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Improving Androgenesis by optimizing conditions of ultraviolet treatment in Nile tilapia, *Oreochromis niloticus* (L.)

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Abstract

The objectives of the present study were to produce homozygous male in Nile tilapia (*Oreochromis. niloticus*), where the male is heterogametic, by using UV-irradiation and heat shock treatments. Nile tilapia, *O. niloticus* brood stock was collected from the Lake Manzala population, Egypt and send to the Institute for Animal Breeding and Genetics, Goettingen, Germany, where this experiment was carried out. Fertilization of ultraviolet (UV) irradiated eggs was carried out by using a dosage of 254 nm at a fixed intensity of 58 mJ/cm^2 for different distances between UV-lamp and eggs via a heat-shock (41°C for 5 min started at 4.5 min post-fertilization) of Nile tilapia, *O. niloticus* with sperm from homozygous *O. niloticus* males induced by mitotic gynogenesis from the second and the third generations of low and high temperature sensitive selection induced. Treatment of eggs at 5 cm distance before fertilization proved to be sufficient to inactivate egg DNA. The present results showed that for the values of a sex ratio of gynogenetic males was mated with normal females (one male x one female) for control group with a lowest percentage of males (14%) and the highest percentage was 44%. For the experimental treatment groups, the results obtained that, at hatching stage (4 days post-fertilization), survival ranged from 0.57% to 3.50%. Although high survival rates were obtained at the hatching stage, they sharply decreased by the yolk-sac absorption stage and dropped by the swim-up stage (30-150 day post-fertilization). Sex ratio of all the progeny (treated groups) significantly differed from 1:1 while their respective controls did not significantly differ from the expected 1:1 ratio ($P>0.05$), with exception of one male which produced 14% male progeny. After testing the homozygous of fishes, they will be used to produce subsequent generation of androgenetic strains. This study may indicate successfully inactivated the maternal genome (nuclear DNA) in Nile tilapia eggs at 5 cm distance using UV-irradiation.

Keywords: Oreochromis niloticus, monosex, sex ratio, androgenesis, haploidy, diploidy

1. Introduction

Androgenesis has been used in numerous genetic studies and breeding programs, including rapid establishment of isogenic, clonal lines (Scheerer *et al*., 1991; Arai *et al*., 1995), sex control through induced YY males (Onozato, 1989; Scheerer *et al*., 1991), generation of nucleocytoplasmic hybrids between two different species (Grunina *et al*., 1991), and recovery of valuable strains from cryopreserved sperm (Thorgaard *et al*., 1990).

Egg chromosomes can be inactivated by C-ray, X-ray, and UV light. Androgens can be produced in two ways. The most common way is to fertilize irradiated eggs with normal sperm. This way aims to produce a haploid androgen and at first cleavage, a shock is given to prevent cell division, and the two haploid (N) nuclei fuse to form a diploid (2N) nucleus. The second technique that can be used to produce androgens is to fertilize eggs whose genetic material has been destroyed by irradiation with sperm from a tetraploid male. Consequently, the sperm pronucleus is diploid rather than haploid, which means the ensuing zygote, will also be diploid. Androgenesis has also been induced in a variety of other ways in different species (Stanley and Jones, 1976; Gervai *et al*., 1980; Briedis and Elinson, 1982; Liu *et al*., 1987).

The production of viable diploid progeny by androgenesis is much more difficult than gynogenesis when UV-irradiation is used. It causes several types of damage, including pyrimidine-dimers, DNA–DNA cross-links, pyrimidine adducts in many species. However, pyrimidine-dimer formation (T–T, C–T, C–C) in adjacent DNA bases is the most common type of UV-damage (Friedber, 1985). Similar damage occurs in RNA with regard to the pyrimidines uracil and cytosin.

Myers *et al*. (1995) performed Southern analyses of mtDNA from control and UV-irradiated eggs of *O. niloticus* to assess the extent of damage, and found no difference between their autoradiograms. However, the positive controls (purified mtDNA irradiated directly

with a 254-nm lamp) revealed extensive damage to the mtDNA. Due to the relative position of the egg pronucleus and the scattered distribution of mitochondria throughout the egg, the pronucleus perhaps suffers greater damage and even total inactivation, while a large number of mitochondria remain intact, partially or totally. Hitherto, the intensity of irradiation ranges from 100 to 7500 ergs/m^2 has been used (Masaoka *et al*., 1995).

Efficient procedures for genetic inactivation of eggs and restoring of the diploid have been studied in fish species, and successful androgenesis has been produced in loach spp. (Arai *et al*., 1992 ; 1995; Masaoka *et al*., 1995), trout spp (Parsons and Thorgaard, 1984 ; 1985; Scheerer *et al*., 1986; May *et al*., 1988; Thorgaard *et al*., 1990; Araki *et al*., 1995), *O. niloticus* (Myers *et al*., 1995), carp spp. (Stanley, 1976; Stanley and Jones, 1976; Grunina *et al.*, 1990; Bongers *et al*., 1994; Cherfas *et al*., 1994; Pooniah *et al*., 1995), amago salmon (Nagoya *et al*., 1996), zebrafish (Corley-Smith *et al*., 1996), and muskellunge (Lin and Dabrowski, 1998) by inhibition of the first mitosis, or by using diploid sperm obtained from tetraploid or fused sperm. However, the effects of UV irradiation on eggs have not been studied extensively. DNA fragments, probably of maternal chromosome residues, were found in haploid androgenetic loach and muskellunge after eggs were inactivated by UV irradiation (Arai *et al*., 1992; Lin and Dabrowski, 1998).

The successful development of androgenetic techniques for production of monosex populations of tilapia species relies on the assumption that sex determination in this species is based on inheritance of sex chromosomes. Therefore, the present study was conducted to induce homozygous male in Nile tilapia (*O. niloticus*), where the male is heterogametic, by using UV-irradiation and heat shock treatments.

2. Materials and methods

Nile tilapia, *O. niloticus* brood stock used for this study was collected from the Lake Manzala population, Egypt and kept at the Institute for Animal Breeding and Genetics, Germany (Müller-Belecke, 1997). Fish were cultured in the warm freshwater recirculation systems; lighting in the systems was adjusted by automatic timer to 12:12 hours (light : dark schedule). The water temperature was maintained at 28 ± 1°C.

Female brood stock was kept in partitioned glass tanks, while male brood stock was kept individually. Normal females (normal population) and homozygous males induced by mitotic gynogenesis (Samavati, 2008), either from the second or third generation of low and high temperature sensitive selection (Wessels, 2006), were stocked for all experimental treatment at sex ratio of (1: 1 male to female).

2.1. Fish breeding, stripping and fertilization of eggs

All experiments were carried out under aquarium conditions, females which are ready to spawn (mature females of *O. niloticus* spawn at approximately two to six weeks intervals) showed a swollen urogenital papilla and pre-spawning behaviour such as nest building and cleaning. After anaesthetizing the female, eggs were collected by applying gentle downward pressure with the fingers from below the pectoral fin to the genital opening of the fish. The eggs were collected and washed carefully with 0.02l saline solution (0.9% NaCl) several times until ovarian fluid and any blood were removed. Milt from Nile tilapia male delivering high quality sperm (high density and motility) was stripped, in a similar way to egg collection using a glass capillary tube, milt contaminated with water and/or urine were rejected, and mixed in saline solution 1:5 (Müller-Belecke and Hörstgen-Schwark, 1995).

Eggs were counted and fertilized by mixing the milt of an appropriate one male with eggs of one female and then 10-20 ml of aquarium water was added. The fertilized eggs were kept in the incubation jars for 4.5 min then transferred to the incubator until hatching.

In all experiments, a normal fertilization was carried out to check the quality of the eggs and sperm.

A batch of eggs was neither irradiated nor shocked and served as diploid control and a portion of the eggs was irradiated but not subjected to heat shock and used as the UV- treated haploid control to ensure complete deterioration of maternal genome. After day nine, fry were counted and transferred into 2 L tanks where first feeding was started with a high protein (47% XP) diet (Mikromin, Tetramin from TETRA-Werke, Germany). After day 30, fishes were counted and only 50 fishes were stocked into 80 L glass aquaria and given trout feed (F-0, Pro Aqua Brut, 57% XP, 15% XL).

When the fish have reached suitable body size, they were counted, sacrificed and their sex were determined by microscopic inspection of gonads. For treatment groups, sex was determined by checking the urogenital papilla.

2.2. Experimental design to optimize UV-irradiation of eggs

Optimization of UV-irradiation was carried out by irradiating 200 - 300 eggs, spread on a 9 cm diameter plastic Petri-dish forming a single layer. To determine the optimal distance that eliminate the female genome corresponding to UV-irradiation duration of 2 min and fertilized with sperm from the homozygous males (Samavati, 2008), the UV-Hand lamp (254 nm) with an intensity of 58 mJ/cm^2 was used at different four distances of 5, 10, 15 and 20 cm between the lamp and the egg samples for five batches, and at other four distance of 5, 7.5, 10 cm for another four batches as preliminary experiments. Egg samples were manually stirred in glass Petri dishes to ensure uniform

irradiation. The entire procedure of irradiation was completed under total darkness (Friedberg, 1985).

After irradiation at the right distance; i.e., which all the fry (without subsequent heat shock) died at day nine, egg samples were immediately mixed with sperm suspension and fertilized by adding enough freshwater (28°C). These eggs were incubated until day four. The exact time when the milt was added to fertilize the irradiated eggs was considered as zero time, which helped in determining the age of the embryo at the time of heat shock.

To depolarize the haploid androgenetic zygotes, the protocol for meiotic gynogenesis according to Puckhaber (1992); Puckhaber and Hörstgen-Schwark (1996) was used. The heat shock by transferring the eggs to thermo-regulator water-bath (HAAKE DC 10) at 41°C was applied for 5 min after 4.5 minutes post activation (Figure 1). Eggs were transferred, including controls, into individual flow-through incubators and its development was monitored through hatching and swim-up. Eggs were counted at three different development stages: hatching (4 days post-fertilization), yolk-sac resorption (9-11 days post-fertilization) and swim-up (30-150 day post-fertilization).

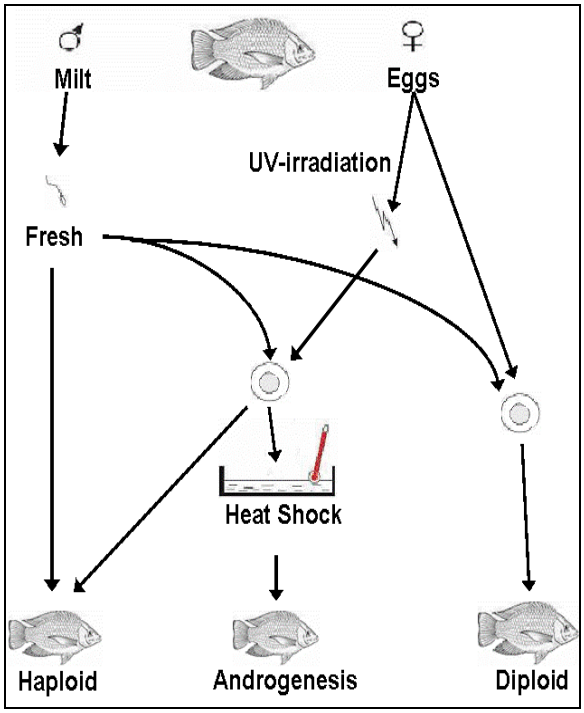

Figure 1. Schematic representation of the interspecific and rogenesis experiment performed for Nile tilapia.

2.3. Parameters recorded and statistical analysis

During all experiments, survival rates at different development stages and sex ratios were measured and recorded. Since the egg quality of each spawn varied greatly within and between females which influenced the number of eggs to be fertilized, the survival of each treatment was calculated relative to the survival of the corresponding diploid control.

Survival rate of the diploid control was calculated as: (number of survived at a given development stage / total number of eggs) x 100 and the results were presented as mean and standard error of mean (Mean ± SEM). Sex ratios were analyzed by a Chi-square test (X^2 goodness of fit) to investigate whether they statistically differed from the expected 1:1 (Gardiner, 1997; Lomax, 2007). All statistical analyses were performed by SPSS 15 software.

3. Results and Discussion

Eggs obtained from the single spawn, irradiated by UV light at 5 cm distances between the eggs and the UV lamp, were conducted to test fertilization (survival to yolk-sac absorption stage). The highest mean fertilization rate values (90.43 ± 1.90%) was obtained in the diploid control while the lowest values (0.75 ± 0.34%) was obtained at 5 cm distance; complete mortality of haploid was recorded at 5 cm at day nine (Table 1).

Sex ratio of five gynogenetic male mated with normal females (one male x one female) are shown in Table (2). The present study showed that the lowest percentage of males was 14%, meanwhile the highest percentage of produced was 44%. Sex ratio of all the progeny was significantly differed from 1:1 ratio, while their respective controls did not significantly differ from the expected 1:1 ratio (p>0.05). At hatching stage, survival in UV-treatment ranged from 0.57% to 3.50% (Table 3). Although high survival rates at the hatching stage were decreased sharply by the yolk-sac resorption stage and dropped by the swim-up stage.

The considerable variability between the sex ratios of the different progenies suggested that the mechanism of genetic sex determination is more complex, either due to polymorphism at the major sex determinant (multi-allelic) and/or the action of minor genetic factor(s), either autosomal or linked to the major sex determinant, as suggested in other studies highlighting such predominant parental influences. Besides genetic factors, environmental effects may influence sex ratio (lower viability of one of two sexes under given rearing conditions) or sex determination in *O. niloticus* (Müller-Belecke and Hörstgen-Schwark, 1995).

There is evidence that in domestic stocks of *O. niloticus*, sex ratios of individual pairs may vary from 50:50. Shelton *et al*. (1983) found that 21% of the pair spawns were outside a 95% confidence interval for an expected 50:50 ratio. Karsina (1993) reported that the percentage of 32% for 22 pair spawns did not conform to an expected 50:50 ratio. Lester *et al*. (1989) found sex ratios from 60 families which were ranged from 2 to 60% males. Abucay (1997) reported sex ratios ranging from approximately 30 to 70% males from

presumed XX females x XY males. Baroiller *et al*. (1993) mated hormone-induced "XX" male *O. niloticus* to normal XX females. The resultant groups of progeny should have been all female, but for fry held at 27 to 29°C they found that 26% of the spawns contained > 10% males and when at 32 to 36°C, 92% of the spawns had > 10% males. However, it is unlikely that the environmental effects have caused the sex ratio observed where the fishes were raised under completely controlled conditions, as explained before.

Table 1. The fertilization rate (relative to the diploid control) of Nile tilapia haploid androgenetic (Mean ± SEM).

Treatment	Statistics	Fertilization (%)
Diploid control [1)]	**Mean**	**90.43 ± 1.90**
	Minimum	82
	Maximum	100
5.0 cm [1)]	**Mean**	**0.75 ± 0.34**
	Minimum	0
	Maximum	3
7.5 cm [2)]	**Mean**	**16.91 ± 5.90**
	Minimum	3
	Maximum	31
10.0 cm [1)]	**Mean**	**25.90 ± 5.81**
	Minimum	9.71
	Maximum	60. 83
15.0 cm [3)]	**Mean**	**44.98 ± 6.47**
	Minimum	26.55
	Maximum	76
20.0 cm [3)]	**Mean**	**79.33 ± 3.24**
	Minimum	71.33
	Maximum	89.82

Note: [1)] n = 9
[2)] n = 4
[3)] n = 5

This study may indicate successfully inactivating the maternal genome (nuclear DNA) in Nile tilapia eggs at 5 cm distance using UV-irradiation. The results demonstrate that androgenetic diploids can be produced in *O. niloticus*, although the percentage was extremely low. Such poor viability was not surprising as it was similar to that of mitotic gynogenetic diploids and tetraploid fish obtained by suppressing the first cleavage by physical shock. The reason for low survival can be attributed to several factors:

1) Radiation can induce structural chromosomal aberrations which may be transmitted from cell to cell during subsequent cleavage (George *et al*., 1991; Lin and Dabrowski, 1998). Bongers *et al*. (1995) suggested that UV irradiation of eggs may damage the maternal RNA which is essential for development to the blastula stage, and thereby affect the differentiation process in embryonic development by altering cell fates and lineages.

2) Low survival might be the result of deleterious effects of the physical shock used to the second polar body. Chromosomal changes such as terminal deletion, exchange type deviation, inter or intra-arm exchange or inter-chromosome exchanges through rapid cell cycles caused by hydrostatic pressure treatment at the first cleavage were reported in gynogenetic *O. masou* (Yamazaki and Goodier, 1993) and in gynogenetic *Misgurnus anguillicaudatus* (Masaoka *et al*., 1995). These changes are similar to those induced by irradiation (Yamazaki and Goodier, 1993), aging or interspecific hybridization by Yamazaki *et al*. (1989)

3) Inbreeding depression arising from homozygosity of deleterious alleles was suggested for the low viability of androgenetic progeny (Bongers *et al*., 1994) and for mitotic and meiotic gynogenetic progeny (Yamazaki and Goodier, 1993).

Table 2. Sex ratio of progeny by mating with gynogenetic diploid males and normal female of Nile tilapia (diploid control and treatment).

No. Gynogenetic male tag number		No. of fish observed	Sex		Male (%)	X^2
			Male	Female		
1 HL25 00-06B8-876E	control	46	18	28	39	2.17
	treatment	1	1	0	100	1.00
2 HH25 00-06B8-96D2	control	39	16	23	41	1.26
	treatment	1	1	0	100	1.00
3 HH25 00-06B8-96D2	control	21	3	18	14	10.71
	treatment	1	1	0	100	1.00
4 HH25 00-06B8-A723	control	48	21	27	44	0.75
	treatment	2	2	0	100	2.00
5 HH25 00-06B8-B62C	control	64	20	26	43	0.78
	treatment	1	1	0	100	1.00

Table 3. Survival (%) of presumed androgenetic Nile tilapia to different development stages where eggs were subjected to UV-irradiation for 5 cm distance (n = 5) (Mean ± SEM).

Treatment	Statistics	Hatching stage (%)	Yolk-sac resorption stage (%)	Swim-up stage (%)
Diploid control (neither irradiated nor shocked)	**Mean**	**97.53 ± 10.23**	**74.29 ± 12.31**	**34.57 ± 5.36**
	Minimum	40	31	20
	Maximum	95.83	95.83	50
Haploid control (UV-irradiated but not shocked)	**Mean**	**0.20 ± 0.20**	**0**	**0**
	Minimum	0	0	0
	Maximum	1	0	0
Treated (UV-irradiated and shocked)	**Mean**	**1.71 ± 0.51**	**1.18 ± 0.18**	**0.68 ± 0.16**
	Minimum	0.57	0.57	0.50
	Maximum	3.50	1.50	1.33

Additionally, based on the hypotheses of Markert, 1982; homozygosity of androgenetic progeny may cause inviability through the disruption of topographic interaction of chromosomes of the interphase nucleus.

Although the obvious success of eggs inactivation (haploid in the preliminary experiments and haploid control as well), homozygosity of the offspring fishes should be insured by analyzing DNA-level polymorphism. DNA microsatellites are the most simplest and useful markers that are widely and efficiently used in the evaluation of the genetic status of different organisms. These markers are highly polymorphic tandem arrays of short nucleotide motifs dispersed throughout the nuclear genome of eukaryotes (Hamada *et al*., 1982; Crooijmans *et al*., 1997). Nevertheless, this study provides a valuable baseline dataset that will provide the background for later more focussed studies.

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