control (C). The 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 photoperiod lengths in the second and fourth weeks did not significantly differ ($P>0.05$), but in six and eight weeks, the LP had higher gene GtH-I than the short photoperiods (SP) and control ($P<0.05$). The highest GtH-II mRNA expression (1.938) observed in the LP group in eight weeks was significantly different ($P<0.05$) from the short photoperiods (SP) and control (C) (Fig. 5). The mRNA expression for 18L:6D (LP) group significantly increased with post spawning periods ($P<0.05$). The GtH-II mRNA expression for other photoperiod lengths in the second and fourth weeks did not significantly differ ($P>0.05$), but in the six and eight weeks, the LP had higher gene GtH-II than SP and control ($P<0.05$).

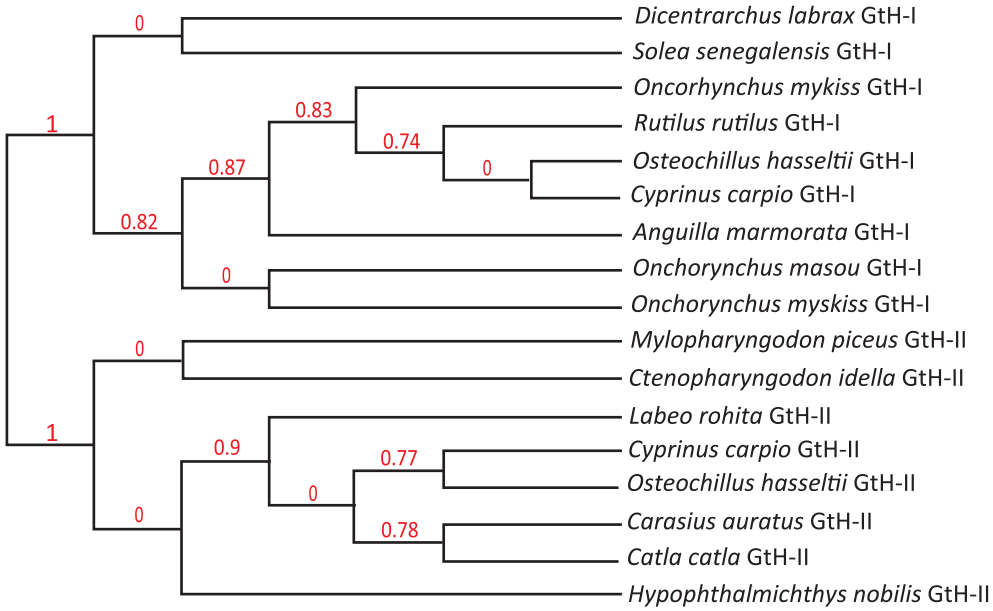

Figure 3 Phylogenetic relationship of precursors derived from known nucleotide encoding gonadotropin hormone (GtH-I and GtH-II). The relationship was generated with CLUSTAL W and the unrooted tree was generated using the Treeview version 1.5.2. The scale bar represents the estimated evolutionary distance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

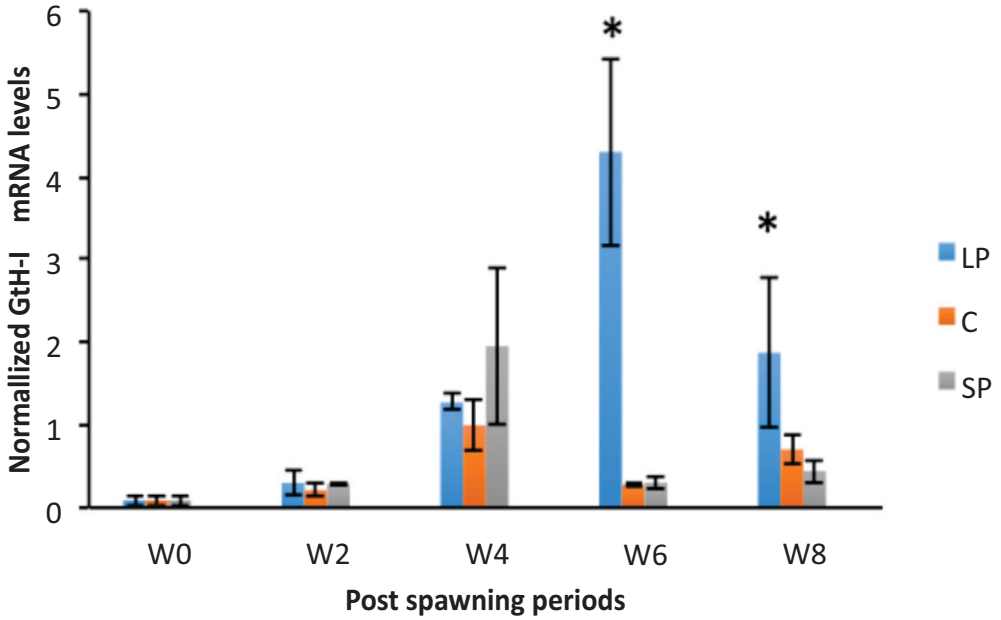

Figure 4 GtH-I gene expression of hard-lipped barb kept under different photoperiod for eight weeks ((C=14L:10D (control), SP=8L:16D (short photoperiods) and LP=18L:6D (long photoperiods)), *: significantly different means analyzed every two weeks

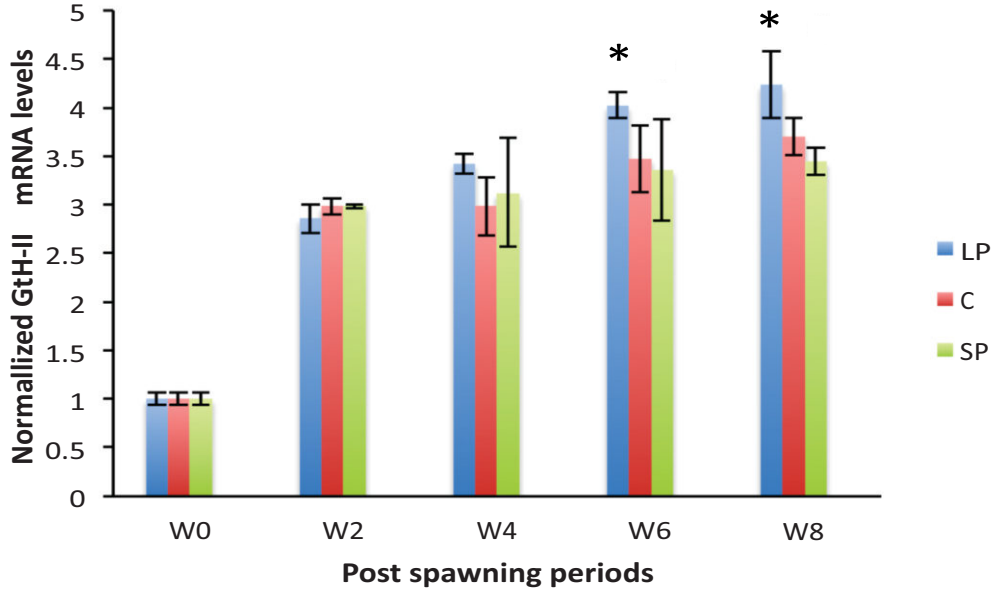

Figure 5 GtH-II gene expression of hard-lipped barb kept under different photoperiod for eight weeks ((C=14L:10D (control), SP=8L:16D (short photoperiods) and LP=18L:6D (long photoperiods)), *: significantly different means analyzed every two weeks

This study reports for the first time the cloning of different cDNAs for GtH encoding of the GtH-I and GtH-II from the pituitary tissues of hard-lipped barbs. Comparison of the nucleotide sequence identity of GtH-I cDNAs was very similar with another variant of 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) (Fig. 6). The same pattern was also reported in GtH-II. The nucleotide sequence identity of GtH-I cDNAs is also very similar with another variant GtH-I like carp and goldfish. The nucleotide sequence identity of GtH-II cDNAs was similar with carp goldfish, roach and with grass carp (Fig. 7).

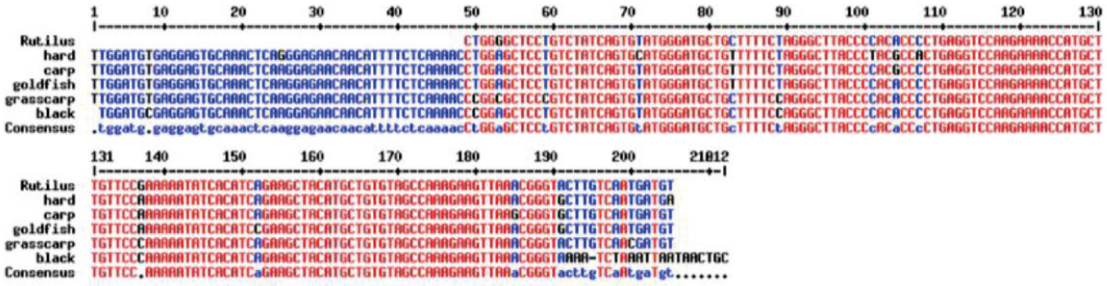

Figure 6 Nucleotides alignment of GtH-I cDNA in hard-lipped barb with another teleost (red color: conserved area; blue and black color: not conserved area)

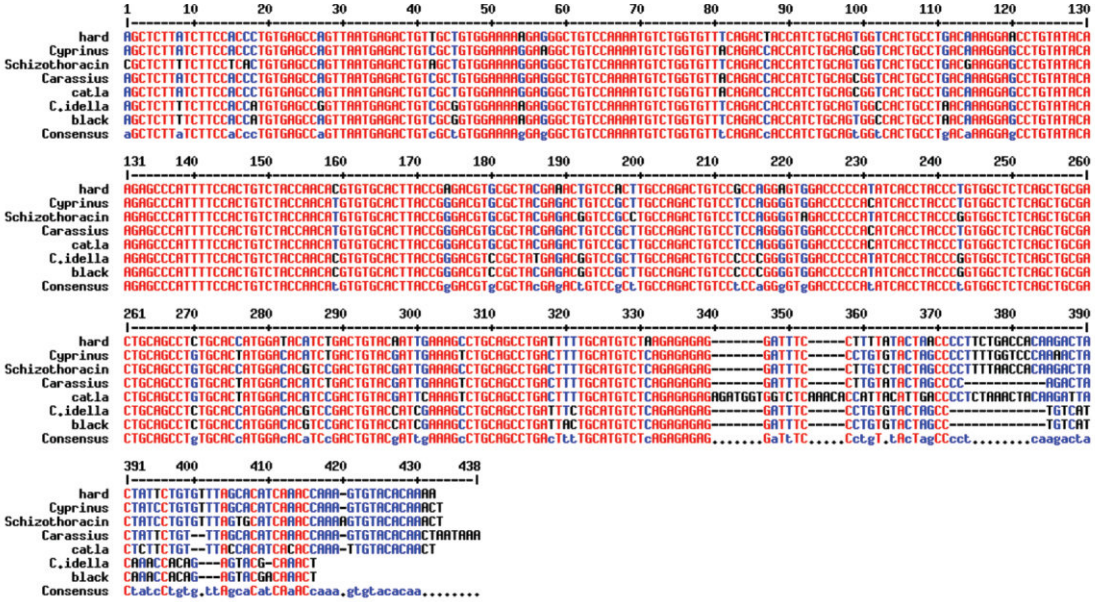

Figure 7 Nucleotides alignment of GtH-II cDNA in hard-lipped barb with another teleost (red color: conserved area; blue and black color: not conserved area)

The partial GtH-I precursor encoded by cDNAs was translated to 67 amino acid residues and was composed of mature peptide (Fig. 1). The amino acid sequences of the hard-lipped barb GtH-I precursors encoded by cDNA were compared with some of the previous GtH-I precursors (Fig. 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, when compared with other teleosts GtH-I precursors, the amino acid homology of GtH-I precursors in teleosts was only 50-71% (Fig. 8). The alignment explained that the GtH-I mature peptide of hard-lipped barb is different from that of another teleost, like the rainbow trout and grass carp, the former having amino acid numbers of 41(R) and 43(N).

The partial GtH-II precursor encoded by cDNAs was translated to 118 amino acid residues and was composed of 1 amino acid signal peptide and 117 mature GtH-II decapeptide (Fig. 2). When these precursors 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% (Fig. 9). The multi-alignment analysis showed that the GtH-II mat-peptide of the hard-lipped barb had different amino acid number 27 (F), number 66 (H), number 111 (K) and number 117 (L) when compared with another teleost like carp (*Cyprinus carpio*). Probably, the peptide function may be changed due to the barb's adaptation or natural selection.

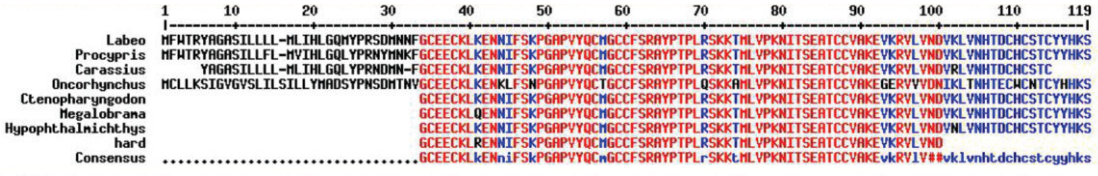

Figure 8 Amino acids alignment of GtH-I cDNA in hard-lipped barb with other teleost (red color: conserved area; blue and black color: not conserved area)

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

The present study provides new evolutionary information on this GtH gene family in the pituitary gland. The GtH in the 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*) (Fig. 3). The GtH of the hard-lipped barb is similar to teleost GtH special with goldfish and carp, indicating that they also have some similar features and functionality. Furthermore, the GtH-I and GtH-II of the hard-lipped barb were similar to goldfish, carp and red crucian carp, considering that the hard-lipped barb had also two subunits of GtH (alpha and beta).

The mature peptide is a minimal structural requirement for the delineation of gonadotropin activity. The GtH-II mature peptide from hard-lipped barb only differs by 4 amino acids from carp, considering that they have similar functionality. The comparative results of amino acid sequences of GtH-II precursors from different vertebrates showed that the mature GtH-II was much conserved. A mature peptide is a peptide chains coding the sequence of the mature or final peptide or protein product following a post-translational modification. So, GtH-II was conserved during the process of evolution. Accordingly, the GtH-II commonly had 12 cysteine residues, which could form six conserved disulfide bridges with three b barrettes. The glycosylation sites in glycoprotein were also important for the synthesis, secretion, ratio metabolism and regulation of hormones (Ulloa *et al.* 1999). The previous studies showed that GtH-II had only one N-linked glycosylation site (Asn number 11). These glycosylation sites would influence the combined activity of the hormone and its receptor (Yu & Peter 1992).

Other studies on teleost showed that extended photoperiod (18L:6D) decreased the melatonin level and increased the gene expression of cGnRH-II, gene expression of sGnRH and the estradiol level (Prayogo *et al.* 2012). This increase resulted from the decreased melatonin production that lead to the increase of GnRH production from the hypothalamus thereby increasing the GtH expression (Miranda *et al.* 2008; Shin *et al.* 2014).

In this study, the changes in the 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 different photoperiods. The results confirm previous studies by the increase in all GtH genes levels during photoperiods manipulation in hard-lipped barb. In addition, this study shows for the first time, the changes in the gene expression levels of GtH genes in correlation with different photoperiod lengths. Although the 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.

This study also showed that GtH-I and GtH-II level increased togther with the increasing photoperiod lengths. This proved that photoperiods influenced the reproduction process through the hypotalamus-pituitary-gonad interaction, which integrates and conveys

input from external and internal cues to the pituitary organs (Veras *et al.* 2013; Minniti *et al.* 2009). Photoperiods regulate the melatonin production and the melatonin mediated cyclical regulation of GnRH mRNA expression involves the protein kinase C and the extracellular signal-regulated kinase 1 and 2 pathways. Regulated-melatonin act through the 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 the GnRH gene expression through the promoter (Qingbo *et al.* 2005; Prayogo *et al.* 2012). The GnRH binds to GnRH receptor and the active G protein-mediated phosphorylation to produce the protein kinase C and synthesize the Gonadotrophin (GtH-I and GtH-II). The GtH-II is then secreted into the blood vessel, to the receptor in theca cell, and activates the G protein and adenylate cyclase to cAMP phosphorylation and to staR protein regulated cholesterol (Martins *et al.* 2015). On the contrary, short photoperiod stimulated the melatonin production and suppressed the hypothalamus-pituitary-gonad axis. Therefore, increasing the photoperiod should, in theory, increase the reproductive activities (Prayogo *et al.* 2012).

CONCLUSION

This study documented the first cDNA sequence of GtH genes variants in hard-lipped barb. The phylogenetic results support the idea that all GtH genes share the same basic structure. This means that GtH-I and GtH-II genes in the hard-lipped barb were much conserved, and assumed to have the same function with other teleosts. The photoperiod regulated the gene expression of GtH-I and GtH-II in the hard-lipped barb. The more extended photoperiod increased the gene expression of GtH-I and GtH-II, via the HPG axis.

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