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Effects of GnRHa on Plasma Sex Steroid Hormones of River Catfish *Hemibagrus nemurus* (Valenciennes 1840)

(Kesan GnRHa Terhadap Hormon Seks Steroid Plasma Ikan Baung *Hemibagrus nemurus* (Valenciennes 1840))

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ABSTRACT

The effect of gonadotropin releasing hormone analogue (GnRHa) on plasma sex steroid hormones of river catfish Hemibagrus nemurus *was studied. Enzyme linked immunosorbent assay (ELISA) was used to measure the hormones. The fish were treated with saline (control) and GnRHa at various doses of 5 µg/kg, 20 µg/kg and 50 µg/kg body weight (BW) of fish. Blood samples were collected at 0, 6, 12 and 24 h post hormone administration. The results showed that GnRHa elevated the plasma sex steroid hormones even at a low dose of 5 µg/kg. Significant increase (*p< *0.05) in plasma sex steroid levels were observed with 20 µg/kg and 50 µg/kg BW GnRHa treatments and a dose of 5 µg/kg BW GnRHa produced a slow response to steroidogenesis. Treatment with 50 µg/kg BW GnRHa produced the best result. The highest level of testosterone, 11-ketotestosterone and 17β-estradiol were observed at 24 h for all treatments. The results indicated that GnRHa increased steroid production in the plasma of* H. nemurus. *Therefore, it can be used as an inducing agent for the control of reproduction in* H. nemurus.

Keywords: Gonadotropin releasing hormone analogue (GnRHa); Hemibagrus nemurus; *testosterone; 11-ketotestosterone; 17β-estradiol*

ABSTRAK

Kesan analog hormon lepasan gonadotropin (GnRHa) terhadap hormon seks steroid plasma ikan baung Hemibagrus nemurus *telah dikaji. Enzim berkaitan imunosorben asei (ELISA) telah digunakan untuk mengukur hormon. Ikan telah dirawat dengan salina (kawalan) dan GnRHa pada pelbagai dos 5 µg/kg, 20 µg/kg dan 50 µg/kg berat badan (BW) ikan. Sampel darah telah dikumpul pada 0, 6, 12 dan 24 jam selepas suntikan hormon. Keputusan menunjukkan bahawa GnRHa meningkatkan hormon seks steroid plasma walaupun pada dos yang rendah, 5 µg/kg. Peningkatan yang signifikan (*p< *0.05) dalam tahap seks steroid plasma telah diperhatikan pada rawatan 20 µg/kg dan 50 µg/kg BW GnRHa sementara dos 5 µg/kg BW GnRHa menghasilkan tindak balas yang perlahan untuk steroidogenesis. Rawatan GnRHa pada dos 50 µg/kg BW menunjukkan hasil yang terbaik. Tahap tertinggi testosteron, 11-ketotestosterone dan-estradiol 17β telah diperhatikan pada 24 jam untuk semua rawatan. Keputusan menunjukkan bahawa GnRHa meningkatkan pengeluaran steroid dalam plasma* H. nemurus. *Oleh itu, ia boleh digunakan sebagai ejen pendorong untuk kawalan pembiakan* H. nemurus.

Kata kunci: Analog hormon lepasan gonadotropin (GnRHa); Hemibagrus nemurus*; testosteron; 11-ketotestosterone; 17β-estradiol*

INTRODUCTION

Reproduction in fish is controlled by several factors, which include sex steroids. Testosterone, 11-ketotestosterone and 17β-estradiol are involved in the regulation of reproductive processes in fish (Kime 1993). The endocrine mechanism of control of reproduction in fish is through the brain-pituitary-gonadal axis. The brain is stimulated by environmental cues like flooding, water temperature, feeding, rainfall and photoperiod to release gonadotropin releasing hormone (Rottman et al. 1991a; Zohar et al. 2010).

Gonadotropin releasing hormone is produced by the hypothalamus which functions in fish maturation and gonadal development (Amano et al. 1997; Ando & Urano 2005; Zohar et al. 2010). The gonads are influenced by gonadotropins released from the pituitary and sex steroids are produced through steroidogenesis in the ovaries and testes (Okuzawa 2002). Ovulation and spermiation are effected as a result of the sex steroids that have been produced. In order to control the amount of hormones that is released into the system, there are positive and negative feedback mechanisms for hormone regulation in fish (Rottman et al. 1991a; Zohar et al. 2010).

Hatchery-bred fish do not reproduce spontaneously, unless they are induced to reproduce (Rottman et al. 1991b). This could be achieved by environmental simulation and hormonal control methods. The reason for lack of reproduction in hatchery-bred fish had been attributed to failure of the pituitary to release gonadotropins which will result in gonadal maturation in fish (Zohar & Mylonas 2001).

Plasma sex steroid hormonal levels normally increase after hormone treatment within few hours to days. Increase in sex steroid hormonal levels is dependent on the initial dose of treatment, the variant of gonadotropin releasing hormone used, water temperature and type of fish species (Harmin & Crim 1993; Mylonas & Zohar 2001; Taufek et al. 2009).

Administration of gonadotropin releasing hormone analogue has been reported to increase levels of plasma sex steroids like testosterone, 11-ketotestosterone and 17β-estradiol in fish (Harmin & Crim 1993; Meng-Umphan et al. 2006; Prat et al. 2001; Semenkova et al. 2002; Zhuo et al. 2011).

The Asian redtail catfish, *Hemibagrus nemurus* is a river catfish that is economically important for commercial fisheries and aquaculture in most Asian countries like Malaysia, Thailand, Vietnam, Indonesia and Cambodia. It belongs to the family of Bagridae. It is valued for food and is high in protein and omega 3- polyunsaturated fatty acid (Mesomya et al. 2002; Rainboth 1996).

It is necessary to study the effect of gonadotropin releasing hormone analogue on plasma sex steroid levels of the Asian redtail catfish *H. nemurus*, so that we are able to observe the changes in the levels of sex steroids and know the timing of spawning in this fish. This paper reports the findings of research conducted on the effect of gonadotropin releasing hormone analogue on plasma sex steroid levels of *H. nemurus*.

Materials and Methods

EXPERIMENTAL FISH AND DESIGN

Fifty-six mature male and female of *H. nemurus* were obtained from stocks kept in the broodstock tank for this experiment. The body weight ranged from 400 to 650 g and body length measured from 28.5 to 38.0 cm (standard length). All fishes were anaesthetized with clove oil (0.1 mL/L) before handling. Mature males and females were identified by the genital papillae. Males had elongated genital papillae with reddish tip and released milt upon pressing the abdomen, while the females had round genital papillae and were identified by ovarian biopsy. The fishes were divided into 4 groups. The first group was injected with 0.5 mL of 0.7% saline solution and was used as control. The second group received 5 μg/kg BW GnRHa hormone treatment, the third group was injected with 20 μg/kg BW GnRHa and the fourth group received 50 μg/kg BW GnRHa hormone treatment. Each group comprised of 14 fish (7 females and 7 males). The fishes were tagged with plastic tags of different colours for identification based on treatment groups.

MORPHOMETRIC MEASUREMENTS

The body weights of fish were measured using a spring balance and the body lengths (standard and total lengths) were measured using a one-meter measuring board. All measurements were recorded.

HORMONE PREPARATION AND ADMINISTRATION

Hormone (D-Ala^6-Pro^9-NET)-LHRH (GnRHa) was purchased from Syndel laboratories, Canada. GnRHa (1 mg) was dissolved in 10 mL of 0.7% NaCl solution. The solution was divided into 10 aliquots and each aliquot was stored in 1 mL glass vial. (1 mL=100 μg GnRHa). The hormone was stored in freezer at -20°C until used. Hormones were administered by intramuscular injections below the dorsal fin of the fish. Both male and female fishes were injected with different doses of GnRHa (5 μg/kg BW, 20 μg/kg BW and 50 μg/kg BW). The different hormone concentrations of GnRHa used were calculated (Rottman et al. 1991b).

BLOOD SAMPLING PROCEDURE

Blood samples were collected from fish at different time intervals (0, 6, 12 and 24 h) before and after hormone administration. Blood (1 mL) was collected from the caudal vasculature using heparinised needle. The samples were stored in eppendorf tubes and kept in ice until centrifugation to separate the blood serum from the plasma. Centrifugation was done at 6000 rpm for 2 min. Aliquots of 100 μL were transferred into 0.5 mL eppendorf tubes and samples were stored in the freezer at -80°C until assay was performed.

SEX STEROID HORMONE MEASUREMENTS

The sex steroid hormones were analysed using enzyme linked immunosorbent assay. (ELISA). This was done by using commercially available enzyme linked immunosorbent assay kits from Cayman Chemical Company, USA. The sex steroids were determined following the assay kit procedures and methods described by Cuisset et al. (1994) and Nash et al. (2000). Testosterone, 11-ketotestosterone and 17β-estradiol were extracted from the plasma using diethyl ether, ethyl acetate/ hexane (50:50) and methylene chloride, respectively. The organic extracts were evaporated to dryness using nitrogen stream gas. The resulting pellets were dissolved in 0.5 mL ELISA buffer. Testosterone, 11-ketotestosterone and 17β-estradiol assay standards were prepared. The plate was set-up; samples and standard absorbance were read at 412 nm wavelength using a microplate reader (Spectramax 190, Beckman Coulter, Canada). The values of absorbance were then analysed by using Softmax Pro Software (Molecular device). Assay precision is shown in Table 1.

STATISTICAL ANALYSIS

All data analyses were carried out using a computer Statistical Analysis Software (SAS 9.2). The data were expressed as mean ± standard error of mean. The one way analysis of variance (ANOVA) followed by Duncan's new multiple range test (DMRT) was used for the variation in treatment means. The two-way analysis of variance (ANOVA) was used for variation due to treatment and time and $p< 0.05$ was selected as the level of significance.

TABLE 1. Enzyme linked immunosorbent assay precision

	Sex			
	Male *H. nemurus*		Female *H. nemurus*	
Hormones	T	11-KT	T	E2
Inter-assay coefficient of variation	7.55	8.50	6.50	7.50
Intra-assay coefficient of variation	8.00	8.13	7.30	6.70

E2 – 17ß estradiol, T – Testosterone, 11-KT- 11-ketotestosterone

RESULTS AND DISCUSSION

CHANGES IN MALE SEX STEROID LEVELS

The results revealed that before hormone administration (i.e. at 0 h) the levels of testosterone in all the treatment groups were not significantly different ($p > 0.05$) and the levels were 0.019 ± 0.006 ng/mL, 0.021 ± 0.009 ng/mL, 0.022 ± 0.003 ng/mL and 0.022 ± 0.002 ng/mL for control, 5 µg/kg BW GnRHa, 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa treatment groups, respectively.

After 6 h of hormone administration, there was a rise in the levels of testosterone. The 5 µg/kg BW GnRHa treatment group had testosterone level increased to 0.040 ± 0.011 ng/mL. In 20 µg/kg BW GnRHa treatment group, testosterone rose to 0.100 ± 0.021 ng/mL and 50 µg/kg BW GnRHa had an increase in testosterone as well, with a value of 0.078 ± 0.029 ng/mL.

A significant increase in testosterone levels was observed at 12 h post hormone administration in the 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa treatment groups. Levels were higher than at 6 h. Peak testosterone levels were observed at 24 h for all the treatment groups. Figure 1 shows the changes in testosterone levels in male *H. nemurus*.

The 11-ketotestosterone also had a similar trend. Gonadotropin releasing hormone induction led to increase in the 11-ketotestosterone levels. For treatment with 5 µg/kg BW GnRHa, the 11-ketotestosterone increased from 0.023 ± 0.009 ng/mL at 0 h to 0.026 ± 0.003 ng/mL after 6 h, then to 0.037± 0.003 ng/mL at 12 h and 0.049 ± 0.002 ng/mL at 24 h. Figure 2 shows the changes in 11-ketotestosterone levels in male *H. nemurus*.

CHANGES IN FEMALE SEX STEROID LEVELS

In females, the results showed that gonadotropin releasing hormone analogue (GnRHa) raised the plasma levels of testosterone and 17β-estradiol after 6, 12 and 24 h post injection. At 0 h, there was no significant difference ($p > 0.05$) in the testosterone levels in all the treatment groups. Six h after injection, the testosterone levels in female fish

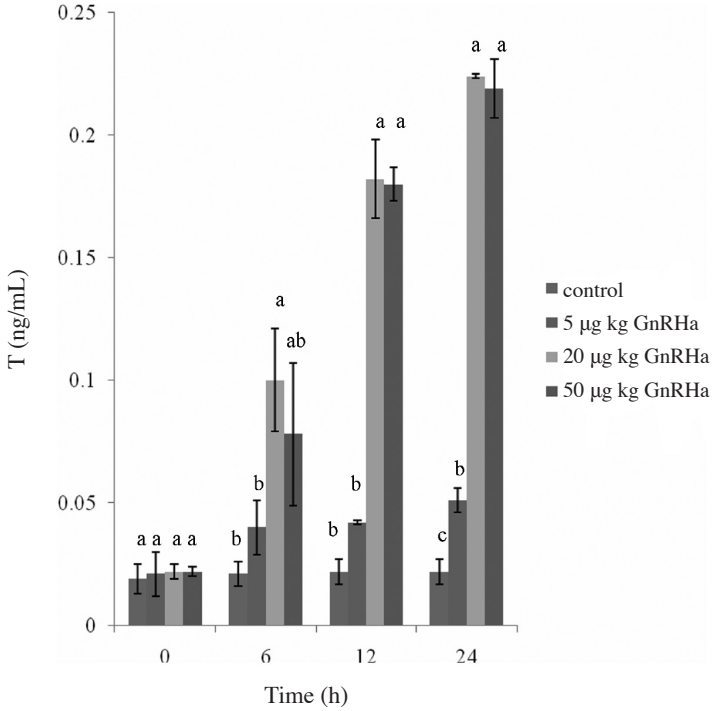

FIGURE 1. Changes in male testosterone levels (Bars with the same letters within groups are not significantly different $p > 0.05$)

treated with 5 µg/kg BW GnRHa, 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa increased. An increase from 0.022 ± 0.002 ng/mL to 0.033 ± 0.010 ng/mL was recorded for 5 µg/kg BW GnRHa treatment group, while 20 µg/kg BW GnRHa group had the testosterone levels increased from 0.024 ± 0.009 ng/mL to 0.060 ± 0.004 ng/mL and 50 µg/kg BW GnRHa treatment group had a rise from 0.023 ± 0.003 ng/mL to 0.068 ± 0.009 ng/mL.

At 12 h, the levels increased further and a peak in testosterone level was observed at 24 h post hormone administration. There was no significant difference (p> 0.05) between the testosterone levels at 0, 6, 12 and 24 h for fish treated with 20 µg/kg and 50 µg/kg BW GnRHa. Figure 3 shows the changes in testosterone levels in female *H. nemurus* after hormone administration.

It was observed that the levels of 17β-estradiol also increased with time, after administration of gonadotropin releasing hormone analogue (GnRHa). This increase was due to the effect of gonadotropin releasing hormone in the endocrine system. Initially, the levels of 17β-estradiol were not significantly different (p> 0.05) among all the treatments. After 6 h, there was an increase in the level of 17β-estradiol in all treatment groups and a significant difference (p< 0.05) was observed in the treatments. Figure 4 shows the changes in plasma 17β-estradiol in female *H. nemurus* after hormone administration.

In this study, there was a slow steroid response when *H. nemurus* was treated with a low dose of GnRHa (5 µg/kg BW GnRHa) based on a slight increase in 11-ketotestosterone levels over the study period at different time intervals. It was observed that 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa treatment groups had elevated levels of 11-ketotestosterone over time.

Semenkova et al. (2002) reported that testosterone levels increased after a few hours of GnRHa administration in male stellate sturgeon *Acipenser stellatus*. A similar result was observed in this study. The levels of testosterone increased after few hours of hormonal treatment. Peak testosterone levels were observed at 24 h for all the treatment groups. Peak value of testosterone was also obtained at 24 h in winter flounder *Pseudopleuronectes americanus* (Harmin & Crim 1993). GnRHa at a dose of 50 µg/kg BW GnRHa was able to elevate plasma testosterone levels in common dentex (*Dentex dentex*) (Greenwood et al. 2001). Testosterone showed significant increase in Indian catfish *Heteropneustes fossilis* at 2, 4, 8, and 12 h and the peak was observed at 8 h (Joy et al. 1998). GnRHa also raised plasma testosterone levels after hormone administration in the Mekong giant catfish (*Pangasianodon gigas*) (Meng-Umphan et al. 2006).

Semenkova et al. (2002) also reported that 11-ketotestosterone increased few hours after GnRHa

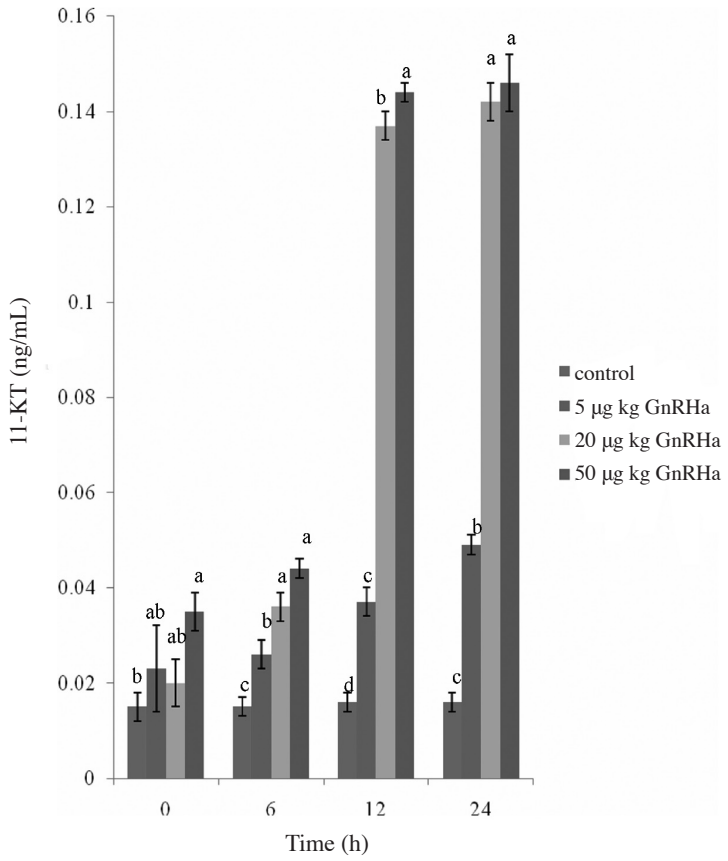

FIGURE 2. Changes in male 11-ketotestosterone levels
(Bars with the same letters within groups are not significantly different p> 0.05)

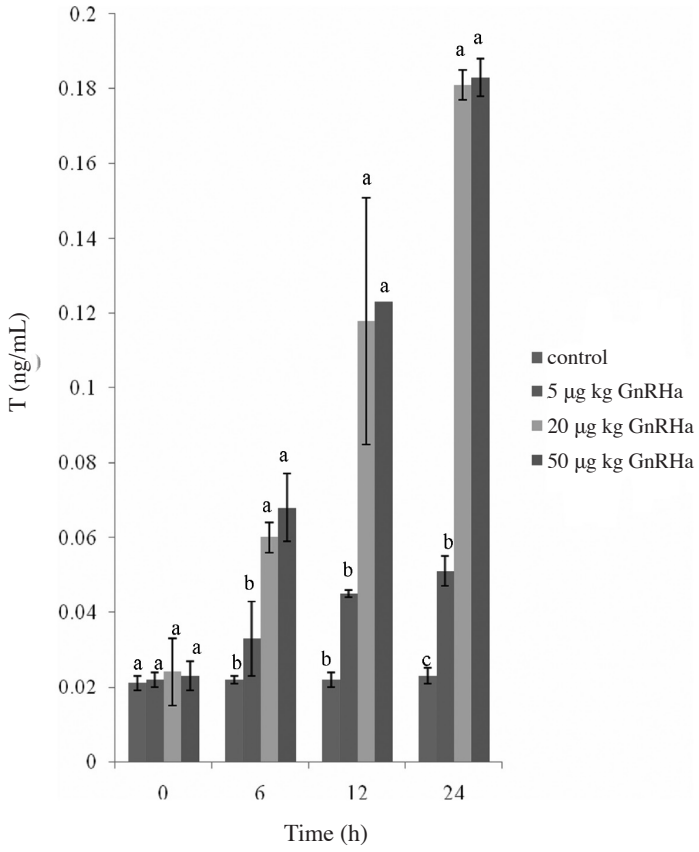

FIGURE 3. Changes in female testosterone levels
(Bars with the same letters within groups are not significantly different $p > 0.05$)

administration in male stellate sturgeon *Acipenser stellatus*. Similarly as obtained in this study, GnRHa at a dose of 50 µg/kg BW GnRHa was able to elevate plasma 11-ketotestosterone levels in common dentex (*Dentex dentex*) (Greenwood et al. 2001). Peak levels of 11-ketotestosterone were observed at 24 h post injection for 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa treatment groups. Peak value of 11-ketotestosterone was also obtained at 24 h in winter flounder *Pseudopleuronectes americanus* (Harmin & Crim 1993). There was a significant difference in the plasma 11-ketotestosterone levels in 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa treatment groups at 12 h in this study.

The use of gonadotropin releasing hormone analogue is effective in raising the plasma sex steroid hormone levels in fish. In this study, doses of 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa were very effective in stimulating steroidogenesis in *H. nemurus*. The levels of testosterone and 11-ketotestosterone were low when treated with 5 µg/kg BW GnRHa compared with high levels obtained when treated with 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa which suggested a delay in steroidogenic response when treated with 5 µg/kg BW GnRHa. Similarly, Harmin and Crim (1993) observed a delay in steroidogenic response in the male winter flounder *Pseudopleuronectes americanus* when treated with a low dose of GnRHa (2 µg/kg BW GnRHa).

The results of this study revealed that response of *H. nemurus* to GnRHa treatment and steroid production was dose dependent. High doses of 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa resulted in quick response compared with the low dose of 5 µg/kg BW GnRHa. A similar result was reported for winter flounder *Pseudopleuronectes americanus* (Harmin & Crim 1993). Fish treated with higher doses of GnRHa at 20 µg/kg BW GnRHa and 200 µg/kg BW GnRHa responded faster compared with treatment with lower doses of 2 µg/kg BW GnRHa (Harmin & Crim 1993). GnRHa was successfully used to speed up testicular maturation in male yellow catfish (*Pelteobagrus fluvidraco*) in captivity (Zhuo et al. 2011).

Apart from mammalian GnRHa (mGnRHa), variants of GnRH like catfish GnRH (cfGnRH) and chicken GnRH-II (cGnRH-II) have been reported to influence plasma sex steroid levels. In African catfish *Clarias gariepinus*, cGnRH-II and cfGnRH were effective in elevating androgen (testosterone and 11-ketotestosterone) levels in the plasma. However, significant increase in plasma androgen was recorded after 24 weeks for 11-ketotestosterone using cGnRH-II and 39 weeks using cfGnRH. When treated with cGnRH-II, testosterone increased significantly over time and no increase was observed using cfGnRH. Generally, increase in androgen plasma were not observed until after three to four weeks; which was probably due to restricted luteinizing hormone responsiveness in the testis at the

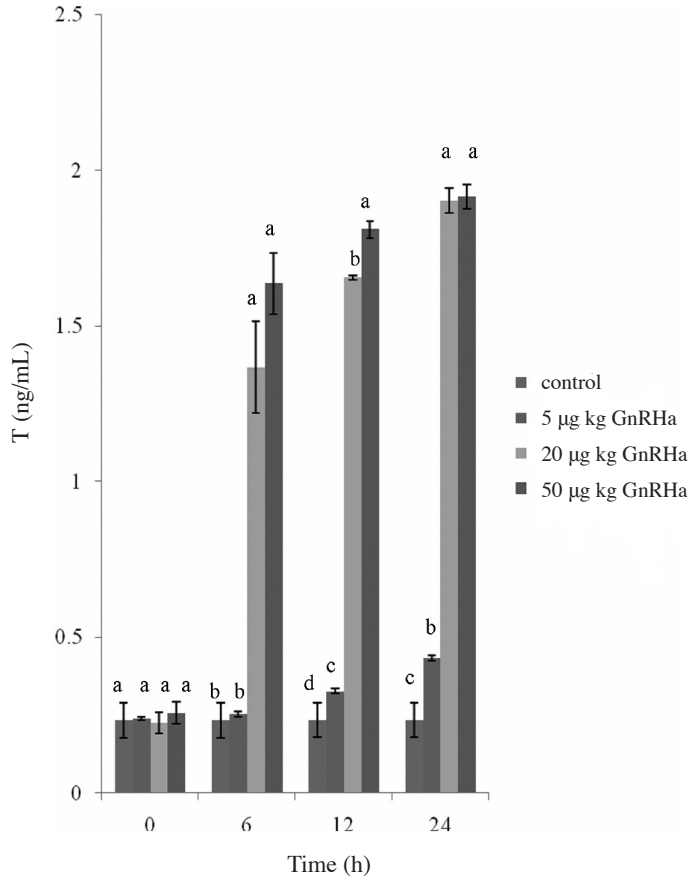

FIGURE 4. Changes in female 17β-estradiol levels
(Bars with the same letters within groups are not significantly different $p > 0.05$)

onset of spermatogenesis which limited further pubertal development of the catfishes (Schulz et al. 1994).

Similarly as observed in this study, the female sea bass had testosterone levels elevated when treated with GnRHa (Prat et al. 2001). Gonadotropin releasing hormone analogue in combination with pimozide treatment elevated testosterone levels in *Clarias gariepinus* with a maximum level of 4 h and declined at 8 h. Levels at 12, 16 and 24 h were lower than the initial level at 0 h (Richter et al. 1987).

An increase in 17β-estradiol levels was also reported in the stellate sturgeon *Acipenser stellatus* few hours after GnRHa treatment (Semenkova et al. 2002). GnRHa also raised 17β-estradiol levels after hormone administration in the Mekong giant catfish (*Pangasianodon gigas*) (Meng-Umphan et al. 2006). Also, GnRHa at a dose of 50 µg/kg BW GnRHa was able to elevate plasma 17β-estradiol levels in common dentex (*Dentex dentex*) and *Engraulis ringens* at 0, 12 and 24 h (Espinoza et al. 2010; Greenwood et al. 2001). In this study on *H. nemurus*, 20 µg/kg and 50 µg/kg BW GnRHa treatment groups resulted in very high values of 17β-estradiol compared with 5 µg/kg BW GnRHa and control groups. At 12 h, the levels of 17β-estradiol was further increased and were 0.326 ± 0.008 ng/mL, 1.656 ± 0.008 ng/mL and 1.810 ± 0.026 ng/mL for 5 µg/kg BW GnRHa, 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa treatment groups, respectively. The highest value of 17β-estradiol was obtained at 24 h after injection. It was observed that *H. nemurus* responded faster to gonadotropin releasing hormones. Steroid production was more with higher doses of gonadotropin releasing hormone analogue (i.e. 20 µg/kg BW GnRHa and 50 µg/kg BW GnRHa) compared with a low dose (i.e. 5 µg/kg BW GnRHa). 17β-estradiol decreased significantly ($p < 0.05$) at 2, 4, 8 and 12 h in Indian catfish, *Heteropneustes fossilis* the 1owest value was recorded at 8 h, then it increased at 16 and 18 h. 0.15 µg/g BW GnRHa induced ovulation at 16 h (Joy et al. 1998). In Asian catfish, *Clarias batrachus* and *Heteropneustes fossilis*, ovarian 17β-estradiol decreased significantly ($p < 0.05$) at 8 h. This decrease was probably due to stimulation of catecholoestrogens synthesis and degradation of 17β-estradiol during GnRHa induced spawning (Senthilkumaran & Joy 2001). In the sea bass *Dicentrarchus labrax,* 17β-estradiol levels were elevated when treated with GnRHa (Prat et al. 2001). 17β-estradiol in African catfish, *Clarias gariepinus* increased at 4 and 8 h and then declined at 12 and 16 h, it later increased, resulting in the highest value at 24 h (Richter et al. 1987).

Based on the results obtained from this study, it is evident that gonadotropin releasing hormone analogue increased the plasma sex steroid hormone levels in male and female *H. nemurus*. It appeared that the effect of gonadotropin

releasing hormone analogue is dose dependent. High dose of gonadotropin releasing hormone analogue leads to fast steroidogenic response while low dose resulted in a delay of steroid production. This is because; a low dose of 5 μg/kg BW GnRHa did not produce a significant ($p > 0.05$) increase in levels of plasma sex steroid hormone. The fact that sex steroid levels were increased after treatment even at a low dose of 5 μg/kg BW GnRHa showed that gonadotropin releasing hormone analogue is effective for stimulating steroid surge in the plasma. It is better to wait for 12 h or more after injection in order to get a good response to hormonal treatment during induced spawning.

CONCLUSION

Gonadotropin releasing hormone analogue will likely bring about the completion of final oocyte maturation, ovulation and spawning and spermiation in *H. nemurus* by elevating the plasma sex steroid hormone levels. Therefore, this hormone can be used for spawning induction in *H. nemurus* in order to control reproduction and quick steroidogenic response would be obtained at high doses of 20 μg/kg BW GnRHa and 50 μg/kg BW GnRHa. For economic reasons, 20 μg/kg BW GnRHa is recommended, since there was no significant difference between the results obtained with 20 μg/kg BW GnRHa and 50 μg/kg BW GnRHa.

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