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Full Length Research Paper

Monosex production of African catfish, *Heterobranchus longifilis*, through gynogenesis and androgenesis

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Chromosome engineering is a biotechnology tool currently used for fish improvement. *Heterobranchus longifilis* gynogens were produced by fertilizing eggs with irradiated sperm of the same species and subjecting the zygote to cold shock at 5°C, 40 min after fertilization for 15 min. To induce androgens, irradiated eggs were fertilized with normal spermatocytes and allowed to incubate for 40 min, then cold-shocked at 5°C for 15 min in a thermoregulated chamber to restore diploidy. Induction of gynogenesis and androgenesis was possible as 50.7 and 6.7% hatchability were obtained, respectively. After six days of rearing, the percentage survival of gynogens (97.8%) was better than that of the androgens (6.25%).

Key words: African catfish, *Heterobranchus longifilis*, polyploidy, gynogenesis, androgenesis.

INTRODUCTION

Biotechnology can be defined as the use of biological processes for the improvement of the characteristics of economically important plants and animals. This has been a tool for the improvement of fish stock employing chromosome manipulation techniques, leading to desirable qualities (Kowtal, 1987). The techniques more commonly employed include polyploid induction (animals having more than two complete sets of chromosome, e.g., triploidy, tetraploidy, etc); gynogenesis (all females; development with only maternal chromosome), and androgenesis (all males; development with only paternal chromosome). Growth improvement has been accomplished through the use of hormones or chemicals (McElwee et al., 2002). However, chemical treatment of food fish has become increasingly constrained due to fear for the safety of human consumption; therefore the use of chromosome manipulation is a most welcome practice (Shelton, 1987; Dunham et al., 2000).

Chromosome engineering which could involve ploidy alteration or euploidy induction with contribution from a single parent is an alternative control mechanism. Gynogenesis involves diploidization of the maternal genome through polar body retention or mitotic interference (McElwee, 2002). Diploidy is restored in androgenesis by interference with the first mitosis for eggs that have been genome-neutralized before fertilization. Diploidy can also result from the suppression of the second meiotic division and the retention of the second polar body, similar to the mechanism proposed for the establishment of triploidy in cold-shocked fish (Svardson, 1945; Ihssen et al., 1990). Temperature treatment interferes with normal action of the spindle and extrusion of the second polar body. Consequently an extra haploid set of maternal chromosomes is retained (Ihssen et al., 1990). Gynogenesis is of high importance in fish biotechnology research. It serves as a tool for monosex production and sexual studies. If the female is the homogenetic sex, all-female lines can be produced by gynogenesis in one generation. All-female gynogenetic diploids have been observed in common carp (Golovinskaya, 1969), rainbow trout and coho salmon *Oncorhynchus kisutch* (Refstie et al., 1982).

Chromosome neutralization could be achieved through different methods. This include ultraviolet (UV) light, gamma ray, etc. (Tave, 1992). UV irradiation is preferred for its simplicity and safety but also because it dimerizes

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Table 1. Methods for induction of gynogenesis and androgenesis in fish.

Methods	Results	References
in loach weatherfish		
X-irradiated sperm 2-4 kGy plus early heat 33-34°C I = 0 mm, D = 4 min	Gynogenetic diploids; up to 36% survival.	Romashov et al. (1960)
X-irradiated eggs	Androgenetic haploids; non-viable	Romashov and Belyaeva (1964)
in common carp		
X-irradiated sperm 1 kGy plus early cold 8-9°C, I = 0 min D = 3.5 h	Gynogenetic diploids; 8% survival	Cherfas (1975)
UV-irradiated common carp sperm	Spontaneous gynogenetic diploids; 2.1% survival up to 3 months	Stanley and Jones (1976)
in brook trout		
1) ^{60}Co gamma-irradiated eggs 0.88 kGy plus late pressure 595 kg/cm^2, I = 7.5 h, D = 3 min	Androgenetic diploids; 37% survival to the eyed-egg stage	May et al. (1988)
i**n rainbow trout**		
^{60}Co gamma-irradiated eggs 3.6 kGy plus late pressure 630 kg/cm^2, I = 5.75 h, D = 1-3 min	Androgenetic diploids; 40% survival to hatching	Parsons and Thorgaard (1985)

Early cold, heat or pressure treatments were applied before or during second meiosis, late treatments were applied just before or during first mitosis. I: Interval between fertilization and start of treatment; D: Duration of treatment; UV: Ultraviolet. Survival was to first feeding, unless otherwise indicated (after Ihssen et al., 1990).

the DNA rather than fragmenting it (Bhise and Khan, 2002). Androgens may be produced in two steps; the first is the activation (fertilization) of irradiated eggs with normal sperm, which produce a haploid androgen unable to survive; thus, a shock is applied following the first cleavage (34 min after fertilization in *Clarias gariepinus* (Aluko, unpublished), to block the subsequent cell division and permit the fusion of the two haploid nuclei to form a stable diploid, which is a pure inbred line (Stanley and Jones, 1976; Bhise and Khan, 2002; Tave, 1992).

Androgenesis is desirable for the production of viable super-males (YY) in male-heterogametic species, inbred isogenic lines and conservation of germplasm. If androgens are successfully raised to sexual maturity and then crossed with normal female, they could produce all-male progenies. This phenomenon had been reported in species like cyprinids, cichlids and salmonids (Bhise and Khan, 2002).

Gillespie and Armstrong (1980) first produced androgenetic diploid vertebrates by UV inactivation of the egg nucleus and hydrostatic pressure to suppress the first mitotic division. Androgenetic haploid fish have been observed repeatedly, but attempts at diploidization by suppression of the first mitotic division have been difficult (Parsons and Thorgaard, 1985; May et al., 1988). Examples and methods of androgenesis and gynogenesis are shown in Table 1. Activation haploid zygoterequires diploidisation, without which it cannot survive long. The timing of shock usually is targeted to coincide with a cytological event such as disruption of the spindle fibers during metaphase to prevent karyokinesis or interference with the cell duplication during cytogenesis. Shock type and intensity, duration and time of application must be optimally combined into a protocol for maximum yield of diploid progeny. Because the shocking temperature usually is near the lethal point, this eventually increases mortality of embryos; moreover, the genomic diploidization reduces fitness by increasing homozygosity; finally, it is that viable, all-male maturity will be quite low.

MATERIALS AND METHODS

Broodstocks of Heterobranchus longifilis (VAL, 1840) were obtained from Onitsha (Nigeria) within the rain forest Anambra River Basin (5.00 to 6.10 N, 6.47 to 7.50 E) and transported to the National Institute for Freshwater Fisheries Research (NIFFR) New Bussa where they were acclimatized for two months. One male and one female were injected with Ovaprim Agrivet Farmcare hormone at a dose of 0.5 ml/kg of fish. After 15 h of latency period, eggs were stripped by a slight pressure applied on the abdomen, and collected into dry Petri dishes. The male was sacrificed and dissected to expose the testes that were removed and kept in Petri dishes until time of use. The Methods for induction of gynogenesis and androgenesis in fish is given in Table 1.

Androgenesis

Batches of 150 eggs (10) each were placed in four 9 cm diameter Petri dishes at 26°C. Eggs were spread to form a single layer to

Table 2. Hatching and survival rate in embryos of not-irradiated eggs and sperm of *H. longifilis* (diploid).

S/N	Treatments	No. of eggs fertilized	Number of hatchlings	Percentage hatchability (%)	Survival at day (%)		
					2	4	6
1	1	150	147	98.2	140 (95.2)	140 (100)	138 (98.6)
2	2	150	145	96.7	138 (95.2)	135 (97.8)	135 (100)
	Mean	150	146	97.4	139 (95.2)	138 (99.3)	136 (98.6)

Table 3. Hatching and survival rate in embryos of irradiated sperm and not-irradiated eggs of *H. longifilis* without cold shock (haploid - gynogenesis control).

S/N	Treatments	No. of eggs fertilized	Number of hatchlings	Percentage hatchability (%)	Survival at day		
					2 (%)	4	6
1	1	150	98	65.3	3 (3.1)	0	0
2	2	150	97	64.3	1 (1.0)	0	0
	Mean	150	98	64.8	2 (2.0)	0	0

ensure that all the eggs were irradiated. Each Petri dish was exposed in turn to 254/366 nm short-wave UV light (Model UVGL-15 Mineralight® multi-band light) from a height of 8 cm. Eggs were manually stirred to ensure uniform irradiation. After irradiation, not-irradiated sperm was used to activate the eggs. The exact time of fertilization was taken as time zero. After 40 min of incubation in glass aquaria maintained at 26°C, eggs were cold-shocked for 15 min in a thermoregulated chamber maintained at 5°C, to restore diploidy. After the shock, developing embryos were transferred back to the aquaria with clean, well-aerated water. Not-irradiated eggs were fertilized with normal sperm to serve as control. Embryos development was monitored under a photomicroscope (Zeiss Stemi 2000-C) fitted with a Contax 167MT camera.

Hatching and early survival rates were determined as described by Olufeagba (1999). Feeding of the hatchlings started on the second day when yolk absorption was almost complete. They were fed with mixed zooplankton obtained from the NIFFR fish-food production unit.

Gynogenesis

The stripping, activation, hatching, survival monitoring and feeding steps were similar to those followed for the androgenesis experiment, except that it was the sperm that was irradiated. The irradiated sperm was used to fertilize normal eggs, and the zygotes were cold shocked at 50C for 15 min starting 40 min after fertilization. Before to from experimental parameter interfering with the first mitosis was a cold in the same batch. These experiments were conducted thrice.

Confirmation of ploidy level

1. Functional analysis (haploid): Irradiated eggs (or sperm) were fertilized with not-irradiated sperm (or eggs); developing embryos were not cold-shocked (that is, no diploidy restoration).
2. Genetic determination: Ploidy level was determined through chromosome count, which was carried out on 10-12 h-old fry, as described by Olufeagba (1999).
3. Morphological appearance: Developing eggs and embryos were observed and monitored under the photomicroscope to compare their appearance with control diploid eggs and embryos (Olufeagba, 1999).

RESULTS

Different crosses resulted in successful activation and embryo development, even if the degree of development, hatching and subsequent survival varied. Details are shown in Tables 2 to 6.

Gynogenesis

Induction of gynogenesis was successful (meiotic gynogenesis). Hatching was first noticed 24 h after activation, which was about 1 h later than their diploid counterparts. The average hatchability was 50.6% with residual survival after 2, 4 and 6 days being 57.0, 97.5 and 97.8%, respectively (Table 6). Just like the haploid control of the androgens, morphological malformations were seen in both. These included curved tail and distorted head and trunk (Plate 1). Photomicrographs of mitotic chromosomes of the haploid and diploid androgens are shown in Plate 2; diploid gynogens chromosome count was 50, and haploid was 25. The morphology of the diploid (meio) gynogens was similar to the conventional diploid control. Some fry are + still alive (at 15/09/2010), and will be raised to maturity for further studies. Diploid control experiment (where not-irradiated eggs and sperm were used) the percentage hatchability rate was 97.4% and embryo development was normal (Table 2). Hatching occurred 23.16 h after fertilization, this high rate indicating a good quality of gametes. Mean residual survival after 2, 4 and 6 days of hatching was

Table 4. Hatching and survival rate in embryos of irradiated eggs and not-irradiated sperm of *H. longifilis* without cold shock (haploid – androgenesis control).

S/N	Treatments	No. of eggs fertilized	Number of hatchlings	Percentage hatchability (%)	Survival at day		
					2	4	6
1	1	150	18	1.2	0	0	0
2	2	150	4	2.7	0	0	0
	Mean	150	11	7.4	0	0	0

Table 5. Hatching and survival rate in embryo of irradiated eggs and not-irradiated sperm of *H.longifilis* with cold shock (androgenesis).

S/N	Treatments	No. of eggs fertilized	Number of hatchlings	Percentage hatchability (%)	Survival at day		
					2	4	6
1	1	150	13	8.7	12 (92.3%)	8 (66.1%)	*1 (12.5%)
2	2	150	7	4.7	0	0	0
	Mean	150	10	6.7	6 (46.2%)	4 (33.3%)	*1 (6.25%)

*that single androgen died 27 days after hatching.

Table 6. Hatching and survival rate in embryos of irradiated sperm and not-irradiated eggs of *H. longifilis* with cold shock (gynogenesis).

S/N	Treatments	No. of eggs fertilized	Number of hatchlings	Percentage hatchability (%)	Survival at day (%)		
					2	4	6
1	1	150	68	45.0	40 (60.0)	38 (95.0)	37 (97.4)
2	2	150	84	56.0	54 (54.0)	54 (100)	53 (98.1)
	Mean	150	76	50.7	47 (57.0)	46 (97.5)	45 (97.8)

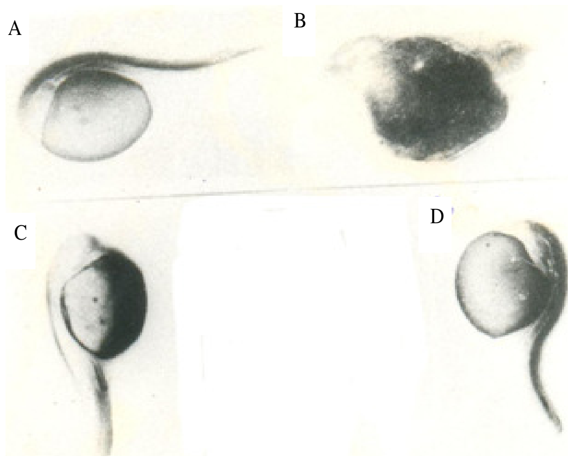

Plate 1. Morphological aberrations in (A) zygotes (B) hatchlings of androgens (C) gynogens and (D) conventional diploids *H. longifilis.*

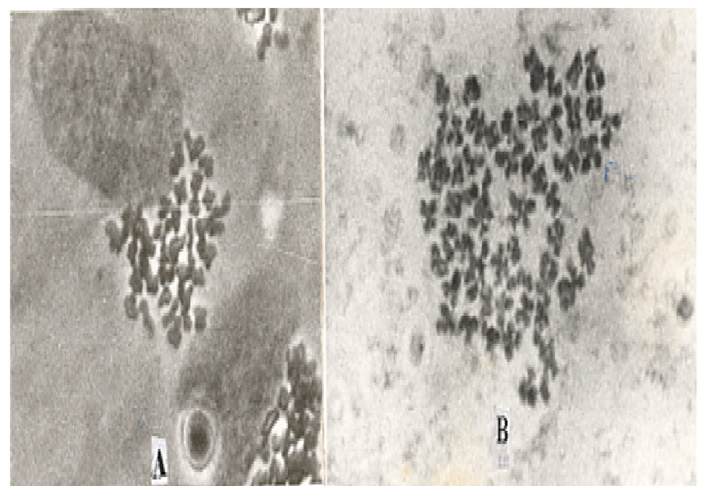

Plate 2. Photomicrographs (× 2600) of mitosis showing metaphase chromosomes in *H. longifilis*. A = haploid (n=25); B = diploid (n=50).

95.2, 99.3 and 98.6%.When irradiated eggs were fertilized with not-irradiated sperm (haploid gynogenesis control) but without cold shock, 64.8% of the eggs hatched; however 98.0% died within 24 h, and within

72 h, all the remaining died too (Table 3). The haploid syndrome caused some hatchlings to have abnormal embryos (Plate 1).

A similar observation was made in treatments where irradiated sperm was used with not-irradiated eggs (haploid androgenesis control) but without cold shock. However, unlike in the above case where some hatchlings survived up to 72 h, all died within 24 h (Table 4). Eggs earlier seen with white, translucid “shields” all died and did not develop further.

Androgenesis

In the first and second treatment, 13 and 7 hatchlings were recorded (Table 5); in a third one, no progeny was obtained. The shocking time in that experiment was delayed for 40 min after fertilization and the treatment failed to yield androgens (mitotic gynogens). It is obvious that androgens are more difficult to produce than gynogens. All cases of mortality, monitored under the photomicroscope, occurred mostly at the gastrula stage which seems to be the most critical phase. Mean hatchability obtained was 6.7% and mean residual survival on 2, 4 and 6 day after hatching was 46.2, 33.3 and 6.2%, respectively (Table 5). It is evident that lower mean hatchability and survival were obtained in androgens compared with gynogens.

DISCUSSION

It is not surprising that higher success level of hatching was obtained in gynogens production. This is due to heterozygosity remaining after diploidy restoration; in fact, Purdom (1969) observed that some crossing-over between genes and the centromere occurs in fish chromosomes, therefore gynogenetic diploids would have some levels of heterozygosity; moreover, studies carried out by Thorgaard et al. (1983) and Allendorf et al. (1986) showed high levels of heterozygosity in gynogenetic diploids. Golovinskaya and Romashov (1966) had already confirmed that gynogenetic diploids were due to meiotic crossing-over in the region between genes and the centromere. This eventually determines the rapidity of production of lines.

According to Thorgaard et al. (1985), UV irradiation had been used extensively because it is easy and safe to apply. Moreover, it leaves less residual paternal inheritance, which may lead to higher mortalities in gynogenetic diploids than using ionizing radiation; on the contrary, UV irradiation had been found to have low genetic inactivation potential on eggs, because of its small penetration power (May et al., 1988).

Low or zero androgen progeny had been reported for common carp (Stanley and Jones, 1976). In *Cyprinus carpio*, Bhise and Khan (2002) observed 100% mortality after 6 h of fertilization. This outcome is similar to our observation in *H. longifilis*, with the gastrula stage being the most critical period. Hatching and survival difficulties could arise because zygotes are diploidized by a late shock, causing all loci to be homozygous, which in turn results in an abrupt increase in the pairing of recessive detrimental or lethal genes. Therefore, even zygotes that survive the trauma of cold treatment may have reduced viability because of genomic influence.

Androgens are more difficult to produce than gynogens. This is because diploidy can better be induced at first cell division. This “window time” is a difficult period to manipulate the embryo. Also, androgens are totally homozygous, presenting a large amount of noxious genotypes (Scheerer et al., 1986).

Abnormally developing embryos of androgens were obtained by Onozato (1984) in coho salmon *Oncorhynchus keta*, but the viability of those embryos was not reported; he suggested that these traits may be the result of homozygous loci for noxious recessive alleles, which should be expected in mitotic gynogenetic diploids because of their complete homozygosity.

Nevertheless, mitotic gynogenetic diploids and androgenetic diploids are of interest because completely homozygous lines (clones) can be produced in two generations, compared to 10 or more generations for meiotic gynogenetic diploids (Nagy and Csanyi, 1982). The practical applications include the production of superior lines by cross breeding and the study of single-locus effects.

Androgenetic diploids could be used to study the phenotypic effects of cytoplasm constituents, and in the conservation of genetic resources because diploid individuals can be recovered using only sperm, and sperm, unlike eggs can easily be maintained in frozen storage indefinitely (Stoss, 1983). Thawed sperm, in conjunction with genetically inactivated donor eggs from a closely related stock or species could be used in the future to produce diploid individuals of an extinct stock or species.

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