INDUCTION OF TRIPLOIDY AND ERYTHROCYTE CELL SIZE ANALYSIS OF TRIPLOID AFRICAN CATFISH, *CLARIAS GARIEPINUS* (BURCHELL, 1822)

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ABSTRACT

In this study, genetic manipulation technique was employed to produce triploid African catfish Clarias gariepinus. Artificial fertilization was carried out using synthetic hormone (Ovaprim®) at 0.5 ml/kg body weight. Eggs numbering 100 ± 10 in quadruplicates from C. gariepinus were fertilized by milt from the male species. The fertilized eggs were subsequently transferred to a thermoregulated refrigerator at 2°C for 20 minutes to suppress cell division 4 minutes after fertilization. Haploid larvae were produced by fertilizing the eggs with ultraviolet (UV) irradiated milt at 30000 µWcm^{-2} for 15 minutes. Fertility, hatchability and survival after one week for triploids were 82.5%, 69.8% and 61.3%; for haploid, 100%, 15%, 0% and diploid (controls), 100%, 93% and 91%, respectively. There was significant ($p<0.05$) variation among the ploidy catfish larvae developed across the parameters of fertility, hatchability and survival determined. The ploidy levels of the triploid species were evaluated by karyotyping and erythrocyte measurements. The chromosome numbers obtained were 28 ± 2, 56 ± 2 and 84 ± 2 for haploid, diploid and triploid treatments, respectively. Erythrocyte cell size measurements of the triploid C. gariepinus species revealed bigger and larger cellular volume of about 1.5 times to that of the diploid.

Keywords: Genetic manipulation, Triploid, African catfish, *Clarias gariepinus,* Ultraviolet, Karyotyping, Erythrocyte, Chromosome

INTRODUCTION

World fish productions contributes about one-fifth of all animal protein in human diet (Olaniyi and Omitogun, 2014). With this, productions need to be increased in a sustainable manner to meet up with the estimated requirement of additional 40 metric tons of fish per year in the next two decades (2030); and even more by the year 2050 when global population is expected to grow to 9.2 billion (UNPP, 2008; Jones and Thornton, 2009). Sustainable aquaculture development will be indispensable to cater for food insecurity resulting majorly from the challenges of declining captured fisheries. Aquaculture development perhaps a panacea to food insecurity based on evaluation and application of appropriate technologies such as genetic engineering and other genomic technologies. These technologies have been employed to enhance the genetic merit of animals such as growth performance, sex control, environmental and disease tolerance etc. Biotechnology techniques such as all-male production or paternal inheritance (androgenesis), all-female production or maternal inheritance (gynogenesis) and production of progeny with additional set of chromosome (induced polyploidy) have really been in use as improved breeding techniques.

Studies have been carried out on ploidy or chromosome set manipulations in many aquatic species (Chourrout, 1980) employing these improved techniques. Reports and reviews on these techniques (Thorgaard, 1983; Don and Avtalion, 1986; Lu *et al.*, 1993; Horvath and Orban, 1995) have contributed significantly to research potential of ploidy or chromosome set manipulations in plants and animals, particularly in fish (Olaniyi, 2014). These studies are development in fish breeding strategies and are used in its management to sustain the ever-increasing human population. Studies on triploidy has been reported to cause sterility in fish (Purdom, 1972; Gervai *et al.*, 1980) due to its chromosome number (3n), and perhaps in turn leads to reduction in gonado-somatic index; that is the energy needed for gametogenesis can be used in flesh production (Don and Avtalion, 1986). This also leads to effective management of some species that usually have precocious sexual development and breed uncontrollably in water bodies (da Silva *et al.,* 2007).

African catfish *Clarias gariepinus* Burchell, 1822 is a model species that has been widely cultured in the tropical and subtropical countries of Africa and Asia (Hecht *et al.*, 1996). However, it has not been fully optimized in many African countries due to lack of continuous targeted selection programme (Volckaert and Agnèse, 1996). Hence, continuous research is still necessary on its aquaculture potentials using recent technologies. This study therefore has employed chromosome set manipulation to induce triploidy in *C. gariepinus* and its ploidy level was verified using cytogenetic and erythrocyte size techniques.

MATERIALS AND METHODS

Experimental Location and Broodstock Management: The study was carried out in the Biotechnology and Wet Laboratories of the Department of Animal Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. *C. gariepinus* broodstock (900 ± 100g) were obtained from reliable commercial fish farms in Ile-Ife, Southwestern part of Nigeria, and maintained in black fibre glass tanks (2m × 1m × 1.5m) for acclimatization. They were fed with 6.0 mm Coppens (Netherlands) pelleted fish feed (45% crude protein) twice daily at about 009 and 17.00 hrs.

Artificial Breeding: Sexually matured broodstock, both female and male were induced to spawning by treating them with ovaprim (Syndel, Canada) hormone. The broodstock were injected intramuscularly with 0.5 ml of the hormone per kg body weight, and left for 12 hours latency period (Olufeagba *et. al.,* 1997; Fafioye and Adeogun, 2005; Coppens, 2011). The testes were dissected out and eviscerated laterally from the male broodstock then macerated to extract the semen. The female broodstock were stripped to get the eggs by gently pressing its abdomen with the thumb from the pectoral fin towards the genital vent, thereby making the ovulated eggs ooze out from the urogenital orifice easily in a thick jet that were collected into a dry bowl (Olaniyi and Omitogun, 2012, 2013).

Fertilization: Good quality greenish olive eggs were randomly chosen for fertilization by simple observation, while the batch with white eggs and those contaminated with blood or excrement were discarded. One hundred (100 ± 10) eggs were randomly scooped with a spatula and measured. The milts i.e. irradiated (through UV-sterilization chamber at 5 cm distance under the UV lamp providing 30000 μW/cm^2 at 254 nM wavelength for 15 minutes) and non-irradiated milts were diluted with 0.9% saline solution, and 0.5 – 1.0 ml milt was used for the fertilization of egg. Diploid control and triploid treatments were fertilized with normal (non-irradiated) milt while the irradiated milt was used for the fertilization of the haploid control eggs. After 3 minutes of fertilization, the control treatments were set in hatching tanks except the triploid treatment that was subjected to 2^{o}C cold shock for 20 minutes to suppress cell division, and was later set in hatching tank. Each of the treatments was carried out in quadruplicates.

Post-fertilization Management: The treatments were incubated in breeding tanks provided with an air-pump for oxygenation. The fertilized eggs were spread in single layers over a suspended nylon meshed net (1 mm). Water parameters such as pH, alkalinity and dissolved oxygen were monitored, and temperature was measured with a Celsius thermometer, while the optimum oxygen level was maintained with RESUN LP- 100 low noise air-pump. At yolk absorption stage the fry were fed with *Artemia naupli* shell free feed, 54% crude protein (INVE Aquaculture Nutrition, Ogden, Utah, USA). Fertility, hatchability and survival were monitored and recorded for a week.

Fertility, Hatchability and Survival: The fertility was evaluated by simple observation of the morula (many-celled stage) 3 hours after fertilization. Percentage hatchability (equation 1) was calculated by counting the number of larvae hatched (N_H) with respect to the number of eggs fertilized (N_F). The survival (equation 2) was calculated at the end of the 7th day for each treatment as the relative percentage of survival multiplied (×) at a particular stage [Day 7 (z)] following Don and Avtalion (1986). % hatchability = $N_H \times 100 / N_F$ (Equation 1) and % survival (X_z) = $n \times 100 / i \times c/100 = n \times 10^4 / i \times c$ (Equation 2), where; c = the absolute percentage of fertile eggs (% of morula at 2 – 5 h); i = is the initial number of the eggs; and n = the number of eggs which survived up to a given developmental stage (i.e. day 7).

Ploidy Evaluation: Post-hatched larvae (1-day old) were incubated in 0.02% Colchicine (BDH) for 3 hours and later treated firstly in distilled water for 5 minutes, and then in a diluted catfish serum 1: 3 with 0.9% NaCl solution for 25 minutes. The fragments were later fixed in acetic acid–methanol mixture (1:4 v/v) and the fixed cells were aged for 4–7 days. Then chromosome preparation was done and stained with 20% GIEMSA (Scharlau) solution in phosphate buffer solution (PBS) for 20 minutes. The labeled slides were later screened under a light microscope (x 40) 4 days after the chromosome preparation and were then photographed at higher magnification objective (x100). The chromosomes were counted and representative spreads were photographed using light microscope (Model N – 800M) mounted with Canon Power Shot A 640/630.

Erythrocyte Cell Analysis: Blood samples were collected from both the diploid and triploid catfish. The blood were drawn from their haemal arch using 0.2 ml sterile hypodermic syringes and immediately released into EDTA bottles. 20 µl anticoagulated blood samples of each of the fish were added to erythrocyte diluting fluid in a 75 × 10 mm tube. These were well-mixed for 2 minutes, thereafter the cell suspensions were charged to Improved Neubauer counting chamber. The cells were allowed to settle before taking the measurements. The length and width of the cells and nucleuses were measured with the aid of biological computer-aided light microscope mounted with digital camera; these measurements were used in their calculated volumes based on the assumption that the cell and nucleus are perfect ellipsoids.

Statistical Analysis: Data recorded for fertility, hatchability, and survival were subjected to statistical analysis using General Linear Model procedure with Duncan option, which runs the two-way analysis of variance (ANOVA) (SAS, 2003). The same analysis was also employed for diploid and triploid erythrocyte cells' size comparison.

RESULTS

Parameters such as pH, alkalinity, dissolved oxygen and temperature average measurements were 7.1 ± 1.0, 112.31 ± 1.14 mg/l, 4.5 ± 0.5 mg/l and 27.0 ± 2.0 ^oC, respectively. Both haploid and diploid controls gave same mean fertility of 100% which were significantly different ($p<0.05$) from the triploid treatment that was 82.5% (Table 1). Hatching commenced in the controls almost at the same time, 26 hours post-fertilization, while hatching of the triploid occurred 30 minutes later.

Table 1: Effects of genetic manipulation on fertility, hatchability and survival in *Clarias gariepinus*

Parameters	Haploid Control	Diploid Control	Triploid
Fertility (%)	100[a]	100[a]	82.5 ± 6.4[b]
Hatchability (%)	15 ± 1.6[c]	93 ± 8.1[a]	69.75 ± 6.4[b]
Survival (%)	0[c]	91 ± 4.0[a]	61.25 ± 9.8[b]
Time of hatching after fertilization (h)	26	26	26. 5

[abc]Means within a row with different superscripts are significantly different (P<0.05).

Table 2: Erythrocyte size measurements of diploid and triploid *Clarias gariepinus*

Parameters	Diploid	Triploid
CL	12.20 ± 0.73[b]	15.75 ± 1.15[a]
CW	10.88± 0.87[b]	11.80 ± 0.99[a]
NL	4.33 ± 0.62[a]	5.66 ± 1.04[a]
NW	3.80[a] ± 0.73	3.85 ± 1.11[a]
CL×CW	132.88 ± 15.96[b]	186.65 ± 28.26[a]
CL+CW	23.08 ± 1.35[b]	27.55 ± 2.04[a]
NL×NW	13.00 ± 4.85[a]	22.49 ± 10.05[a]
NL+NW	7.13 ± 1.38[a]	9.50± 1.99[a]
CL/CW	1.13 ± 0.09[b]	1.34 ± 0.06[a]
NL/NW	1.12 ± 0.09[a]	1.54 ± 0.35[a]
CL/NL	2.86 ± 0.41[a]	2.87 ± 0.55[a]
CW/NW	2.95 ± 0.67[a]	3.37 ± 1.29[a]
Vc	428.38 ± 84.70[b]	655.98± 155.41[a]
Vn	19.63 ± 9.88[a]	28.29± 20.77[a]
Vc/Vn	27.02± 15.26[a]	40.89± 43.14[a]

[ab]Means, ± SD within a row with different superscripts are significantly different (P<0.05). CL = Cell Length; NL = Nucleus Length; CW = Cell Width; NW = Nucleus Width; Vc = Cell Volume; Vn = Nucleus Volume. Cells were assumed to be perfect ellipsoids for calculated cell and nucleus volumes, thereby, $V = 3n\ ab^2/4$; where a = CL/2 or NL/2; b = CW/2 or NW/2.

Hatchability was significantly different ($p<0.05$) in all the treatments, it was highest in diploid control (93%), then followed by triploid (69.75%) and lowest in haploid control (15%) that were observed not to survive up to yolk-absorption stage. Mean survival were all statistically different ($p<0.05$) in all the treatments (Table 1). The diploid control gave the highest mean survival of 91%, triploid had 61.25%, while there was zero survival for haploid control. Ploidy chromosomal number were n = 28 ± 2, 2n = 56 ± 2 and 3n = 75 ± 2 for haploid control, diploid control and triploid treatments, respectively (Figure 1).

The erythrocyte sizes of diploid and triploid catfishes produced were significantly different ($p<0.05$) in all the parameters except in nucleus length and width, combined parameters - NL × NW, NL + NW and calculated nucleus volume, Vn (Table 2). The cell volume (Vc) was the most significant that particularly characterized the triploid catfish from the diploid (Figure 2). In this study, the Vc and Vn of triploid was about 1.5 times than that of diploid.

DISCUSSION

The differences in fertility data primarily rest on fecundity, egg size and egg quality though egg size determines its quality (Bromage and Roberts, 1995). Some fish species are reported to produce at least two kinds of eggs by a single female simultaneously.

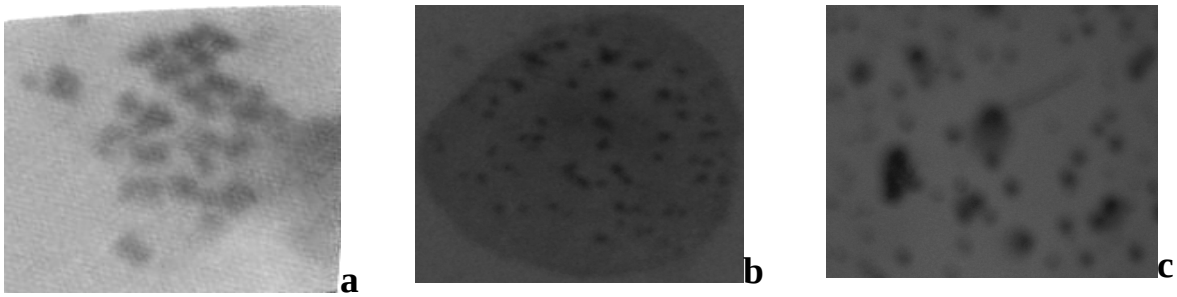

Figure 1: Representative metaphase chromosome spreads (X1000) from 1 day old larvae of *Clarias gariepinus,* (a) haploid larva*,* n = 28 ± 2 (b) diploid larva, *2*n = 56 ± 2 (c) triploid larva, 3n = 84 ± 2

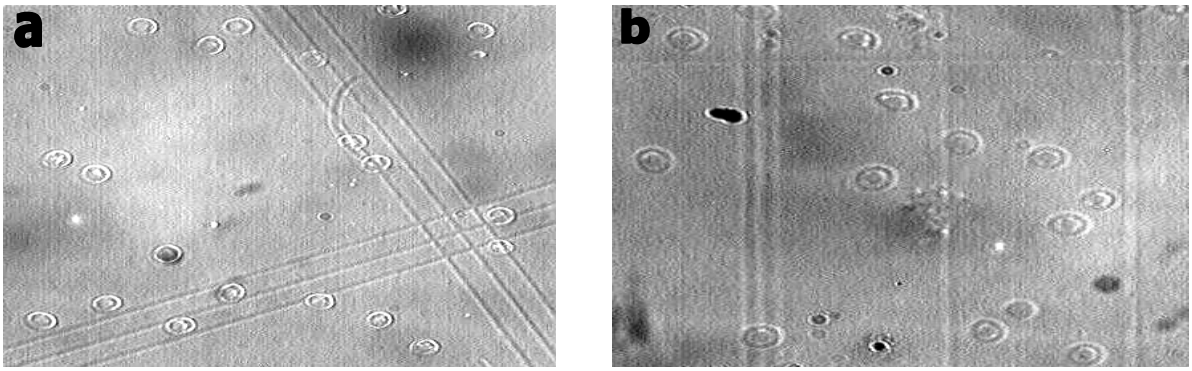

Figure 2: Erythrocyte cells from (a) diploid; and (b) triploid, *Clarias gariepinus* (x400)

It was reported that a polyploid (3n) loach simultaneous produces small, intermediate and large eggs carrying n, 2n and 3n genomes (Pandian and Koteeswaran, 1998). The differences in hatchability can be explained in terms of biological developmental process from fertilization to hatching, which is dependent on water temperature. The higher the water temperature the faster the eggs hatched and with better survival (de Graaf and Janssen, 1996; Olaniyi and Omitogun, 2013, 2014). The temperature of 27 ± 2^{o}C employed in this study falls in the recommended optimal temperature of 25 – 29 ^{o}C for African catfish's egg incubation in the hatcheries (Legendre and Teugels, 1991). Early study on temperature of the egg incubation of African catfish reported no hatching at 21 ^{o}C and the duration of 26 – 33 hours at 25 ^{o}C (Legendre and Teugels, 1991). Moreover, Olaniyi and Omitogun (2013) reported the hatching time of 17 hour at temperature of 28.5 ± 0.5^{o}C, and hatchability of 85% in their embryological study of *C. gariepinus.*

The kind of inductors applied, cold shock and UV-irradiation significantly affected the hatchability of triploid and haploid control treatments, respectively (Pandian and Koteeswaran, 1998). The most significant difference of the diploid control over other treatments was due to the effect of inductors applied, that is, cold shock and UV-irradiation. These ultimately affected survival of triploid and haploid control treatments (Pandian and Varadaraj, 1990; Pandian and Koteeswaran, 1998). However, these inductors have been employed and proved to be the best for genetic manipulation study being that UV-irradiation is safe and inexpensive compared to other forms and sources of irradiation (Stanley, 1981; Thorgaard, 1983; Bongers *et al.,* 1995; Karayücel and Karayücel, 2003). Other sources of irradiation such as gamma ($_{Y}$) and x rays have high penetrating power but they induce chromosome breakages (Thorgaard, 1983; Myers *et al.,* 1995). Cold shock has been reported to be the effective method of induction to suppress cell divisions in freshwater fish

(Tiwary *et al.*, 2004). Cold temperature (0 – 4 ^{o}C) has good effect over heat temperature in the induction process in warm water fish like *C. gariepinus*, and reverse is the case for cold biotype fish (Olaniyi, 2008).

The erythrocyte cell analyses showed that the triploid cells and nuclear sizes are bigger, and their volume was 1.5 times more than that of diploid. This corroborate earlier studies that erythrocytes cells' sizes analyses can be employed to identify ploidy levels in fish (Benfey and Sutterlin, 1984; Don and Avtalion, 1986; Thorgaard and Allen, 1987; Liu and Cordes, 2004). The result from this study is also in line with the studies of Valenti (1975) and Don and Avtalion (1986) that respectively reported the approximate polyploidy / diploid ratio of 1.5 and 2 for nuclear and cell volumes in *Tilapia* species. The ploidy levels of the embryos from the treatments as revealed by the karyotype were in line with the reports of Teugels' (1982), Eyo (2005) and Ojo *et al.* (2011) that the ploidy chromosomal number (2n) of *C. gariepinus* is 2n = 56. However, we had few variations in the expected triploid chromosome number (3n) to be ~84 since n = 28 ± 2, this may be due to karyotypical polymorphism, therefore further research on the karyotype and corresponding morphological characteristics (metacentric, submetacentric, subtelocentric, acrocentric etc) especially for different strains from respective areas and localities will be more beneficial.

The successful production and survival of triploid catfish produced in this study employing a simple technique of using cold shock (2^{o}C for 20 minutes) in a thermoregulated refrigerator showed great promise. The ploidy determination which revealed odd number of chromosome sets for triploidy is expected to cause gametogenetic failure (gonadal sterility). This is expected to result in faster growth rate of triploids that are required to shunt energy from gamete production to somatic growth. Hence further study is needed to follow the triploids to adult or matured stage to fully ascertain the observed faster growth rate and their sterility.

ACKNOWLEDGEMENTS

The financial grant of World Bank/Federal Government Science and Technology Education Post Basic, Innovators of Tomorrow [STEPB (IOT)] (Cr. 4304-UNI) Abuja, Nigeria is gratefully acknowledged. The authors thank Mr. Akinlolu Babatunde of Biotechnology Laboratory, Department of Animal Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria, for his technical assistance on the study.

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