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Full Length Research Paper

Effect of chicken gonadotropin-releasing hormone (cGnRH-II) on plasma steroid hormone, maturation and ovulation in African catfish, *Clarias gariepinus* (Burchell)

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Nowadays, many studies on the effects of native GnRHs and various agonists have been evaluated on the spawning of African catfish, *Clarias gariepinus*. For the current study, three experiments have been conducted to determine the effectiveness of chicken gonadotropin-releasing hormone II (cGnRH-II) in stimulation of maturation and ovulation of African catfish. The first experiment concerned the graded dosage treatment; the second experiment was designed specifically to determine the response of dopamine inhibitor to accelerate the maturation process. Finally the third experiment was carried out to compare the effectiveness of cGnRH-II against leutinising hormone releasing-hormone analogue (LHRHa) and salmon gonadotropin-releasing hormone analogue (sGnRHa) in inducing maturation and ovulation. Plasma steroid levels (17ß-estradiol (E2) and testosterone) were determined and germinal vesicle (GV) was observed prior to hormone administration at (0), 6, 12 and 24 h post injection. From the first experiment, cGnRH-II 200 µg/kg was found to be effective to induce ovulation in African catfish. 100% ovulation was also observed for the fish treated with cGnRH-II 200 µg/kg with the combination of pimozide based on the second experiment. Finally in the third experiment, sGnRHa was proven to be more efficient compared to cGnRH-II, only 20 µg/kg was administered intramuscularly in single injection. For the group that showed ovulation, that is cGnRH 200 µg/kg alone and with the combination of pimozide and also sGnRHa, plasma level of E2 and T increased significantly with the association of germinal vesicle migration and ultimately ovulation. The concentration reached the peaked after 12 h, then decreased dramatically at 24 h post treatment. In contrast to the non-ovulated group that is cGnRH-II 2 and 20 µg/kg, the plasma level showed small increased after the injection and throughout the experiment. The present study indicates that administration of cGnRH-II is effective for sexual maturation and ovulation in African catfish.

Key words: Chicken gonadotropin releasing-hormone II (cGnRH-II), African catfish, GnRH, ovulation, plasma steroid hormone level.

INTRODUCTION

Gonadotropin hormone releasing hormone (GnRH) is a peptide hormone that is responsible for stimulating the release of gonadotropins from pituitary and consequently influence the steroid hormones production level in the ovary (Peter and Yu, 1997). Thus, administration of GnRH particularly the superactive GnRH analogues (GnRHa), has often been used to induce ovulation and spawning in several species of fish.

Various hormones preparations have been used on African catfish, *Clarias gariepinus*, to stimulate maturation and ovulation including mammalian Lutenizing Hormone Releasing Hormone Analogue (LHRHa), a commercial preparation of GnRH such as ovaprim (De Leeuw et al., 1985a, b), Carp pituitary extract (CPE) and Human Chorionic Gonadotropin (HCG). While most commonly

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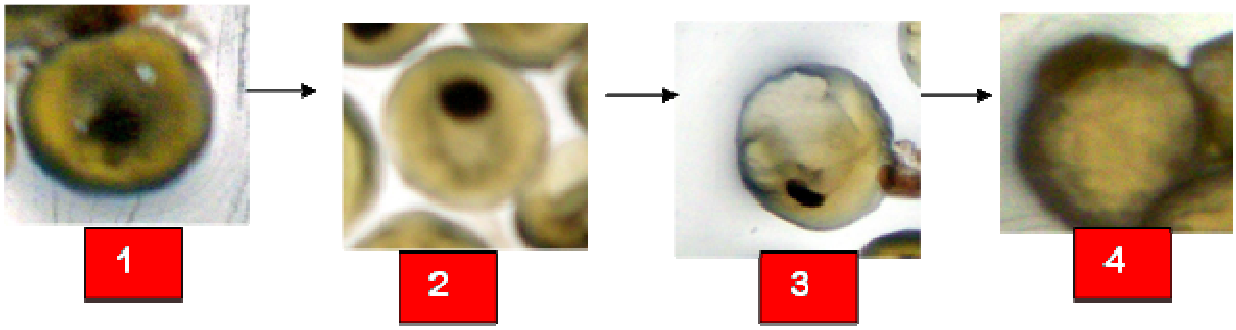


Figure 1. The GV stage.

used GnRHs are originally from mammalian was used for manipulation of the reproductive cycle of fish, still there appears to be many other forms of GnRH present in the fish species. Among other forms of GnRH are salmon GnRH (sGnRH), mammalian GnRH (mGnRH), catfish GnRH (cfGnRH), seabass GnRH (sbGnRH), chicken GnRH-II (cGnRH-II), herring GnRH (hrGnRH), medaka GnRH (mdGnRH) and pulsatile GnRH (pGnRH) have been identified in the brain of different teleosts (Montaner et al., 2001). Yamamoto et al. (1995) reported that cGn RH-II system were predominant in midbrain. Fish share two forms of GnRH namely (mGnRH) and (cGnRH-II) with other vertebrates, but nine variants have been identified in fish (Philippa and Sherwood, 2005).

One of the reasons for lack of ovulation in cultured fish is the failure of pituitary to release gonadotropin (GtH-II) to stimulate maturation and ovulation (Zohar and Mylonas, 2001). Dopamine inhibitor such as pimozide and combination with GnRHa has been commonly used to ensure the efficiency of the treatment in common carp (Lin et al., 1988) and other cyprinids (Peter et al., 1987). It induces a much higher lutenizing hormone release from the pituitary than using GnRHa alone.

The desired outcome of this respective work is to determine the effectiveness of cGnRH-II as an inducing agent for maturation and ovulation in African catfish, *C. gariepinus,* a commercially important food fish in Malaysia. To achieve this outcome, it requires the understanding of the potent dosage needed to bring ovulation. Following that, we determined the impact of cGnRH-II and pimozide alone or in combination and finally compare the effectiveness of cGnRH-II with other hypothalamic hormones such as sGnRHa and LHRHa. In addition, plasma sex steroid levels of 17ß-estradiol and testosterone were measured to obtain *in vivo* steroidogenic activity responses following hormone administration.

MATERIALS AND METHODS

Experimental fish and system

Sexually mature post-vitellogenic females with a body weight between 450-550 g were used in the experiments. Fish were acquired from commercial farmers and transported back to our aquaculture research centre in Puchong, Selangor. They were acclimitised with ambient water temperature of 27°C ± 1 and exposed to a normal photoperiod. The animals were fed with dry pellets containing 34% protein at 2.0% body weight (BW) twice a day.

Hormone administration

All hormones; cGnRH-II (Research Biolabs), sGnRHa and LHRHa (Syndel, Canada) were dissolved in saline (NaCl 0.7%) while Pimozide (PIM), (Sigma-Aldrich) were diluted in methyl sulfoxide and propylene glycol (Omeljaniuk et al., 1987). Each group consists of six sexually mature female African catfish. The experimental parameters observed were ovulation, hormonal changes following treatment and germinal vesicle (GV) migration. Females were given with single intramuscular injection .

Ovulatory response

Fishes were examined for GV position at the same time as blood sampling by exposing ovarian fragments to clearing solution (ethanol: formalin: acetic acid, 6:3:1 v/v) for about 1 min. About 30 - 40 oocytes from the ovaries were cleared of yolk to identify the GV stage. Fish with central GV were selected and tagged. A system to stage the position of the germinal vesicle (GV) under the microscope was as follow: (1) GV in the central position, (2) GV slightly off-centre, (3) GV migrated midway to animal pole, (4) germinal vesicle breakdown (GVBD) and ovulation (Harmin and Crim, 1992). Figure 1 describe the GV stages in clear detail.

In order to calculate the GV stage in every fish, the following calculation have to be used (for each fish, 2 sub samples were needed):

Fish 1 petri 1
Stage 1: 5 x 1 = 5
Stage 2: 12 x 2 = 24
Stage 3: 3 x 2 = 6
Stage 4: 0
Total eggs = 20
5 + 24 + 6 = 35
35/20 = 1.8
Mean GV stage: 1.9

Fish 1 petri 2
Stage 1: 3 x 1 = 3
Stage 2: 14 x 2 = 28
Stage 3: 4 x 3 = 12
Stage 4: 0

Total eggs = 21
3 + 28 + 12 = 43
43/21 = 2.0

Steroid hormone analysis

Blood samples were extracted from the caudal vein; the blood were centrifuged at 6000 rpm for 2 min and plasma were drawn off and stored at -20°C until used. Plasma steroid extracted from plasma samples were analysed for 17ß- estradiol and testosterone using a commercial kit of enzyme immunoassay (Cayman Chemical Company).

Egg quality determinations

After final oocyte maturation (FOM) has been detected, eggs were stripped from the ovulated fish. Free flowing eggs were then fertilised with sperms sacrificed from the males. The fertilized eggs were mixed together gently and divided into 100 - 200 eggs (0.1 g) per petri dish for 3 replicates. Fertilisation rate and hatching rate were observed and recorded after 3 and 24 h, respectively.

Experimental design

Three experiments were carried out where fish were separated into four groups in each experiment. The first experiment (Expt 1) concerned the effect of graded dosage of cGnRH-II. Three different concentrations have been given to the 3 groups (2, 20 or 200 µg/kg) and saline used as control. The second experiment (Expt 2) was conducted to determine the effect of pimozide in combination with cGnRH-II to accelerate the maturation and ovulation. The treatments involved in this experiment were 200 µg/kg cGnRH-II alone, 200 µg/kg cGnRH-II alone + 5 mg/kg pimozide, 5 mg/kg pimozide alone and saline Finally, the third experiment (Expt 3) determine the effectiveness of 20 µg/kg cGnRH-II when compared to LHRHa and sGnRHa with the same dosage. Ovulatory response, plasma steroids level and germinal vesicle (GV) stages were observed at 0 h (prior to hormone administration), 6, 12 and 24 h after injection.

Statistical analysis

Data were presented as a mean ± standard error of the mean (S.E.M). Differences between experimental and control groups in GV stages and plasma levels of steroids were determined by one-way analysis of variance (ANOVA), $p < 0.05$, followed by Duncan's multiple comparison test using SPSS.

RESULTS

Experiment 1

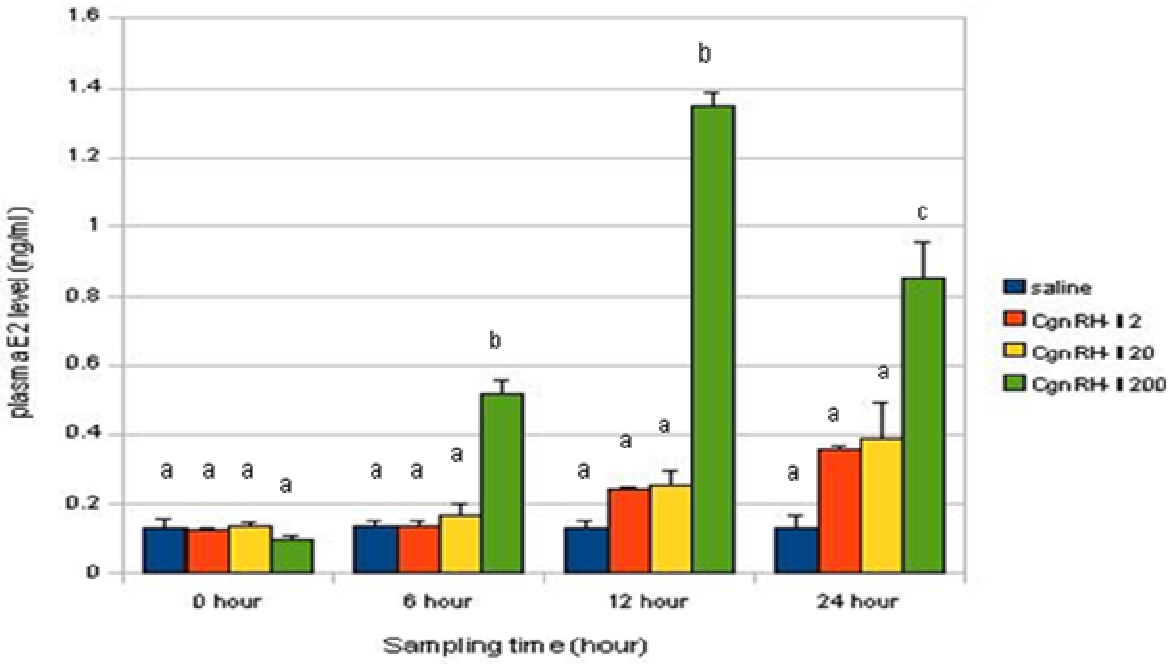
In the first experiment, 50% of the fish ovulated in the group treated with 200 µg/kg cGnRH-II. The fertilisation and hatching rate were 76 and 62%, respectively. Plasma concentration of E2 and T for the first experiment were shown in Figures 2a and 2b, respectively. Similar pattern of changes was observed in both hormone. Steroid level for saline injected fish remained the same throughout the experiment. Initially the plasma of steroid level for fish treated with 200 µg/kg cGnRH-II was low (E2: 0.14 ± 0.01 ng/ml and T: 0.04 ± 0.01 ng/ml respectively), the level increased after 6 h and reached the highest peak at 12 h with the level (E2: 1.35 ± 0.01 ng/ml and T: 0.068 ± 0.01 ng/ml). However the level decreased after 24 h (E2: 0.85 ± 0.04 ng/ml; and T: 0.07 ± 0.03 ng/ml) but the other treated groups still remained increasing until the end of the experiment.

The GV determination observed was shown in Figure 2c. Saline group remained at the 1st stage throughout the experiment while the other treated group showed significant changes ($P < 0.05$) in GV movement. Mean GV for 200 µg/kg cGnRH-II moved from stage 2.41 ± 0.21 at 6 h to stage 3.13 ± 0.25 at 12 h and continuously migrated to stage 3.20 ± 0.24 after 24 h. For the other treated group (2 µg/kg cGnRH-II and 20 µg/kg cGnRH-II), there are significant different ($P < 0.05$) from the saline group where GV moved from stage 2.10 ± 0.09 at 6 h to 2.6 ± 0.16 at 12 h and finally 2.29 ± 0.18 at 24 h for the group treated with 2 µg/kg cGnRH-II. The similar trend was observed in 20 µg/kg cGnRH-II but slightly higher in GV migration where they migrated from 2.37 ± 0.16 at 6 h to 2.83 ± 0.07 and the final stage at 2.96 ± 0.06.

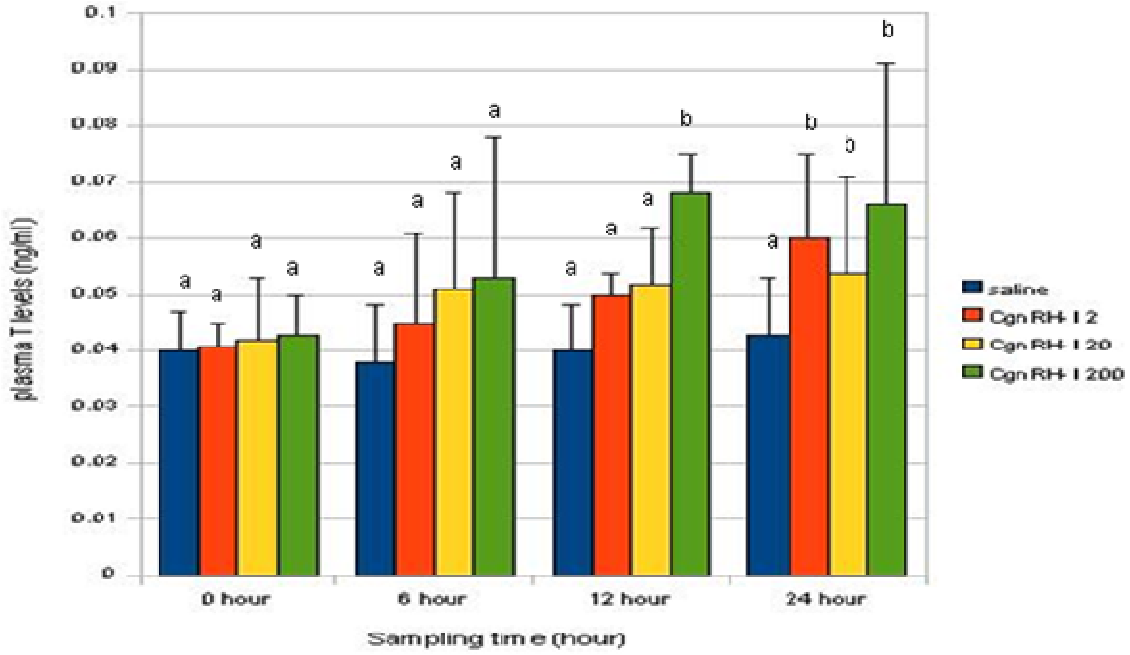
Experiment 2

In this experiment, fish receiving pimozide and saline did not show any ovulation; however, the ovulation rate for both treatment of cGnRH-II alone and cGnRH-II + pimozide showed equal result with 100% ovulation after 12 h. Fertilisation rate of both treatment are 73 and 80% for cGnRH-II alone and cGnRH-II + pimozide, respectively. In addition to that, the hatching rate constituted of 58% for cGnRH-II alone and 67% for cGnRH-II + pimozide. However, nonsignificant fluctuations of plasma steroid concentration were observed in the control group throughout the experiment (Figure 3). In contrast, significant higher concentrations of the steroid level of E2 and T were seen in group treated with cGnRH-II alone and cGnRH-II with combination of pimozide which reached the peak level at 12 h after injection and consequently decreasing after 24 h (Figure 3c) Plasma concentration of cGnRH-II alone was found to be higher than cGnRH-II + pimozide when it reached the peak at 12 h (E2: 1.77 ± 0.4 ng/ml; T: 0.36 ± 0.06 ng/ml) for cGnRH-II alone and (E2: 1.54 ± 0.22ng/ml; T: 0.29 ± 0.04 ng/ml) for cGnRH-II + pimozide. The level decreased to (E2: 0.85 ± 0.17 ng/ml; T: 0.04 ± 0.01 ng/ml) for cGnRH-II alone and (E2: 0.91 ± 0.19 ng/ml; T: 0.03 ± 0.02 ng/ml) for cGnRH-II + pimozide after 24 h.

For the group injected with saline, GV stages did not show any changes throughout the experiment but there are significant changes in GV stages where the GV migrated from stage 1.3 ± 0.05 and 1.4 ± 0.06 at 6 hrs to stage 4 after 12 h for both group of cGnRH-II alone and cGnRH-II + pimozide respectively. The pimozide treated



Figures 2a. Effect of graded dosage of cGnRH-II on plasma steroid level of 17ß-estradiol in African catfish. Bars represent mean ± SEM. Mean with different letters differs significantly ($P < 0.05$; One-way ANOVA followed by Duncan's multiple range test).



Figures 2b. Effect of graded dosage of cGnRH-II on plasma steroid level of testosterone in African catfish. Bars represent mean ± SEM. Mean with different letters differs significantly ($P < 0.05$; One-way ANOVA followed by Duncan's multiple range test).

group also demonstrated the GV migration from 1.5 ± 0.09 at 6 h to 1.6 ± 0.12 at 12 h and elevated to 1.8 ± 0.16 after 24 h.

Experiment 3

sGnRHa proved to be the best inducing agent among the

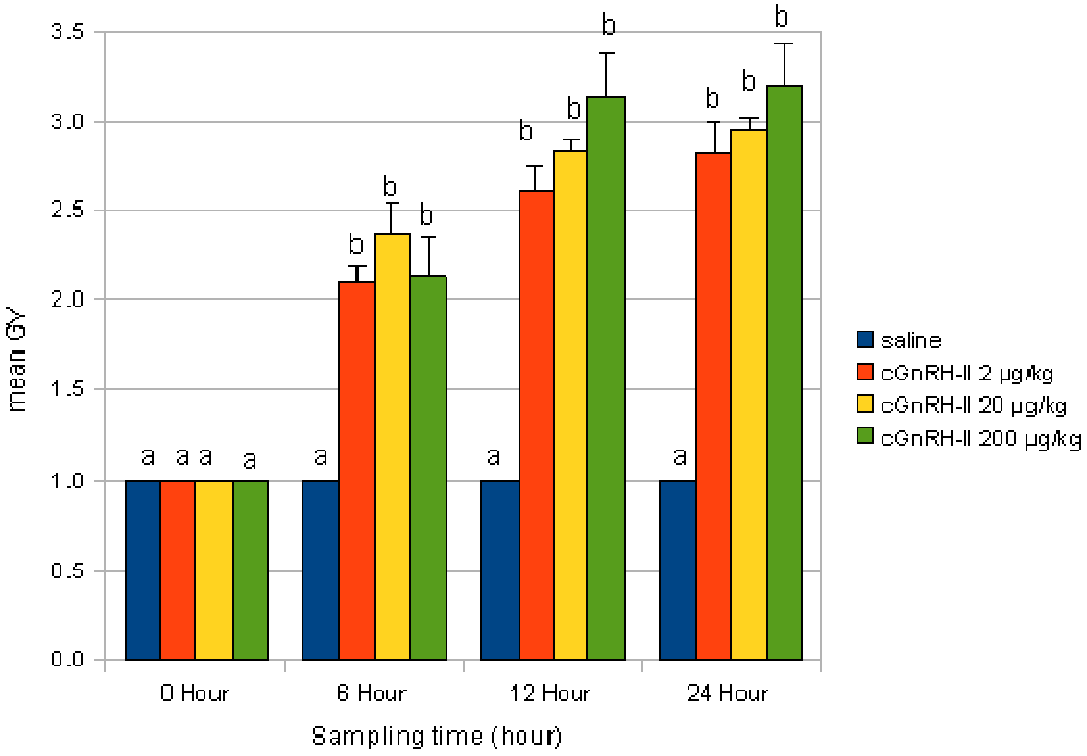
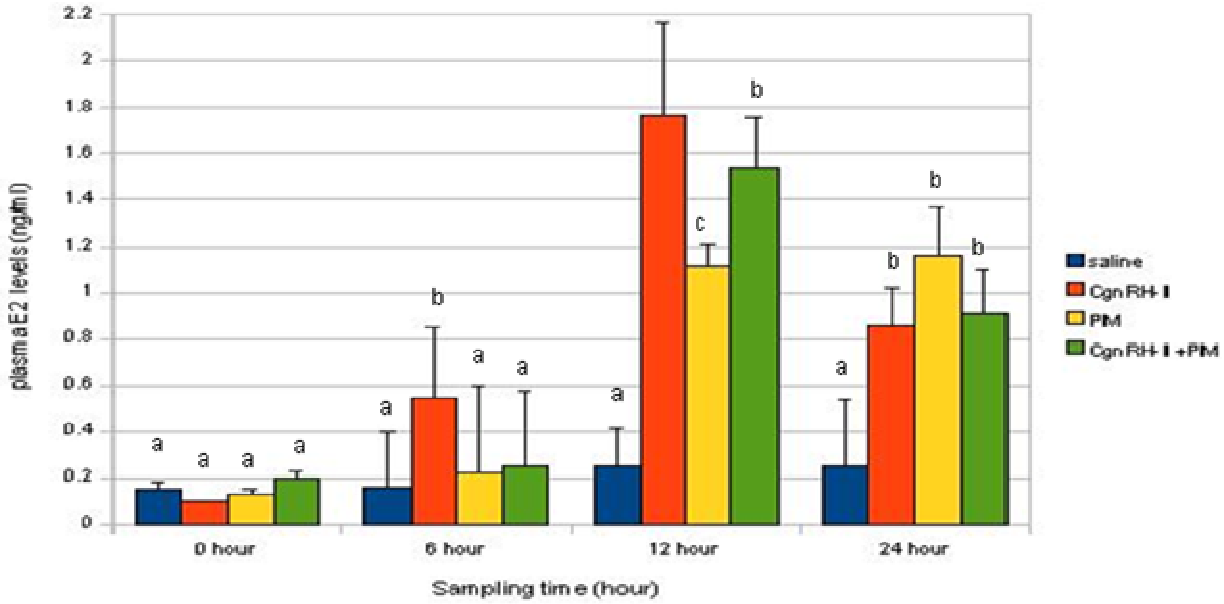
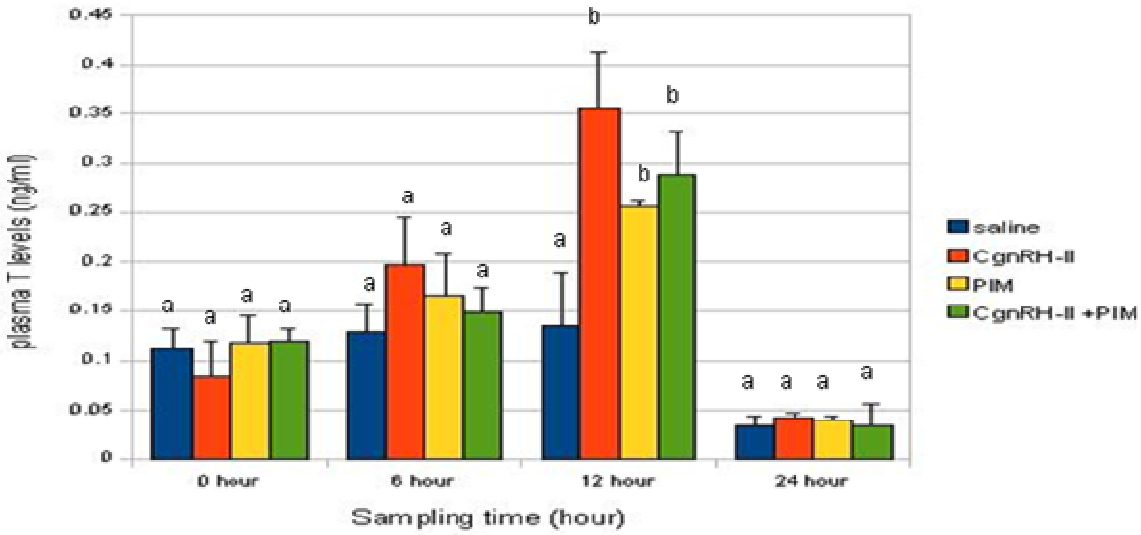


Figure 2c. Germinal vesicle (GV) determination on graded dosage of cGnRH-II in African catfish. Each value represents mean ± SEM. Mean with different letters differs significantly (P < 0.05; One-way ANOVA followed by Duncan's multiple range test).



Figures 3a. Effect of dopamine inhibitor on plasma steroid level of 17ß-estradiol in African catfish. Bars represent mean ± SEM. Mean with different letters differs significantly (P < 0.05; One-way ANOVA followed by Duncan's multiple range test)

other hormones. It was demonstrated by the only treatment that succesfully induce final oocyte maturation. Four out of six fish reached GVBD at 12 h after injection. Egg quality determination observed in this group were the



Figures 3b. Effect of dopamine inhibitor on plasma steroid level of testosterone in African catfish. Bars represent mean ± SEM. Mean with different letters differs significantly (P < 0.05; One-way ANOVA followed by Duncan's multiple range test) .

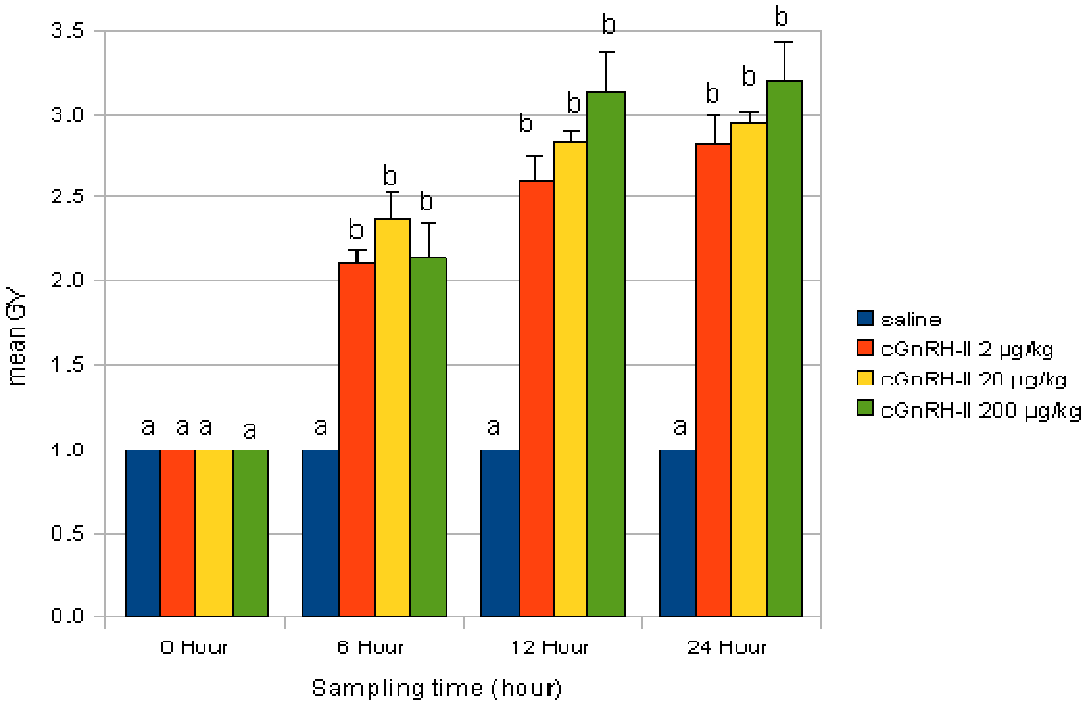
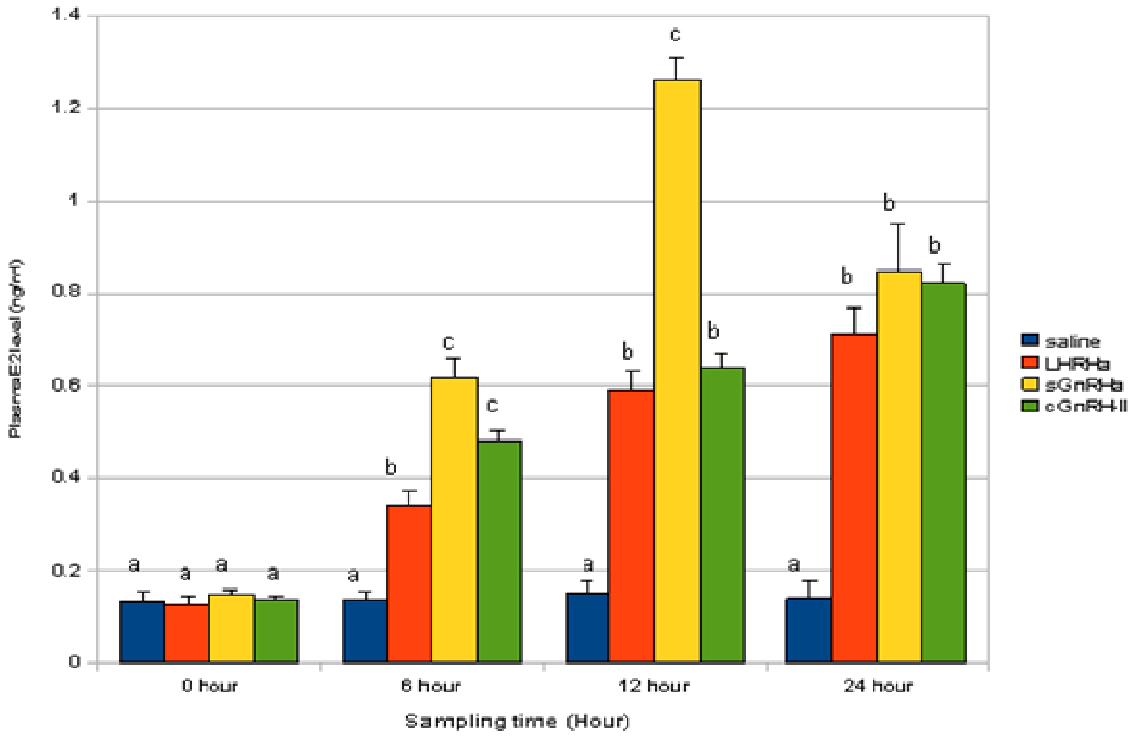


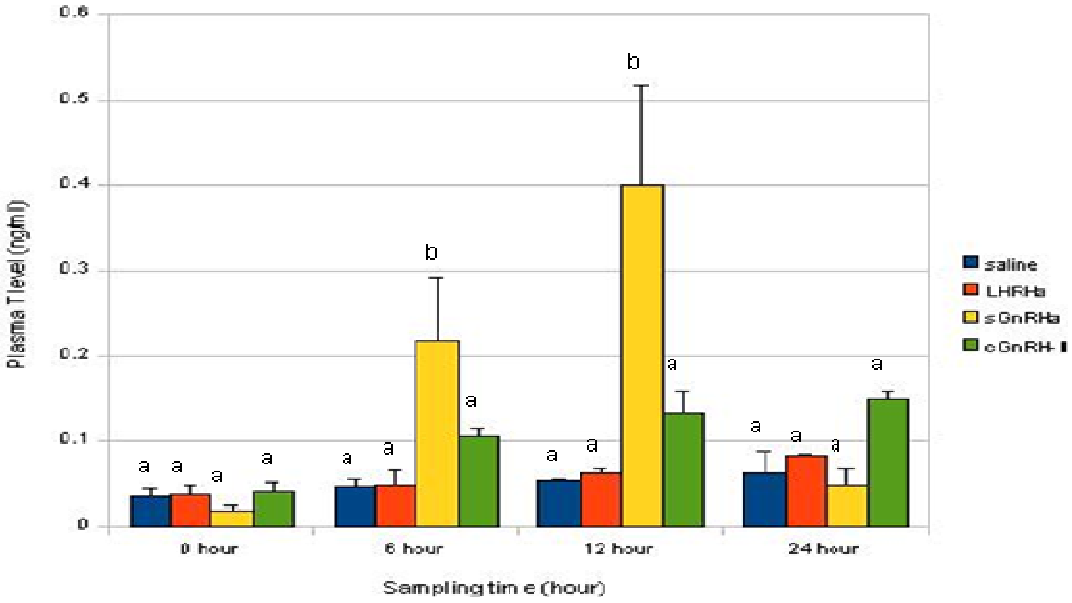
Figure 3c. Germinal vesicle (GV) determination on fish treated with 200 µg/kg cGnRH-II alone, 200 µg/kg cGnRH-II + pimozide , pimozide alone and saline in African catfish. Mean with different letters differs significantly (P < 0.05; One-way ANOVA followed by Duncan's multiple range test).

best among the other treatments that show ovulation with 95 and 78% of the fish were found to be fertilised and hatched, respectively.

Plasma steroid level for sGnRHa increased after 6 h (E2: 0.62 ± 0.05 ng/ml, T: 0.22 ± 0.07 ng/ml) and elevated significantly (P < 0.05) towards the peak level at 12 h (E2: 1.26 ± 0.08 ng/ml, T: 0.4 ± 0.12 ng/ml) but the concentration decreased after 24 h (E2: 0.85 ± 0.14 ng/ml, T: 0.05 ± 0.02 ng/ml) . However, there was no ovulation for cGnRH-II and LHRHa, therefore the plasma



Figures 4a. Changes in plasma 17ß-estradiol in response to fish treated with 20 µg/kg LHRHa, 20µg/kg cGnRH-II, 20 µg/kg sGnRHa and saline in African catfish. Bars represent mean ± SEM. Mean with different letters differs significantly ($P < 0.05$; One-way ANOVA followed by Duncan's multiple range test).



Figures 4b. Changes in plasma testosterone in response to fish treated with 20 µg/kg LHRHa, 20µg/kg cGnRH-II, 20 µg/kg sGnRHa and saline in African catfish. Bars represent mean ± SEM. Mean with different letters differs significantly ($P < 0.05$; One-way ANOVA followed by Duncan's multiple range test).

steroid level continue to increase until the end of the experiment (Figure 4).

GV determination shows concurrent effect as the plasma steroid level when GV stages migrated throughout the experiment (Figure 4c). Saline injected fish did not show any significant changes on the GV migration during the

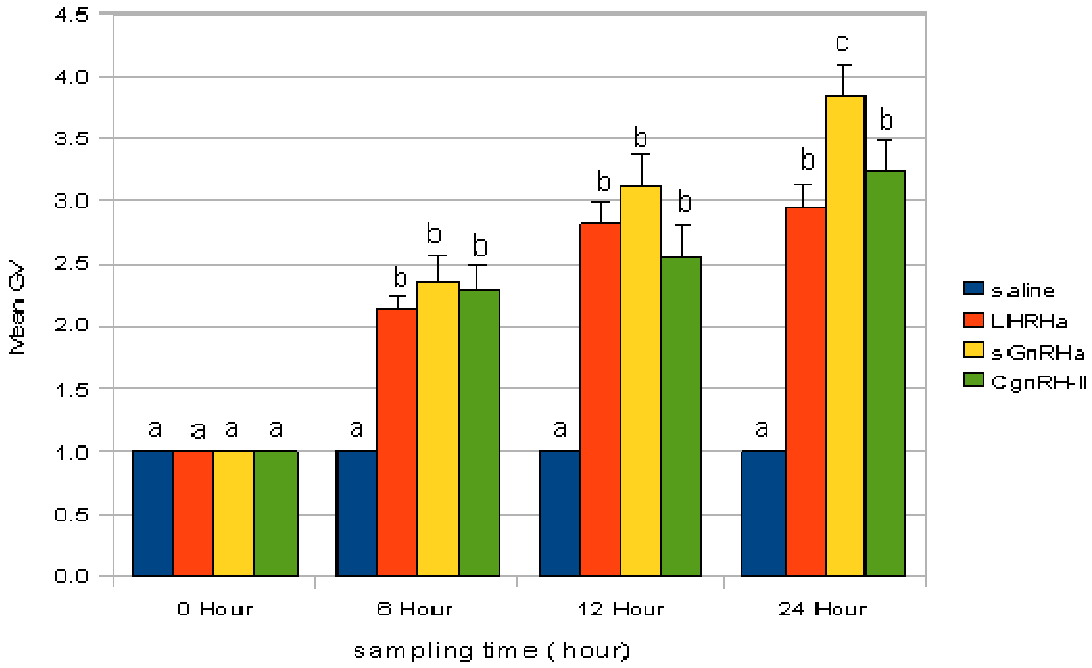


Figure 3c. Germinal vesicle (GV) determination in fish treated with 20 µg/kg LHRHa, 20 µg/kg cGnRH-II, 20 µg/kg sGnRHa and saline in African catfish. Bars represent mean ± SEM. Mean with different letters differs significantly ($P < 0.05$; One-way ANOVA followed by Duncan's multiple range test).

experiment. Progressive GV analysis of oocytes during the experiment indicated that GnRH treatment accelerated the GV migration for sGnRHa at 6 h, the mean stage was 2.4 ± 0.12 and moved to 3.2 ± 0.09 at 12 h. Mean GV for sGnRHa was the highest since ovulation occured at this hour and finally after 24 h mean GV advanced to 3.7 ± 0.14. The GV of oocyte for LHRHa and cGnRH-II continuously migrated from 6 h (mean GV stages 2.2 ± 0.02 and 2.3 ± 0.04, group LHRHa and cGnRH-II respectively) to the next stage at 12 h (mean GV stages 2.8 ± 0.04 and 2.6 ± 0.06, group LHRHa and cGnRH-II respectively). At the end of the experiment, GV stages had advanced to stages 2.9 ± 0.03 and 3.3 ± 0.05 group LHRHa and cGnRH-II ,respectively.

DISCUSSION

The use of cGnRH-II is effective to induce oocyte maturation in African catfish as supported by Szabo et al. (2007). However in this study, cGnRH-II is only effective to induce ovulation in African catfish with a higher dosage which is 200 µg/kg. In the present study, saline-injected control group did not ovulate in captivity while fish treated with cGnRH-II 200 µg/kg completed ovulation within 12 h after hormone administration. The other treated groups (cGnRH-II 2 and 20 µg/kg) did not show any ovulation. It was also observed in a study by Alok et al. (1999) that proved cGnRH-II manage to induce 100% ovulation in Indian catfish *Heteropneusteus fossilis* with 200 µg/kg. cGnRH-II has the most potent GtH releasing activity in fish exogenously (Zohar et al., 1995) although it does not deliver directly by anatomy to the pituitary to induce GtH release (Gothilf et al., 1996).

The result clearly showed that the use of 200 µg/kg cGnRH-II alone is sufficient to induce ovulation due to the fact that dopaminergic inhibition plays a minor role in the regulation of ovulatory gonadotropin secretion in African catfish (De Leeuw et al., 1985) as well as other species; coho salmon, *Oncoryhncus kisutch* (Van der Kraak *et al*., 1986), *Oncorhynchus keta* (Park et al., 2007), sea bass, *Dicentrarchus labrax L*. (Prat et al., 2001) and loach, *Paramisgurnus dab*ryanus (Lin et al., 1985). In the present study, plasma T and E2 levels in cGnRH-II + pimozide treated group were relatively lower than those of cGnRH-II only treated group. These results indicate that there is the possibility that dopamine antagonist has a direct effect on stroidogenesis in ovarian follicles (Kumakura et al., 2003).

The comparison of sGnRHa, LHRHa and cGnRH-II resulted into ovulation in the group treated with sGnRHa. Therefore, only a smaller amount of this hormone (20 µg/kg) is required to bring ovulation in African catfish compared to a large amount of dosage needed for cGnRH-II which is 200 µg/kg . Native cGnRH-II peptides were shown to rapidly degraded by peptidase whereby these enzyme cleave GnRH decapeptide at specifically position which consequently getting smaller and inactive fragments (Zohar et al., 1990). The analogue sGnRH was able to enhance the potency of spawning induction due to

the modifification of the native GnRH with dextrorotatory amino acid which not only resistant to enzymatic degradation but also increasing receptor-binding afinity (Habibi and Peter, 1991). Besides, sGnRHa is more resistant of the enzymatic degradation when compared to native cGnRH-II since their maximum half life in blood circulation does not exceed 23 min *in vivo* (Gothilf and Zohar, 1991). cGnRH-II is the only native form of GnRH that was found to be present in all vertebrate species (King and Millar, 1995 ; Chow et al., 1998). It was observed in the present studies that this particular hormone has sucessfully induced spawning in African catfish. Therefore, the use of a potent cGnRH-II agonist for the improvement of spawning induction therapies is suggested.

In the third experiment, mGnRHa or LHRHa does not perform well as an inducing agent although it is the most extensively used GnRH peptide for fish seed production even in the African catfish. Suprisingly, in this experiment, at dosage of 20 µg/kg, the LHRHa hormone did not induce final oocyte maturation in African catfish. LHRHa was found to be degraded faster than sGnRHa by cytosolic enzymes of the pituitary, kidney and liver as found in the gilthead seabream (Zohar et al., 1990). That is the possible reason for the ineffectiveness of mGnRHa in the third experiment.

The changes of the plasma levels of reproductive hormones with gonadal condition has proved to be a valuable tool in the development of an understanding the endocrine control of reproduction in teleost. The plasma steroids levels elevated after the hormone administration within few hours to a few days depending in the specific initial dose, fish species, specific GnRHa and water temperature (Crim et al., 1988; Harmin and Crim, 1993; Zohar et al., 1995; Mylonas and Zohar, 2001). In the present study, plasma level of E2 and T increased 6 h after injection and peaked at 12 h for the ovulated groups, that is cGnRH-II (200 µg/kg) , cGnRH-II (200 µg/kg) + PIM (5 mg/kg) and sGnRHa (20 µg/kg). An increased in plasma E2 during GVBD induced by hormonal or environmental stimulation has been commonly found in teleost with asynchronous oocyte development such as goldfish (Kobayashi et al., 1987,1988), the bitterling (Shimizu et al., 1985) and common carp (Yaron and Levavi, 1986).

The conversion of testosterone into 17ß-Estradiol by the aromatase enzyme in the granulosa layer of the vitellogenic oocytes is under the stimulation of GtH which stimulate the hepatic synthesis and secretion of vitellogenesis . The hormones tend to decline preceding ovulation where the steroid synthesis pathway shift from the production of E2 to 17,20 ß-P which is not measured in these particular studies (Nagahama, 1987). The decrease of plasma T after ovulation was due to the loss of postvitellogenic oocytes which are the main producer of T (Kobayashi et al., 1988).

In the three experiments, the fish were treated with single injection of hormone and ovulated by 12 h. The short period of half life for native GnRH in the blood circulation may be related to the enzymatic degradation (Mikolajczyk et al., 2004; Kaminski et al., 2004). This situation is probably the reason why a single injection and a huge amount of dosage of cGnRH-II is required to bring 100% ovulation. Therefore, multiple injection of hormonal therapies for this hormone is strongly suggested and as a matter of fact, reducing the amount of hormone dosage might be useful when using this method.

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