Effect of Season on Oocyte Development and Serum Steroid Hormones in LHRHa and Pimozide-injected Catfish *Clarias macrocephalus* (Günther)

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Oocyte and blood samples were taken from gravid female catfish *Clarias macrocephalus* at 4-h intervals to monitor the stage of oocyte development and serum steroid hormone profiles after injection of luteinizing hormone-releasing hormone analogue (LHRHa) and pimozide (PIM) during the off-season (February) and the peak of the natural breeding period (August). Results showed that the onset of final oocyte maturation (12 h) and ovulation (16 h), and levels of serum estradiol-17 β (E₂) did not vary with season in LHRHa + PIM-injected fish. In February, ovulated eggs were stripped from three and two hormone-treated fish at 16 h and 20 h post-injection, respectively. In August, ovulation was observed in all hormone-treated females ($n=5$) at 16 h post-injection but stripping of the eggs was possible only 4 h thereafter. Serum E₂ levels were significantly different only with varying time post-injection; a marked increase occurred at 12 h, but the elevation was higher in fish induced to ovulate during the peak (16.8 ng/ml) than off-season (7.7 ng/ml). Hormone-treated fish showed higher serum testosterone (T) levels during the peak season (17–23 ng/ml) than those injected during the off-season (10–20 ng/ml) at 4–12 h post-injection. Serum 17 α ,20 β -dihydroxy-4-pregnene-3-one (DHP) levels of hormone-treated fish during the off-season were only about half the level (0.29 and 0.52 ng/ml) of those treated with the same hormones during the peak season (0.54 and 0.9 ng/ml) at 8 and 12 h post-injection, respectively. Development of oocytes and serum steroid hormone profiles after LHRHa + PIM-induced ovulation provide basic understanding of the processes that mediate final oocyte maturation and ovulation in captive *C. macrocephalus*.

Key words: season, oocyte development, steroid hormones, catfish

Teleosts show seasonal regularity in the timing of reproductive development and spawning. Environmental factors such as photoperiod, temperature, salinity, food availability, presence of vegetation or substrate provide the stimuli for these reproductive events. When captive broodfish are exposed to an environment different from their natural habitats, reproductive cycles are generally disrupted, and many of these species do not complete their gametogenic cycle. Studies on the reproductive biology and physiology of fishes provide baseline information for developing methods to control reproduction under culture conditions.

The Asian catfish *Clarias macrocephalus* is a native species in the Philippines but is fast becoming scarce in the natural freshwater habitats.¹⁾ It is a favorite food fish due to its tender and delicious meat. Feral catfish reach sexual maturity within the first year, and spawn with the onset of the rainy season.²⁾ Captive catfish also attain sexual maturity, but the oocytes are arrested at the post-vitellogenic stage that remain inside the body cavity year-round,³⁾ and undergo further maturation and ovulation only after hormone administration.⁴⁾ Ovarian development during an annual reproductive cycle,³⁾ as well as the reproductive and larval performance when catfish were induced to spawn at

different seasons,⁵⁾ are already established. However, the influence of season on ovulatory response of the fish to hormonal treatment has not been investigated.

The present study evaluates the effectiveness of a hormone protocol developed for inducing final maturation and ovulation in *C. macrocephalus*⁴⁾ during the off- and peak of the spawning season. The seasonal effects of luteinizing hormone-releasing hormone analogue (LHRHa) and pimozide (PIM) administration will be assessed by monitoring changes in the development of oocytes and serum profiles of steroid hormones in the catfish. The aim of this investigation is to improve the efficiency of manipulation of reproduction in captive *C. macrocephalus* at different phases of its annual cycle.

Materials and Methods

Fish

Two- to three-year old hatchery-bred gravid female catfish *C. macrocephalus*, with a mean body weight of 296.08 ± 12.21 g, were used in this study. The fish were maintained under ambient temperature and photoperiod (about 12L:12D), and alternately fed fish by-catch and pelleted feed containing 36% protein⁶⁾ at 5% of the body

weight (BW). Catfish broodstock were reared in 10,000- l $9.5 \times 1.5 \times 1$ m concrete tanks lined with mud substrate at the bottom. A day before the experiment, gravid females were anaesthetized with 400 ppm of 2-phenoxyethanol, wiped dry, weighed to the nearest 0.01 g, and stocked individually in 60- l oval fiberglass tanks filled with 20 l of dechlorinated tap water and lined with mud substrate. The experiment was conducted during the off- (February) and peak seasons (August).³⁾

Hormone Preparation and Administration

D-Ala⁶ Pro⁹-luteinizing hormone-releasing hormone-*N*-ethylamide analogue (LHRHa) was purchased from Lam Hua Dragon Co. limited (Wanchai, Hongkong). Pimozide (PIM) was a gift from Janssen Pharmaceuticals Limited (Beerse, Belgium). A combination of 0.05 μ g LHRHa and 1 μ g PIM/g BW⁴⁾ was prepared a day prior to the experiment and stored at 4°C until use. LHRHa was dissolved in 0.9% NaCl, while PIM was first dissolved in dimethylsulfoxide (DMSO) to which propylene glycol (PG) was added at a ratio of 1:9 (v/v).⁷⁾ The simultaneous injection of LHRHa and PIM was administered at 06:00 h on alternate sides of the dorsal musculature at an injection volume of 1 μ l/g BW. Fifteen of the broodstock were injected with hormones, while the other fifteen fish were injected with the control vehicles 0.9% NaCl and DMSO:PG.

Oocyte Maturation and Ovulation

Sampling of oocytes and blood in a batch of five fish each of the hormone- and control vehicle-injected groups was carried out at 0 (before), 4, 8, 12, 16, 20, and 24 h post-injection. This means that the first five fish from both control and hormone-treated groups were sampled at 0 h, the sixth to tenth fish from both groups were sampled at 4 h, samples were obtained from eleventh to fifteenth fish at 8 h. Thereafter, samples were again taken from the first five fish at 12 h, etc.

Starting at 4 h post-injection and thereafter, the abdomen of each fish was massaged to check for ovulation. If no oocytes were obtained after manual stripping, ovaries were biopsied by inserting a Silastic medical grade tubing (O.D. = 1.96 mm, I.D. = 1.47 mm; Dow Corning Corp., Michigan, U.S.A.) into the genital pore, and aspirated by mouth as the tube was slowly withdrawn. Fifty to one hundred cannulated oocytes were mounted on plexiglass troughs, to which Serra fluid (37% formalin, 95% ethanol and glacial acetic acid, 6:3:1 v/v) was added. When oocytes were cleared, the position of the nucleus or germinal vesicle was examined under the microscope at 40 $\times$ magnification. Development of the oocytes was classified according to the position of the germinal vesicle.⁸⁾ Each maturation stage was expressed as a percentage of the total number of oocytes examined at each sampling period.

Steroid Radioimmunoassay

Two- to three-milliliters of blood was taken from the caudal peduncle of each broodfish using a 24 gauge $\times$ 1 needle fitted to a 5-ml glass syringe. Blood was kept on ice for 3–4 h before centrifuging at 4°C, 3000 rpm for 15 min. The sera were then stored at –70°C until radioimmunoassay. Radioimmunoassay procedures on testosterone (T) and estradiol-17 β (E₂) developed by Kagawa *et al.*⁹⁾ and

17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) by Young *et al.*¹⁰⁾ were followed. Detectable levels were 30 pg/ml for T and E₂, and 50 pg/ml for DHP.

Statistical Analyses

Analysis of variance (ANOVA) was done separately on T, E₂ and DHP of hormone-treated and control fish, since mean values of all steroid hormones were significantly different between the two groups. Two-way ANOVA was then conducted on T, E₂ and DHP of each group according to season or time of injection and the interaction of season and time. T, E₂ and DHP values were common log-transformed prior to ANOVA, followed by Duncan's multiple range test at $P=0.05$.

Results

Since only five fish each from the hormone-injected and control groups were sampled at 4-h intervals, it is assumed in this study that ovulatory response of these ten fish at each sampling period represents the physiological status of all the experimental and control fish to the injected solutions.

Oocyte Maturation and Ovulation

Percentage of oocytes at various stages of maturation after injection of LHRHa and PIM during the off- and peak seasons is presented in Fig. 1. Oocytes cannulated from gravid females injected with LHRHa + PIM during the off- and peak seasons similarly were all in the central germinal vesicle (CGV) stage before (0 h) until 4 h post-injection. At 8 and 12 h post-injection, most of the oocytes were in the GVM and GVBD stage, respectively. In catfish injected with LHRHa + PIM during the off-season, three out of the five hormone-injected females can be manually stripped of ovulated eggs at 16 h post-injection. Four hours thereafter, eggs were also stripped from the other two fish. In catfish injected during the peak season, presence of ovulated eggs from the five fish sampled was recorded at 16 h; however, stripping of these eggs was possible only at 20 h post-injection.

In contrast to the LHRHa + PIM-injected fish, gravid females injected with the control vehicles during the off- and peak seasons cannot be stripped at all. Cleared oocytes were all at the CGV stage.

Serum Steroid Hormone Profiles

Two-way ANOVA showed that significant differences due to season and time after hormone injection were noted on serum levels of testosterone (T). Serum T levels during the off- and peak seasons were similarly low before injection (4 ng/ml; Fig. 2). However, catfish induced to ovulate with LHRHa + PIM during the peak season had significantly higher serum T levels than those injected during the off-season at 4–12 h post-injection. Thereafter, serum T levels were similar in fish treated during the off- and peak seasons. Serum T levels in control vehicle-injected fish ranged from 4.3 to 7.24 ng/ml in February and 4.24 to 8.38 ng/ml in August.

No seasonal variations were found in the serum estradiol-17 β (E₂) levels of LHRHa + PIM-treated females (Fig. 3). Significant differences were observed only at

Fig. 1. Percentage of *Clarias macrocephalus* oocytes at different stages of development: central germinal vesicle (CGV), germinal vesicle migration (GVM), germinal vesicle breakdown (GVBD) and ovulated egg (OVUL) at several hours after a simultaneous injection of luteinizing hormone-releasing hormone analogue (LHRHa) and pimozone (PIM).

Fractions are number of females stripped of ovulated eggs to the total number of females that were injected with LHRHa+PIM. Values in each column indicate the mean of five fish. A: off-season (February), B: peak season (August).

varying times after hormone injection. A marked increase occurred at 12 h post-injection, although the elevation was much higher during the peak (16.8 ng/ml) than when fish were induced during the off-season (7.7 ng/ml). Serum E_2 levels in control vehicle-injected female catfish were 2.3–5.1 ng/ml in February and 1.7–3.9 ng/ml in August.

Serum $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) levels in fish treated with LHRHa+PIM were significantly different with season and time after injection. DHP levels during the off- and peak seasons were not detectable at 0–4 h post-injection (Fig. 4). Serum DHP levels similarly began to increase at 8 h post-injection in both seasons. At 8 and 12 h post-injection however, serum DHP levels of hormone-treated fish during the off-season were only about half the level (0.29 and 0.52 ng/ml) of fish induced during the peak season (0.54 and 0.90 ng/ml). Serum DHP levels significantly decreased after 12 h post-injection in fish induced to ovulate at both seasons. Serum DHP levels in con-

Fig. 2. Mean levels of serum testosterone (T) in catfish *Clarias macrocephalus* injected simultaneously with luteinizing hormone-releasing hormone analogue (LHRHa) and pimozone (PIM) or their vehicles during the off-season (February) and peak season (August).

Asterisks indicate significant differences among means at different hours during the off- and peak season in LHRHa+PIM-injected fish. off-tr, hormone-injected fish during off-season; peak-tr, hormone-injected fish during peak season; off-veh, control vehicle-injected fish during off-season; peak-veh, control vehicle-injected fish during peak season.

Fig. 3. Mean levels of serum estradiol-17 β (E_2) in catfish *Clarias macrocephalus* injected simultaneously with luteinizing hormone-releasing hormone analogue (LHRHa) and pimozone (PIM) or their vehicles during the off-season (February) and peak season (August).

Asterisks indicate significant differences among means at different hours during the off- or peak season in LHRHa+PIM-injected fish. off-tr, hormone-injected fish during off-season; peak-tr, hormone-injected fish during peak season; off-veh, control vehicle-injected fish during off-season; peak-veh, control vehicle-injected fish during peak season.

control vehicle-injected fish were below detectable levels (<50 pg/ml) during a 24-h sampling at different seasons.

Fig. 4. Mean levels of serum 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) in catfish *Clarias macrocephalus* injected simultaneously with luteinizing hormone-releasing hormone analogue (LHRHa) and pimoide (PIM) or their vehicles during the off-season (February) and peak season (August).

Asterisks indicate significant differences among means at different hours during the off- and peak season in LHRHa+PIM-injected fish. off-tr, hormone-injected fish during off-season; peak-tr, hormone-injected fish during peak season; off-veh, control vehicle-injected fish during off-season; peak-veh, control vehicle-injected fish during peak season.

Discussion

A simultaneous injection of 0.05 μ g LHRHa and 1 μ g PIM/g body weight in gravid female catfish during the off- and peak seasons similarly resulted in oocyte maturation and ovulation, and a rise in serum levels of steroid hormones. In this species, LHRHa and PIM were simultaneously administered because a previous study showed that LHRHa or PIM alone did not result in final oocyte maturation and ovulation.⁵ As in common carp,¹¹ goldfish,¹² catfishes,^{13,14} a dopamine antagonist such as pimoide is administered to effect an adequate increase in GTH concentrations to induce ovulation in *C. macrocephalus*. In contrast, dopaminergic inhibitory system for GTH secretion is probably absent in Atlantic croaker¹⁵ and orangemouth corvina,¹⁶ hence administration of LHRHa alone induced ovulation and spawning.

As reported in the carp,¹⁷ goldfish,¹⁸ and a related Asian catfish *C. batrachus*,¹⁹ serum T levels were the first of the steroids examined to increase 4 h after hormonal injection and had the highest absolute value. These results indicate that the function of endogenous GnRH, i.e., to stimulate a surge in gonadotropin (GTH) that subsequently resulted in increased testosterone levels, can be mimicked by LHRHa and pimoide during both seasons. At 12 h post-injection, gravid catfish injected at different seasons exhibited similar responses to exogenous hormone treatment. Oocytes reached the GVBD stage, serum T levels started to decrease while serum E₂ and DHP levels reached maximum levels. Although treated fish in both seasons had ovulated eggs at 16 h post-injection, absolute levels in

serum T and DHP of the fish in response to hormone treatment during the peak season were generally higher than in fish induced to ovulate during the off-season. These results suggest seasonal differences in the steroidogenic capacity of post-vitellogenic eggs in captive *C. macrocephalus*. The higher levels of serum T at 4–12 h and DHP at 8–12 h post-injection in fish injected during the peak season probably reflect an extended occurrence of final maturation among the more numerous post-vitellogenic oocytes during the peak season. A decline in serum T observed concomitant with the highest serum levels of E₂ during the peak season likewise suggest a more efficient conversion of T to E₂ at this time of the year.

In this study, a similar dose of LHRHa and PIM was injected during the two seasons of the annual cycle. While higher production of steroid hormones could be obtained with the administration of a higher hormone dose, it may not always result in elevation of GTH, steroid hormones or improved reproductive and larval performance, as reported in other catfish species. No significant rise in GTH levels was reported in *Heteropneustes fossilis* after injections of mammalian gonadotropin-releasing hormone analogue (mGnRH) and PIM alone or in combination during the preparatory (equivalent to off-season in the present study) phase.²⁰ In contrast, GTH levels were significantly elevated when the same hormone doses were given during the prespawning and spawning phases. Furthermore, a low mGnRH was not effective in the early spawning phase but was effective in the later spawning season. In *C. gariepinus*, injection of a high dose of LHRHa alone caused significant elevation in plasma GTH and ovulation in 80% of treated fish when the hormone was given close to the natural breeding period but not at other times of the year.²¹ Reproductively active winter flounder readily responded to gonadotropin releasing hormone (GnRH) treatment throughout the year, except during a period of gonadal quiescence when the gonads were regressed.²²

Serum E₂ levels in hormone-injected fish were comparable at different seasons of the reproductive cycle, while serum DHP in control fish were below detectable levels over 24-h even at the peak season. The absence of seasonal differences in serum E₂ levels suggests the termination of vitellogenesis since E₂ is actively synthesized by smaller vitellogenic oocytes.^{23,24} Synthesis of E₂ is much reduced in bigger post-vitellogenic oocytes, that form the bulk of oocyte population in the captive, gravid *C. macrocephalus*.³ The undetectable serum levels of DHP, identified as a maturation-inducing-steroid in *C. macrocephalus*,²⁵ further confirm the observation that final oocyte maturation and spontaneous ovulation do not occur at all in this species under captive conditions.

Serum E₂ and DHP similarly peaked at 12 h post-injection. The less pronounced shift in E₂ to DHP and the low absolute DHP levels in both seasons may be due to the group-synchronous development of oocytes in *C. macrocephalus*.³ In this type of development, the various size-groups of oocytes could mask the changes in hormone levels, such as those observed in orange roughy *Hoplostethus atlanticus*²⁶ and the New Zealand snapper *Pagrus auratus*.²⁷ In contrast, seasonally breeding teleosts with synchronous development of the oocytes showed a distinct shift from E₂ to DHP during ovulation.^{28–30}

Handling stress from repeated cannulation, stripping and bleeding at different intervals may have affected ovarian steroidogenesis and the stripping time of ovulated eggs. Absolute T (4.3–7.24 ng/ml in February, 4.24–8.38 ng/ml in August) and E₂ (2.36–5.12 ng/ml in February, 1.70–3.96 ng/ml in August) levels in this experiment were much lower than the T (37.78 ng/ml in February, 54.74 ng/ml in August) and E₂ (9.07 ng/ml in February, 11.88 ng/ml in August) levels observed in fish sampled only once monthly during the annual reproductive cycle.³⁾ Variability in steroid secretion due to handling caused very high T levels in the sea bass *Dicentrarchus labrax*³¹⁾ or reduced steroid levels in the gudgeon *Gobio gobio*.²⁴⁾ The inhibitory effect of capture stress on ovarian steroidogenesis in female New Zealand snapper caused a decline in plasma T and E₂.³²⁾ Treatment with exogenous cortisol, which is elevated during stress, resulted in decreased T, E₂, vitellogenin and gonad size.³³⁾ Previous data showed that *C. macrocephalus* induced during the peak season are usually stripped of ovulated eggs at 16 h (Tan-Fermin, unpublished data). In this study, ovulated eggs were obtained at 16 h post-injection, yet manual stripping of these eggs was possible only 4 hours after. The delayed response may be due to repeated stress. Interruption of reproductive behavior in rainbow trout showed a marked effect on plasma GTH, T, E₂ and DHP levels, but mainly in relation to the time of ovulation.³⁴⁾ Furthermore, repeated exposure of female rainbow trout to acute stress was observed to cause smaller egg size, delayed ovulation and lower larval survival compared to unstressed fish.³⁵⁾

In fish induced to spawn during the off-season, early and partial ovulation at 16 h could be due to precocious ovulation of underripe eggs, while lower serum steroid hormone levels may be due to decreased steroidogenesis caused by changes in the hypothalamo-hypophysial-gonadal axis.^{36–40)} Significantly lower fertilization rates were reported in the brown trout *Salmo trutta* that ovulated early in the reproductive season after GnRH_a or vehicle administration.⁴¹⁾ Egg production, fertilization, hatching and larval survival rates of catfish induced with LHRH_a + PIM during the off-season were also lower than those induced before, at the peak and end of the spawning season.⁵⁾

Altogether, the present data showed that the onset of GVBD and ovulation in captive *C. macrocephalus* occurred at almost the same time after LHRH_a + PIM injection during the off- and peak seasons. However, there was a higher production of serum T and DHP during the peak season. Serum E₂ did not show any seasonal variation but a marked increase occurred at 12 h post-injection with such an increase being more elevated during the peak than off-season. Although ovulation was artificially induced in this study, changes in oocyte maturation stages and serum steroid hormone profiles provide basic understanding of the processes that mediate final oocyte maturation and ovulation in captive, hatchery-bred *C. macrocephalus*.

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