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Research Paper

Effect of Administration of Serially Diluted Ovaprim and Clomiphene Citrate on the Ovulation and Hatching of C*larias gariepinus*

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ABSTRACT: *This study was designed to determine the effects of using serially diluted Ovaprim®, a Gonadotropin hormone-releasing hormone analogue (GnRHa) with an antiestrogen - Clomiphene citrate (Clomid®) in artificial spawning of Clarias gariepinus. Treatments administered include T1: 0.5ml/kg, T2: 0.4ml/kg, T3: 0.3ml/kg and T4: 0.2ml/kg of Ovaprim® together with 0.9mg/kg of Clomid®. Parameters determined include egg numbers, latency period, fertilization rate, hatching rates and survival to first feeding. The results obtained demonstrated that the use of Ovaprim® (GnRHa) together with the antiestrogen clomiphene citrate (Clomid®) successfully induced ovulation in the experimental Clarias gariepinus broodfish. There was no significant difference in egg weights stripped from each treated group. The application of 0.5ml/kg of Ovaprim® (T1) resulted in earlier synchronization of ovulation (Latency period; 10 hours). Results of the fertilization percentage indicated that dilution in dose of Ovaprim® did not significantly affect fertilization rate in treated groups of broodfish. Overall, there was no significant difference (p>0.05) in fertilization, hatching and survival from all treatments administered.*

KEYWORDS*: fertilization, ovulation, hatching, hormone, egg*

I. INTRODUCTION

Artificial breeding of fish, especially *C. gariepinus*, remains the most capable and reliable way to make sure obtainability of good quality fish larvae [1]. *Clarias gariepinus* hardly reproduces in captivity [1]. In order to make *C. gariepinus* to reproduce faster throughout the year, induced breeding technique is applied to the male and female fish in order to produce sperms and eggs in control condition.

Induced breeding through hormone treatment and artificial incubation of fertilized egg has advantages of better rate of fertilization and hatching, better conditions for growth and better protection of larvae against unfavourable environmental condition and predators [2]. Therefore, study on the usage of hormonal dosage could help to determine the best concentration of hormone that could increase the production of larvae.

There are various hormones that can be used in induced breeding for seed production including pituitary extract or hypophysis from similar or different fish, Deoxycorticosterone Acetate (DOCA), Ovaprim, Ovulin, Ovatide, Human Chorionic Gonadotropin (HCG), Ovopel, Dagin and Aquaspawn [1]. The commercially available synthetic inducing hormones in readymade containing GnRH and dopamine blocker receptor such as ovaprim, ovopel, dagin and aqua spawn are becoming very popular and found to be efficient in successful spawning of fishes [1].

Ovaprim is one of the most popular and effective hormones to stimulate the maturation of male and female adult fish. Ovaprim is used as an effective spawning inducer for artificial breeding of fishes [3]. Ovaprim is made from Salmon Gonadotropin Releasing Hormone (SGnRGH) and Dopamine antagonist in a stable solution, prepared in glycerine and alcohol at certain quantity [4]. Ovaprim is used to quicken the ovulation process in female catfish and make sperm more fertile in male fish. Ovaprim can accelerate final oocyte maturation and induced spawning [5]. Fish treated with Ovaprim have been reported to exhibit increased gonadosomatic index, egg diameter, and wet weight relative to controls with fish that were injected with high dosage of hormones producing large number of eggs [5].

Clomiphene citrate (clomiphene), is the most commonly used drug for ovulation induction, since it is inexpensive, highly effective and user-friendly. It was first synthesized in 1956 and has been commercially available since 1961 [6]. It constitutes the treatment of choice in hyperandrogenic chronic anovulation and other forms of anovulation with an adequate oestrogen reserve [7]. It is also used in the functional assessment of the gonadal axis, may be combined with gonadotropins in the therapy of selected cases of controlled ovarian hyperstimulation, and can be used with or without gonadotropins in assisted reproductive techniques [8].

Due to the structural resemblances of clomiphene citrate to estrogen, it binds competitively with nuclear receptors of estrogen. Through decreasing the negative feedback of estrogen, it stimulates mechanism that alters the releasing trend of GnRH, which enhances pituitary gonadotropin hormones. This mechanism finally stimulates ovarian follicles to develop [9].

The increasing cost of GnRHa hormones in the market has led to trials of serially diluted hormones on the African catfish *Clarias gariepinus* [10]. However, the efficacy of diluted hormones has not been boosted by addition of cheaper ovulating agents such as Clomiphene citrate. This experiment is therefore based on the need to increase potency of serially diluted GnRHa using a cheaper ovulation inducer.

II. MATERIALS AND METHODS

Study Area

The study was carried out at the fishery Hatchery of the Department of Fisheries and Aquaculture, Joseph Sarwuan Tarka University Makurdi.

Source of Broodstock

The brood stocks were from Obedience fish farm No 28 Lucy Aluor Stre*et al*ong New Otupko Road Makurdi, Benue State. A total number of Twelve [12] fish, eight [8] females and four [4] males ware purchased. All brood stocks were selected by external morphological characteristics using the method of Ayinla, et al. [11]. The broodstock were acclimatized for Two [2] days

Source of Hormone

Ovaprim was acquired from J-Climax Agro Limited Ado U-turn Karu Local Government Area of Nasarawa State and Clomid was acquired from MedPearle Pharmacy Makurdi, Benue State.

Experimental Design

Preparation of Hormone

Ovaprim® obtained was manufactured with a concentration of 20µg/ml of salmon gonadotropin-releasing hormone analog (sGnRHa) and 10mg/ml of domperidone [12]. Each treatment was prepared by adding designated quantities of normal saline to make up to the recommended dose of 0.5ml.kg^{-1}. Two tablets (100mg) of Clomid were removed from the pack and pounded using a porcelain mortar and pestle. The volume of the powdered product was determined to be 4ml. This was made up to 20ml by adding 16ml of normal saline. Following the use of 1mg.kg^{-1} by Worthington, et al. [13] to induce ovulation in carp, the current trial utilized 0.9mg.kg^{-1} in each diluted ovaprim treatment. The current trial utilized four different dosages of Ovaprim®: 0.5ml.kg^{-1}, 0.4ml.kg^{-1}, 0.3ml.kg^{-1}, and 0.2ml.kg^{-1}. The dose of Clomid® was fixed at 0.9mg.kg^{-1} of bodyweight. From the foregoing, the following concentrations volumes were used in all cases:

Table 1: Dose of Hormones (Ovaprim® and Clomid®) and constituents administered to female *C. gariepinus*

Treatment	Dose of Ovaprim (ml.kg^{-1})	Volume of diluent (ml)	Concentration of constituent		Clomid (mg.kg^{-1})
			sGnRHa(µg.ml^{-1})	Domperidone® (mg.ml^{-1})	
T1	0.5	-	20	10	-
T2	0.4	0.1	16	8	0.9
T3	0.3	0.2	12	6	0.9
T4	0.2	0.3	8	4	0.9

Hormone Administration

The female brood stock was collected from the holding tanks by using a scoop net after which the weight of the fish was taken using a Salter® weighing scale. The weighed fish was then covered with clean towel and injected intramuscularly above the lateral line towards the dorsal section and pointed towards the ventral side. After withdrawal of the needle the fish was finger rubbed to avoid flow of the injected fluid. The injected females were returned separately into their respective plastic bowls.

Stripping and Fertilization

Injected female broodstock were removed from plastic bowl after 10-13 hours and stripped in dry bowl by holding the fish at the head and tail by an assistant. The ovulated eggs oozed out on slight pressure by thumb into the dry plastic bowl and 10g of eggs were collected from each sample into a petri-dish for counting so as to

know the total number of eggs produced from each of the female brood stock. The male broodstock were removed after dissecting them and the milt was collected by laceration of the testes with a clean razor blade. The sperm was then used to fertilize each treatment by mixing both eggs collected and sperm with a plastic spoon before adding distilled water. The bowl was vigorously shaken for a few seconds to improve fertilization.

Incubation

Incubation of the fertilized eggs was carried out in 60 liters plastic bowl containing about 45 liters of clean water which was equipped with water aerators. Nylon mesh size (1mm) was suspended above the floor in the plastic bowl for spreading of fertilized eggs. The fertilized eggs were spread in a single layer on the suspended nylon meshed net for incubation. Upon hatching (about 24 hours after incubation), the nylon meshed net was removed with the egg shells while the hatched larvae clustered at the bottom of the incubation tank.

Determination of Fertilization Rate

Fertilization rate was determined using 750 eggs from each cross. The eggs were covered in the dry, labeled Petri dish and were kept with labels. The number of eggs were estimated using the gravimetric method (number of eggs/g). The translucent eggs containing embryonic eyes at the time of polar cap formation 10 - 20 minutes after fertilization were considered fertilized and counted to estimate fertilization rate [14].

Hatchability

Eggs were incubated in plastic aquaria with a water volume of 40L and mosquito mesh as substrate. Percentage hatchability was estimated 24 hours after hatching was completed. This was estimated using the volumetric method. To do this, the incubation bowl was stirred gently to disperse the larvae evenly in the water. A beaker (100ml) was used to collect water from the bowl with the dispersed larvae swimming freely inside. The number of larvae in the volume of water was counted. This was repeated three times and the average number was taken. The value was then estimated to cover 40 litres water volume using mathematical relationship. The hatching rate was determined using a modified version of formula provided by Adebayo and Popoola [15] as:

$$Hatching\ Rate = \frac{Total\ Number\ of\ Hatched\ Eggs}{Total\ Number\ of\ Incubated\ Eggs} \times 100$$

Survival

The survival rate of larvae was estimated four days after hatching i.e. post yolk sac absorption. The volumetric method was employed in determining survival rate. Here water in the holding tanks was stirred to ensure even dispersion of fry using a glass rod. After this, a representative sample of the water (100ml) was taken in a beaker and fry within the water volume were counted. This was repeated three times and the average was taken. The population was then estimated to cover the entire water volume (40,000ml). Therefore, the following equations were used:

$$A_{100} = \frac{\sum No.\ of\ fry\ in\ three\ samples}{3}$$

$$Survial\ rate = \frac{A_{100} \times 40000ml\ /_{100ml}}{No.\ hatched} \times 100$$

Water Quality Parameters

Water quality parameters such as pH, Electrical Conductivity, Total Dissolved Solids (TDS) and Dissolved Oxygen of the water were monitored using Hanna Multiparameter Water Quality Probe Model HI-98129. A mercury in glass thermometer was used to take temperature readings.

Statistical Analysis

Data was analysed using R version 4.0.0 [16]. Descriptive statistics for hatching success were obtained using Rmisc package in R [17] and reshape2 [18]. Differences in the hatching rates across the treatments were determined using one-way ANOVA in R via agricolae and emmeans packages [19, 20]. Mean separation was done using the Tukey HSD method implemented in multcomp package [21] and viewed using multcompView [22]. Graphs were drawn using the ggplot2 package in R [23].

III. RESULTS

Fecundity

Fecundity of female broodstock to be induced with a combination of Ovaprim® and Clomid® (Figure 1) shows that broodstock used for the 0.2ml.kg^{-1} ovaprim dose had the highest fecundity followed closely by

broodstock allotted to the 0.5ml.kg^{-1} dose while broodstock selected for the 0.3ml.kg^{-1} dose had the least fecundity.

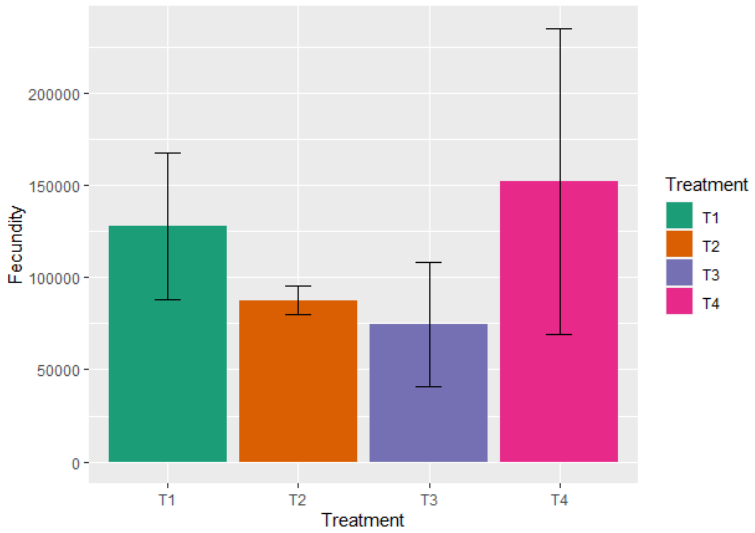

Figure 1: Fecundity of Female Broodstock of *C. gariepinus* stripped under each treatment

Breeding Performance

The effect of each dose administered on respective breeding parameters (Table 2) shows that the weight of eggs stripped from each female for each treatment was not significantly different ($p>0.05$) and also reflective of the fecundity (Figure 1). Fertilization rates did not differ across the treatments ($p>0.05$). Latency period differed significantly ($p<0.05$) among the treatments with the least period of 10 hours being recorded for fish treated with 0.5ml.kg^{-1} of ovaprim®. Hatchability did not differ significantly across the treatments ($p>0.05$) with the highest hatchability (54%) being observed for fish administered 0.3ml.kg^{-1} of Ovaprim® and 0.9mg.kg^{-1} of Clomid®. Survival at yolk sac absorption was not significantly different among the treatments ($p>0.05$).

Table 2: Egg and breeding parameters of *C. gariepinus* induced using serially diluted ovaprim supplemented with Clomiphene citrate

Treatment	Egg_Wt	Latency	Fertilization	Hatchability	Survival.rate
T1	217.25 ± 74.80	10.0 ± 0.0^{a}	84.00 ± 7.00	29.50 ± 27.50	68.5 ± 8.5
T2	154.70 ± 19.50	11.0 ± 0.0^{b}	93.00 ± 3.00	28.00 ± 6.00	76 ± 1
T3	137.10 ± 83.80	11 .0± 0.0^{b}	79.00 ± 13.00	54.00 ± 33.00	67 ± 10
T4	177.00 ± 95.80	12.0 ± 0.0^{c}	87.50 ± 2.50	47.50 ± 29.50	64.5 ± 4.5
p-value	0.881	$<2.0\times10^{-16}$	0.651	0.861	0.699

Means in the same column followed by different superscripts differ significantly ($p<0.05$)

Water Quality

Water quality in the incubation tanks (Table 3) reveals that the pH, temperature, Total Dissolved Solids (TDS) and Dissolved Oxygen (DO) were not significantly different among the treatments ($p>0.05$). Electrical Conductivity (EC) was highest in incubation tanks used for 0.3ml.kg^{-1} dose of Ovaprim® and least in the tanks used to incubate eggs that were derived from fish treated with 0.4ml.kg^{-1} Ovaprim® plus 0.9mg.kg^{-1} of Clomid®.

Table 3: Water quality parameters in aquaria used for incubation of *C. gariepinus* eggs

Treatment	pH	EC	TDS	Temp	DO
T1	7.53 ± 0.01	115.5 ± 0.50^{a}	70.5 ± 3.50	26.55 ± 0.05	2.65 ± 0.15
T2	7.5 ± 0.07	105.0 ± 5.00^{a}	66.0 ± 10.00	26.00 ± 0.30	2.95 ± 0.05
T3	7.46 ± 0.09	143.0 ± 7.00^{b}	56.0 ± 2.00	26.15 ± 0.15	2.8 ± 0.1
T4	7.54 ± 0.04	112.0 ± 3.00^{a}	66.0 ± 8.00	26.30 ± 0.60	2.85 ± 0.25
p-value	0.775	0.019	0.541	0.723	0.628

Means in the same column followed by different superscripts differ significantly ($p<0.05$)

Relationship between Water Quality and Breeding performance

Correlations between water quality parameters and breeding parameters: latency period, hatchability and survival (Figure 2) shows that there was only one significant correlation ($p<0.05$) between the water quality parameters themselves (TDS vs Temperature) but no significant correlation between water quality and the breeding parameters.

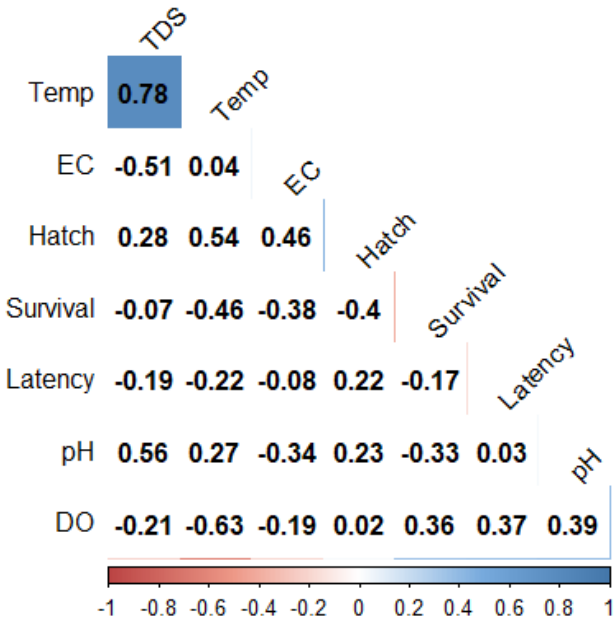

Figure 2: Correlation plot for water quality parameters and hatchability/survival of *C. gariepinus* fry (Increasing colour intensity signifies increasing p-values and correlations without colour are not significant ($p>0.05$); Blue colour = positive correlation and Red colour = Negative correlation)

IV. DISCUSSION

Fecundity recorded in the Ovaprim treatment alone (T1) and the lowest diluted Ovaprim® + Clomid® treatment (T4) were 127975 and 152359 respectively. These results compare favourably with the report of [24] where 145981 eggs were recorded for the same species induced with 0.5ml.kg-1 of Ovaprim®. Ovaprim® was also reported to elicit superior fecundity in carp relative to pituitary extract [25]. In sharp contrast, Onyia, et al. [26] reported that the use of homoplastic pituitary extract yielded high fecundity (124,000 eggs) compared to Ovaprim® (65,000 eggs). The results obtained from co-administration of diluted Ovaprim® and clomid in the current study: 87789 (T2) and 74648 (T3) compares favourably with the results obtained by Onyia *et al*., (2021). Recent trials with clomiphene citrate are not readily available. However, earlier trial of clomid to induce ovulation in the Asian catfish *Clarias batrachus* by Chandrasekhar and Rao [27] revealed that ovarian weight increased progressively with time of administration of either $25ug.kg^{-1}$ or $50ug.kg^{-1}$ doses of clomid over 90 days.

Latency period as recorded in the current trial indicated that there was increasing period with the level of dilution of Ovaprim®. Ovaprim® administered alone and at the correct dose ($0.5ml.kg^{-1}$:T1) gave the shortest latency period of 10 hours while the most diluted treatment 0.2ml.kg-1 gave the highest latency period. [28] reported a similar trend with latency period of 8 hours in normal dose of Ovaprim® as against 11 hours and 14.67 hours for $0.4ml.kg^{-1}$ and $0.3ml.kg^{-1}$ dilutions respectively. However, Ameer *et al*., (2021) reported a latency period of 13.42 hours for normal dose Ovaprim® induced *C. gariepinus*. This latency period is quite high compared to the result from the current study. A significant factor that affects latency period apart from the hormone used is the water temperature. Mean water temperature recorded in the tanks from the current study was about 26°C while water temperature recorded by Ameer *et al*., (2021) was 28.05°C. Even though the temperature in the current trial was lower, latency period was faster than that in the report by Ameer *et al*., (2021). This can be attributed to the dynamics between the TDS and temperature as revealed by the correlation matrix (Figure 2). The TDS levels in the current study are quite lower that that reported by Ameer *et al*., (2021) and could offer a reason why the temperature was low since there was a significant positive correlation between TDS and water temperature.

One important parameter that shows the accuracy of hormonal inducement for ovulation or spermiation is fertilization rate. Fertilization rate was observed to be higher (93%) in $0.4ml.kg^{-1}$ (Ovaprim®) + $0.9mg.kg^{-1}$

(Clomid®) treated fish (T2) compared to other treatments. This result varies sharply from that reported by Ahmed and Talib (2018) where the normal dose of Ovaprim® gave the best fertilization rate (95%) followed by 89.33% for 0.4ml.kg-1 and 74.33% for 0.3ml.kg-1. However, the current trial gave a fertilization rate of 84% for normal dose Ovaprim®. This result is quite higher than the value of 73.72% reported by Ameer *et al*., (2021). In a trial using doses of Ovaprim® between 0.4 to 0.6ml.kg^{-1} on common carp, More *et al*., (2016), reported fertilization rates between 92% and 96.02%. This is quite high compared to the current study which varied between 79% and 93%. The reason for this lies in biological quality of the eggs [29].

The current study has shown that diluted Ovaprim® co-administered with Clomid® elicits success of ovulation and hatching in *Clarias gariepinus*. The hatching rate ranged from 28.5% (T2) to 54% (T3). According to Olaniyi and Akinbola [2], Ovaprim® at normal dose gave a hatchability of was 46.3%. This value falls within the range currently reported but is far higher than the value of 29.5% recorded for normal dose of Ovaprim®. Hatching rate of 81.77% as reported by Ameer *et al*., (2021) also surpasses the current value obtained. There are two possibilities that can explain the disparity of results. Firstly, failure of the hypophysis to release luteinizing hormone has been reported as a factor behind spontaneous deposition of eggs that are not fully matured [30]. Secondly, the administration of clomid in the groups treated with diluted Ovaprim® would be effective if a follow up dose was administered to ensure full development of the eggs [31]. However new frontiers in the process of final ova maturation and ovulation of viable eggs calls for further research [32] (Robker *et al*., 2018).

The range of survival rate of larvae at the first feeding stage recorded in the current study compares favourably with 75.42% reported by Ameer *et al*., (2021) as well as 79.68% for Ovaprim® treated fish as reported by Onyia *et al*., (2021). In a report on induced breeding of wild-caught scaper (*Capoeta trutta*) using Ovaprim® and domperidone, Zadmajid, et al. [5] achieved 95.08% survival rate at 6 days post hatching. This result is quite high especially for a wild-caught spawning trial. According to Bromage, et al. [33], the establishment of sustainable culture for a species relies heavily on availability of "high-quality" gametes because survival potential of hatchlings is hampered by poor gamete quality. Bonnet, et al. [34] defines gamete quality as the ability of eggs and sperm to generate developing embryos with normal features. This however is a function of maternal factors such as szie, age and condition factor [35, 36]. Since there was no significant difference in the survival rates of first feeding fry, hormone administration played no part in the survival of fry. This runs contrary to the report by Okere, et al. [37] who posited that hormone administration affected fry survival. In addition, water quality is critical to fry survival [38]. There was no significant correlation between any of the water quality parameters and the survival rate recorded in the current experiment. This suggests that water quality played little role in the survival of fry to first feeding.

The use of diluted Ovaprim® co-administered with Clomiphene citrate has been successfully demonstrated to be a viable option for inducing Final Ova maturation (FOM) for artificial stripping and fertilization in the African catfish (*Clarias gariepinus*). The use of 0.4ml.kg-1 up to 0.2ml.kg-1 of Ovaprim® together with 0.9mg.kg-1 dose of Clomid® are as effective as 0.5ml.kg-1 dose of Ovaprim® alone.

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