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Effect of Photoperiods on Melatonin Levels, the Expression of cGnRH-II and sGnRH Genes and Estradiols Level in Hard-Lipped Barb (*Osteochilus hasselti* C.V.)

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Abstract: Fish reproduction is influenced by photoperiods through regulation on endocrine gland activities in producing hormones necessary for gonadal growth and development, gametogenesis and reproductive cycles. Research needs to be conducted to find out effect of photoperiod on nilem's (Hard-lipped barb) reproductive performance by manipulating photoperiod. Design experimental with four photoperiod treatments namely 14L:10D, 16L:8D and 18L:6D and control were conducted. Four aquaria consisting of nine fishes each were served as replicates. Fishes were kept under these photoperiods for 4 months. The observed variable was the end point pineal-hypothalamus-pituitary-gonad axis activities. Pineal activity was evaluated by measuring melatonin, hypothalamus activity was evaluated by measuring gene expression of cGnRH-II and sGnRH using Real Time PCR, Gonadal activity was evaluated by measuring Gonado somatic index (GSI) and serum level of estradiol was assayed using ELISA. The average of melatonin in each experimental group significantly decreased according to time ($P<0.05$) and there was significantly different was observed between each experimental groups. The average of cGnRH-II and sGnRH in each experimental group significantly increased according to time ($P<0.05$) and there was significantly different was observed between each experimental groups. Similar patterns were also observed for estradiol serum that in each experimental group significantly increased according to time ($P<0.05$) and there was significantly different was observed between each experimental groups ($P<0.05$). The average of GSI in each experimental group significantly increased according to time ($P<0.05$) but there was not significantly different was observed between each experimental groups. These results indicated that photoperiods had stimulatory effect in improving nilem reproductive performance.

Key words: Photoperiods · Melatonin · cGnRH-II · sGnRH · Estradiol · Nilem

INTRODUCTION

Reproductive activities in fish are regulated by several environmental and physiological factors [1]. Photoperiod exert it role on reproduction through pineal that integrates and conveys input from external and internal cues to the hypothalamus via melatonin [2] regulating the synthesis and secretion of gonadotropins releasing hormone (GnRHs). GnRHs regulating the synthesis and secretion of gonadotropins (GtHs) [3]. The GtHs regulate the two main activities of the gonads i.e. hormone and gamete production [4, 5]. Ovarian hormones especially estradiol and progesterone play an important role in maintaining and promoting gamete production [6, 7]. Photoperiod is one of important cues for the timing of spawning in many fish species as gold fish [8], atlantic salmon [9], Atlantic Halibut [10], Atlantic cod [11], Eurasian Perch [12], sturgeons [13] and *Oreochomis nilaticus* [14].

The majority of studies were conducted on temperate-zone fishes in which photoperiod strictly differ between seasons. Studies on influence of photoperiod on tropical fishes are still limited. It is interesting to examine whether photoperiod will induce a similar respond in

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tropical fishes as the case for its temperate-zone counterpart. In this study, hard-lipped barb (*Osteochilus hasselti* C.V.) was used as a model since hard-lipped barb is an indigenous tropical fish and widely cultured in Java [15]. Therefore studies to improve understanding of its biological aspect will be benefit not only for research but also for the fish farmers.

Hard-lipped barb is a synchronous batch spawner fish capable of spawning several time during the peak of the spawning period [15]. Under a suitable environmental setting, this fish capable to spawn in 60 days after the previous spawning. Hard-lipped barb has been adapted to a photoperiod of 12L:12D to 14L:10D. The present study was examined the effect of different photoperiods length on melatonin level, gene expression of cGnRH-II and sGnRH and estradiol level of the hard-lipped barb.

MATERIALS AND METHODS

Animals: 144 sexually mature female hard-lipped barb weighing of 100 g in average were maintained at Laboratory of Fisheries and Marine, Jenderal Soedirman University. They were induced to spawn using ovaprim 0.5 ml/kg body weight [16]. The day of spawning was designated as day zero post spawning period. The post-spawned females were divided into 4 groups. Each group consisted of 4 aquaria with 9 fish/50 L water.

In this study, three types of photoperiods namely 14L:10D, 16L:8D and 18L:6D and control have been tested toward concentration of melatonin, GnRH gene expression and concentration of estradiol. The aquaria were covered with light proof black polybag. The light source provided from 25 Watt (*Phillips*) bulb regulated by otomatic timers 24 hours cycles which were placed at the top of each aquarium. In photoperiod of 14L:10D, light was turned on since 06.00 am until 08.00 pm and in 16L:8D light was turned on since 06.00 am until 10.00 pm and in 18L:6D light was turned on since 04.00 am until 10.00 pm local time. Then, the fish were reared for four months at the laboratory of Aquaculture Department of Marine and Fisheries, university of Djenderal Soedirman.

During the research, fish were fed on commercial pellet (protein 37% and fat 10%) as much as 3% of total body weight daily [17]. The water was siphoned regularly to mantain water quality. The water temperature, disolved oxygen, pH and carbondioxide were monitored every months. Every sampling time, 9 fish were bled for Melatonin and Estradiol 17-β measurement. The brain were collected from 3 fish of each group and were snap-freezed on liquid nitrogen for GnRH expression study. For measuring melatonin and Estradiol, blood resulted from 9 fish were withdrawn, then serum were assayed using enzyme-linked immunosorbent assays (ELISA) at the Laboratory of clinical pathology, faculty of veternary medicine, Gadjah Mada University. The expression of GnRH was evaluated using Real Time PCR applying generated primers derived from cGnRH-II and sGnRH. Real Time PCR was conducted at the Laboratory of Gene Function Animal, Nara Institute Science and Technology, Japan.

mRNA Isolation and DNAse Treatment: Total mRNA was extracted from whole brain using blue Sepasol R-RNA super-1 reagent, based on Ethanol-phenol-chloroform extraction method [18]. The integrity of the RNA was verified in a denaturing agarose gel, stained with ethidium bromide. The RNA samples were treatment with DNAse free RNAse (Takara). The quality and concentrations of total RNA were determined by agarose gel electrophoresis and optical density reading at 260 and 280 nm (Figure 1), the RNA were aliquoted in batches and frozen at -70°C.

RT-PCR: Total mRNA samples (1µl) were reverse transcripted using cDNA synthezis kit (PrimeScript™ Reverse Transcriptase, Takara Bio.Inc) using Random 6 mers (sequence pd $(N)_6$, 50µM) primers and prime script R-tase with manufacture instruction.

Quantitative Real Time Analysis: The primers were designed based on cGnRH-II (accession numbers JN867720) and sGnRH (accession numbers JN867721) using the Primer 3.0 software. The generated primer used in this study were as follows: sGnRH forward 5-TGGTGTGTGTGTTGGAGG TT -3, sGnRH reverse 5-AATGTTGCCTCCACTTCACC-3; cGnRH-II forward 5-CATCTGCAGGCTGTTTGTGG-3, cGnRH-II reverse 5-TGCTGAGAG CTGGCAAAC TG -3. Goldfish actin (GenBank accession number AB039726.2), used as endogenous control, was amplified by the following primers-actin forward 5-GAGCTATGAGCTCCCTGACGG-3, actin reverse 5- AAACGCTCATTGCCAATGGT-3-and were used to normalize variations in RNA (Figure 2C). After optimization, PCR reactions were performed in a 10 µl volume containing 2 µl cDNA, 5 µl SYBR mix (Applied Biosystem), 0.3 µl forward primer, 0.3 µl reverse primer and 2.4 µl 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 15 s. The results were analyzed using the standard curve mode, according to the manufacturer's recommendations (Applied Biosystems).

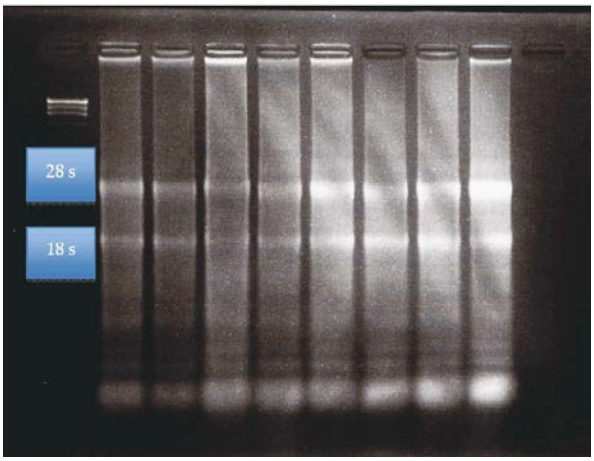


Fig. 1: RNA isolated from the brain of hard-lipped barb kept under different photoperiod for 4 month

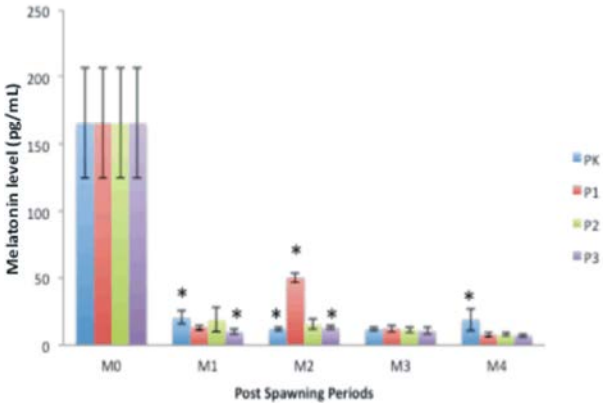


Fig. 2: Melatonin Levels of hard-lipped barb kept under different photoperiod for 4 month. (PK=control, P1=14L:10D, P2=16L:8D, P3=18L:6D, M=Month). (*: Significantly different)

Measurement of Melatonin Levels by Enzyme Linked Immunosorbent Assays: Melatonin levels were determined by ELISA using commercial kits (REF EIA-1431, DRG diagnostic) and extraction colomn C18 RP.1cm^3/100mg (REF KEME761) according to manufacture instructions. Each samples were analyzed in duplicate.

Measurement of Estradiol Levels by Enzyme Linked Immunosorbent Assays: Serum estradiol (E2) levels were determined DRG Estradiol ELISA (EIA- 2693). according to manufacturer's instruction. Duplicate were provided for each measurement.

Gonado-Somatic Index (GSI): Was calculated as the weight of the gonads relative to the total body weight, expressed as a percentage [19, 20].

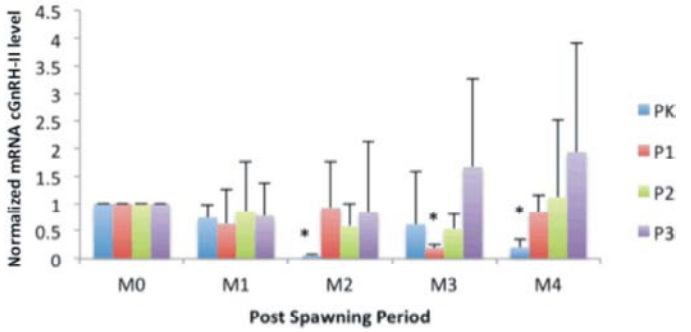
Data Analysis: The mRNA levels for each photoperiods treatment form were expressed as the ratio of GnRH mRNA to actin mRNA and then normalized as a percentage of M0 (pre treatment) group. Levels of mRNA corresponding to sGnRH and cGnRH-II were calculated as relative to those of β-actin and expressed as a percentage of either the M0 groups. The data from melatonin level, estradiol level, Levels of mRNA sGNRH and cGnRH-II and GSI were subjected to ANOVA followed by Tukey's multiple-comparisons tests. Differences were considered significant when $P< 0.05$ [21].

RESULT AND DISCUSSION

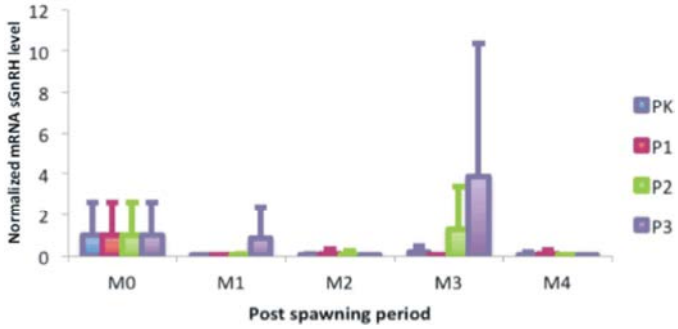
Melatonin Levels: Melatonin level at zero months were 45.51-189.83 pg/ml and in the fourth month were 5.45-23.12 pg/ml. Melatonin level in hard-lipped Barb kept under different photoperiods (14L:10D, 16L:8D and 18L:6D) decreased throughout the experimental period, using statistical methods would be showed that there is significantly different unless in the third month (Figure 2). In the first month control treatment, melatonin level of the control group was higher than those of 14L:10D and 18L:6D groups (P<0,05). In the second month melatonin levels of 14L:10D group was higher than those 16L:8D and 18L:6D groups (P<0.05). In the third month, melatonin levels were not significantly different photoperiod among the groups (P>0.05) and in the fourth month, for control treatment had a highest melatonin level than other photoperiods treatments (P<0.05).

Expression of cGnRH-II mRNA and, sGnRH mRNA: In hard lipped barb females, relative cGnRH-II mRNA expression level in four month were 0.049-1.938 (Figure 3A). The highest cGnRH-II mRNA expression (1.938) was observed 18L:6D group in fourth month significantly different (P<0.05). mRNA expression for 18L:6D group increased with post spawning periods (P<0.05). The cGnRH-II mRNA expression for other treatment photoperiods in first month and second month had non significantly different (P>0.05), but in third month and fourth month photoperiods 18L:6D had higher gene cGnRH-II yang than photoperiods 14L:10D and photoperiods 16L:8D. (P<0.05).

The relative level of sGnRH mRNA expression throughout the study were 0.0037-3.832 (Figure 3B). The highest sGnRH gene expression were (3.832) were found in 18L:6D group at the third month (P>0.05). The sGnRH mRNA expression for photoperiods treatment in first



A



B

Fig. 3: mRNA levels of cGnRH-II (A) and sGnRH (B) of hard-lipped barb kept under different photoperiod for 4 month. (PK=control, P1=14L:10D, P2=16L:8D, P3=18L:6D, M=Month). (*: Significantly different)

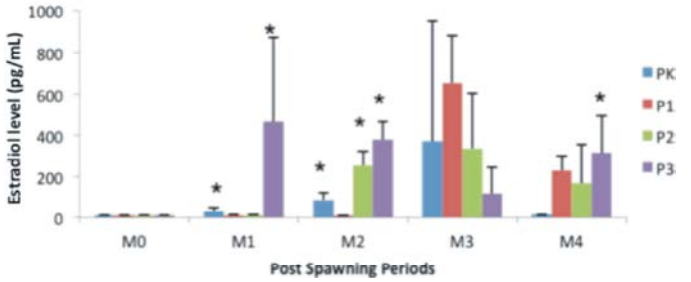


Fig. 4: Serum Estradiol Levels of hard-lipped barb kept under different photoperiod for 4 month. PK=control, P1=14L:10D, P2=16L:8D, P3=18L:6D, M=Month). (*: Significantly different)

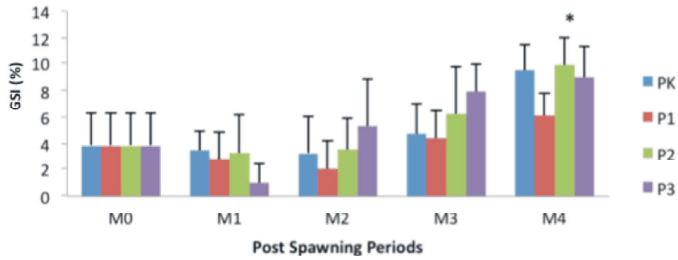


Fig. 5: GSI of hard-lipped barb kept under different photoperiod for 4 moths. PK=control, P1=14L:10D, P2=16L:8D, P3=18L:6D, M=Month). (*: Significantly different)

month untill fourth month had non significantly different (P>0.05), but in third month sGnRH gene expression in 18L:6D group at third month were higher trends than photoperiods 14L:10D and photoperios 16L:8D (P>0.05).

Serum Estradiol 17β Levels: Estradiol 17β level in zero month were 11.46-11.49 pg/ml and in the fourth months were 12.53-511.24 pg/ml (Figure 4). Estradiol level in hard-lipped Barb kept under different photoperiods

(14L:10D, 16L:8D dan 18L:6D) increased throughout the experimental period. Average estrogen 17β levels in third month not significantly different (P>0.05), but for the first, second and fourth months, photoperiods18L:6D, Estradiol level was higher than estradiol level in photoperiods 14L:10D and 16L:6D (P< 0.05).

Gonado-Somatic Indexs (GSI): GSI in first month were ranging from 2.25-9.95% and in the fourth month were ranging from 3.86-13.87% (Figure 5). GSI increased throughout the experimental period (P<0.05). The highest GSI in each group indicated that gonadal development was not maximum since GSI of post spawning hard-lipped barb cultured in ponds reached >16%[15]. Average GSI photoperiods treatment was not significantly different (P>0.05).

The correlation GSI with Serum Estradiol 17β Levels (r: 0.57) and melatonin (r:-0.49). The same pattern was seen in cGnRH-II and sGnRH. The correlation between cGnRH-II and melatonin were relatively high (r = -0.781) and Estradiol (r:-0.89). Different pattern was also seen in the correlation between sGnRH and melatonin (r:-0.044714081) and sGnRH with estradiol (r: 0.07925624).

DISCUSSION

In this study, changes in melatonin level, gene expression of cGnRH-II and sGnRH, Estradiol level in hard-lipped barb were analyzed to characterize the role of several neuropeptides in the control of reproduction under photoperiods manipulation. This study confirms previous results from fisheries and marine laboratory showing decrease in serum melatonin levels during photoperiods manipulation in hard-lipped barb. In addition, we report for the first time, changes in the gene expression levels of several neuropeptides in correlation with hormone secretion. 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 showed that melatonin level decreased equivalent with the long photoperiod increased. This is proved that photoperiod exert it role on reproduction through pineal that integrates and conveys input from external and internal cues to the hypothalamus via melatonin level [2]. Photoperiods also regulated gene expression cGnRH-II in hard lipped barb, the result of this research confirmed that long photoperiods enhaced gene expression cGnRH-II in hard lipped barb, similar in pejjerey fish which treatment with longer photoperiods increased the expression of cGnRH-II gene [7]. Result of gene sGnRH expression in hard-lipped barb in this research, can be determined that long photoperiods can enhaced trends of sGnRH expression in hard-lipped barb althought not significantly different. Average estrogen 17β levels in third month not significantly different (P>0.05), but for the first, second and fourth months, photoperiods18L:6D, Estradiol level was higher than estradiol level in photoperiods 14L:10D and 16L:6D (P< 0,5). This is proved thats photoperiods can enhaced estradiol levels in nilem. For the average GSI amongst photoperiods was not significantly different (P>0,05). Another research demonstrated that different photoperiods and water quality in carp C*atla catla* did not effect reproductive performance [22], photoperiods only influence steroidogenesis in fish. Increasing GSI in post spawning periods caused by Estradiol [6]. Normally photoperiods in tropical area was 14L:10D. Introducing an extreme photoperiods without adaptation caused stress on fish. Hard-lipped barb needed 2 month for every reproduction cycles, hence in every photoperiods studies, the first cycles was not significantly different between the treated groups [14, 23].

This study showed that melatonin level decreased every month contrary to expression cGnRH-II, Serum Estradiol levels and GSI level increased but not for gene expression sGnRH. GSI increased in line with Serum Estradiol 17β Levels and decreased of melatonin. The same pattern was seen in cGnRH-II and sGnRH. The cGnRH-II decreased in line with serum melatonin and increased in line with serum estradiol. There is no correlation between sGnRH and melatonin and sGnRH with estradiol. This result indicated that the melatonin can suppress gene expression cGnRH-II and alter to suppress estradiol, but not for gene expression of sGnRH. Melatonin mediated cyclical regulation of GnRH mRNA expression involve the protein kinase C and the extracellular signal-regulated kinase 1 and 2 pathways [24]. 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 [25]. GnRH binds to GnRH receptor and active G protein mediated phosphorilation to protein kinase C and shyntezis Gonadotrophin (GtH 1 and GtH2). GtH 2 secreted into blood vessel, to reseptor in theca cell actived G protein and adenilat cyclase to phosphorilation cAMP and activation staR protein. staR protein regulated cholesterol

into inner membram mitochondria and actived enzyme cleaved chain side changed cholesterol to pregnenolon. Androgen hormone from theca cell had aromatisase enzym in granulosa cell and transform to estrogen [26].

Oocyte development resulted in an increase in ovarian size and weight as well as the production of reproduction hormone by the ovarium [6]. In a particular condition, GSI might not altered by photoperiod but the Estradiol level was significantly affected as those in Catla catla [22] and Tilapia [14].

Previous studies in teleost showed that extended photoperiod decreased melatonin and increased gene expression cGnRH-II, gene expression of sGnRH and estradiol level. These increased was resulted from the decreased of melatonin production lead to the increased of GnRH production from hypothalamus [7, 23]. Such condition will stimulate pituitary activities in producing gonadotrophin which in turn stimulate gonadal activities including estradiol production [9]. In the contrary, short photoperiod stimulated melatonin production and suppress the hypothalamus-pituitary-gonad axis [9]. Therefore increasing photoperiod should in theory increase the reproductive activities [5].

CONCLUSION

Photoperiod affected regulation of melatonin level, gene expression of cGnRH-II, sGnRH and estradiol level in the hard-lipped barb. The longer photoperiod decreased melatonin level, increased gene expression of cGnRH-II, but not for sGnRH and increased estradiol level.

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