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INDUCTION OF DIPLOID ANDROGENETIC NILE TILAPIA *(OREOCHROMIS NILOTICUS)*

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ABSTRACT

According to the concise definition of Thorgaard (1986) androgenesis is an all paternal type of inheritance, where the genetic material of the egg cell does not contribute to that of the embryo. Therefore, androgenesis is a technique that could facilitate the rapid production of completely homozygous isogenic lines of fish.

Androgenesis was induced in *O. niloticus* by fertilizing UV-irradiated eggs with untreated sperms and then blocking the first cleavage division with heat shock. The optimized UV exposure period, based on the complete absence of diploids 48 hours post fertilization, was 15 minutes when a distance of 27.5 cm being kept between the UV lamp (245 nm) and the eggs. The haploidization nature of the embryo was tested by chromosome counts in 48 hour after fertilization embryos. Diploidy was restored by suppression of the first cleavage division using a heat shock of 41^{o}C. Heat shock treatment of 4.5 minutes duration applied after 26 minutes from fertilization gave 0.68% survival to yolk sac resorption stage.

INTRODUCTION

The tilapia are species of major economic importance in tropical and sub-tropical countries throughout the world. These species have not yet reached their full potential because of the problem of uncontrolled reproduction which often results in the

over reproduction of production ponds with young fish. The induction of androgenetic tilapia fish could be used for the production of YY males which can be used for the production of XY all males progeny to prevent the unwanted reproduction and this method is considered as an alternative to the use of hybridization and hormones as means of producing monosex male fry. Therefore, androgenesis can be utilized for producing monosex population, inbred strains and for different genetic investigations. Totally homozygous androgenetic diploids are considered genetically equivalent individuals and can be used for producing inbred strains for the investigation of fish growth and diseases.

The creation of androgenetic fish offers a method of producing high levels of inbreeding, potentially 100% in a single generation. Fig (1) summarizes the production of homozygous androgenetic *O. niloticus*. The principle androgenesis is to eliminate the genetic contribution of eggs, followed by artificially doubling the haploid genome. This can be achieved by treating eggs before fertilization with UV-irradiation which initiates the formation of thymidine-dimeres in adjacent base-pairs, rendering the DNA inactive (Thorgaard, 1983). After irradiation of maternal genome, it become inactivated and haploid zygotes are produced (Fig 1b). Without any further treatment development proceeds, but haploids die around the moment of hatching. However, the haploid state of the zygote can be changed into a diploid state by suppressing the first cleavage using physical shocks (cold, heat or pressure) applied at the metaphase of the first mitosis (Fig. 1c). After this treatment, a new cell cycle is initiated, starting with DNA-replication. Because an exact copy of the DNA is made, all homologues are fully identical, thus a 100% homozygous individuals will be generated.

Gynogenesis (all female inheritance) involves the irradiation of the paternal genome. This technique has been extensively studied and homozygosity can now be induced in several species, rainbow trout (Goryczko, *et al*. 1991), zebra fish, (Hörstgen-Shwark, 1993); common carp (Cherfas *et al.* 1993); Nile tilapia (Müller-Belecke and Hörstgen-Shwark, 1995) and African catfish (Volckaert *et al.* 1997).

Androgenesis, (all male inheritance) is achieved after irradiating the maternal genome. Irradiating eggs is more complicated than irradiating a sperm suspension due to the relatively large size and adhesive chorion (Bongers, 1997). Therefore, Androgenesis has been applied with much less success than gynogenesis.

The doubling of the paternal chromosome set to obtain androgenetic diploid Nile tilapia, *O. niloticus* should lead to the production of both XX and YY individuals since males are the heterogametic sex in these species.

Androgenetic rainbow trout (*Onchorhynchus mykiss*) were produced by gamma-ray inactivation of the female genome, followed by diploidizationm of the male genetic material by a pressure shock (Scheerer *et al.* 1986). An alternative method of producing androgenetic diploid individuals of the same specie was reported by Thorgaard *et al.* (1990), who used sperm of tetraploid males for fertilization, which made the diploidization step unnecessary.

Androgenetic common carp (*Cyprinus carpio*) was produced by irradiating the eggs by X-rays and 2-3 min of heat shock at 40.5-41^{o}C followed fertilization (Grunina *et al.* 1990), but Bongers (1997) used UV irradiation for the production of androgenetic common carp.

In this study, we describe the UV irradiation treatment (duration required for haploidization) and also, the optimization of the heat shock conditions (timing of application, duration of shock and temperature level) used in suppressing first cleavage in androgenetic *O. niloticus.*

MATERIALS AND METHODS

Broodstock Collection of gametes:

At the beginning of the experiment all fish (12 males and 60 females) were individually marked. The pre-stripping treatment procedures for gamete collection and artificial

insemination were the same as described by Müller-Belecke and Hörstgen-Schwark (1995).

Induction of androgenesis:

1. Inactivation of the female genome:

For the inactivation of the maternal genome, UV irradiation (254 nm) was used, eggs were divided into two groups, the first one was used as a control (without irradiating the eggs), where the eggs were fertilized with normal sperms. The second group was added with 5 ml saline solution (0.9% NaCl) in a petri dish. Eggs were irradiated for 16-30 minutes (with 2 min intervals). During irradiation, eggs were shaked in the petri dish to assure a homogenous irradiation of all pronuclei. A distance of 27.5 cm was kept between the lamp and the eggs.

The effectiveness of eggs irradiation was tested by chromosome counts (Kligerman and Bloom, 1977 adapted by Puckhaber and Hörstgen-Schwark, 1996) in few embryos of the irradiated eggs. These haploid controls were established by activating the irradiated eggs with normal sperms, without exposing them to a shock treatment. Complete mortality of haploid controls indicated successful inactivation of the maternal genome.

2. Mitotic inhibition:

Mitotic androgentic *O. niloticus* was obtained by using heat shock treatment to inhibit first mitosis after the activation of irradiated eggs with normal sperms. Irradiated eggs (for each female) were divided into 6 groups, the first one was activated with normal sperms without heat shock (control) and the other five groups were shocked after 22.5, 25, 26, 27 and 28 min from activation with normal sperms to indicate the most suitable time for applying the heat shock. Prior to a shock treatment, eggs were kept in water at a temperature of 28^{o}C. In the course of optimizing the shock treatment, heat shocks of 41-42.5^{o}C (with 0.5^{o}C interval) were applied for 4.5-7.5 min (with 1 min interval) starting from 22.5 to 28 min post egg activation.

On growing and data collection:

Treated and untreated batches of eggs were incubated in 35 cm^3 hatching jars with 28^oC warm circulating water. On day 9, fry were counted and transferred to 2L glass aquaria where first feeding started with a high protein diet. After reaching body weights of approximately 2 g, fish were stocked in 80 L glass aquaria and were fed ad libitum. At a body weight of approximately 30 g fish were counted and individually marked. Afterwards fish were kept in 700 L tanks until sexing.

RESULTS AND DISSCUSSION

1- Inactivation of the female genome:

UV irradiation was effective in disrupting nuclear DNA in tilapia eggs (Table, 1), as observed in UV irradiation experiment with spermatozoa (Müller-Belecke and Hörstgen-Schwark, 1995). UV radiation is seen to have a number of potential benefits, the low penetrance of UV irradiation reduces potential health risks to user relative to the other types of radiation. Furthermore, the effect of UV irradiation on chromosomes does not result in residual fragments in contrast to gamma irradiation (Chourrout, 1984). Results of table (1) revealed that, the optimum irradiation period for egg denucleation was 15 minutes, thus the diploids reached zero.

The analysis of the embryos survived beyond the 48h stage using the chromosome counts technique in 21 embryos showed that all of them was haploid containing 22 chromosomes coming from the males. According to Myers *et al.* (1995), where the quality of eggs obtained was good (control survival to 48 h post fertilization >30%), the success of the denucleation procedure was directly dependent on the duration of the UV irradiation.

Fig (2) indicated that a 15 min irradiation, using 254 nm UV lamp with a distance of 27.5 cm being kept between the lamp and the eggs, provided the best haploid percent 48 h after fertilization (Table 1 and Fig 2). The complete absence of diploid in the irradiated eggs (15 min) indicated the elimination of the female genome. The 3 and 6 min

irradiation did not contain any haploid embryos but all embryos were diploids (according to the chromosome counts technique used). In the 9, 10, 12 and 15 min irradiation periods, haploid embryos percentage increased gradually from 0 to 100% and diploid embryos had the opposite trend (Fig. 2).

Chromosome counts technique of irradiated eggs shows that the 15 min UV treatments gave the optimum dose for egg denucleation. Different UV doses have been needed to produce androgentic haploid common carp, *Cyprinus carpio* L., with an optimum UV doses of 2500 J/M^2 yielding 53.9% androgenetic haploids relative to the controls (Bongers *et al.* 1994). In other study, Kowtal (1987) found that using of UV as a denucleation treatment for the white sturgen, *Acipenser transmontanus*, the treatment duration was 40 min. Myers *et al.* (1995) indicated that, differences in the thickness, composition and optic qualities of eggs chorions, egg size and shape, and the relative position of the female pronucleus prior to fertilization from specie to another make it difficult to compare egg irradiation treatments required for the complete denucleation of egg. Mair (1993) stated that UV irradiation treatments for tilapia spermatozoa show a similarly wide variation in intensity, 200-2750 J/m^2 which is due in part to variation in sperm concentration in such experiments. He added that, female differences exist in an irradiation period required for the denucleation, this difference perhaps is due to the differences in egg size and the other egg quality.

2. Duplication of the male genome:

Our results demonstrate that androgenetic diploids can be successfully produced in *O. niloticus* using heat shock to block the first mitotic division. As described in fig (3) after activation of irradiated eggs by normal sperms, the hatching percentage increased gradually from 0 to 0.68% when heat shock was applied after 22.5 to 26 minutes from egg fertilization and then the hatching percentage decreased from 0.26 to 0.18% as heat shock was applied after 27 and 28 min from egg fertilization and the maximum percentage was obtained when heat shock was applied after 26 min (0.68%) from egg fertilization. Therefore we can conclude that, the peak survival occur when a heat shock, 41^{o}C lasting for 4.5 min is applied for 26

minutes post-fertilization and the heat shock conditions used in the present experiment are similar to those reported for suppression of first mitotic division in gynogenetic *O.niloticus* by Müller-Belecke and Hörstgen-Shwark (1995).

The low survival levels for inducing androgenetic *O. niloticus* fish are common in all studies. In diploid androgenetic rainbow trout, *Oncorhynchus mykiss*, the average survival to yolk-sac absorption varied from 7.2% to 9.5% for trout strains (Scheerer *et al.* 1986). Similar results were obtained for the induction of androgenetic diploid common carp, 8.1-9.3% (Bongers, 1997). Arai *et al.* (1995) had less than 1% of androgenetic loach (*Misgurnus anguillicaudatus*). With Nile tilapia *O. niloticus*, Karayucel *et al.* (1997) had a percentage of 0.14% survival to yolk sac resorption stage. Several factors potentially can contribute to this poor viability including homozygosity, genotype of the sperm, damage of the egg due to irradiation, and damaging effects of treatments suppressing the first cleavage division (Scheerer *et al.* 1986).

The viability of YY individuals in fish appears to be variable depending on species, Yamamoto (1975) found complete YY viability in the goldfish. Hunter *et al.* (1982) found good viability in coho salmon (*O.kisutch*), while Johnstone *et al.* (1979), could draw no firm conclusion regarding YY viability in rainbow trout. Parsons and Thorgaard (1985) observed that YY rainbow trout are viable to the eyed stage, suggesting that the differences between the X and Y chromosomes in rainbow trout may not reflect loss on any genetic material necessary for development to this stage.

The completely homozygous androgenetic *O. niloticus* and the potential for the use of androgenesis in the production of inbred lines and in genetic studies indicate that androgenesis may become a valuable tool in fish research and breeding.

To date a total number of 18 viable androgenetic diploids (5 males and 13 females) have been produced. These fish are under study to evaluate the potentially deleterious impact of the homogenous genome on their growth and fertility. Additionally, the YY males will be used as a broodstock for the production of all males XY progeny as an alternative method for the sex reversal

method used for the production of all male progeny by treating fry with hormonal treatment.

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Table (1): Relation between the UV irradiation period (min.) and the hatching percentage of androgenetic haploid and biparental larva of *O. niloticus*.

	Time (min.) of UV irradiation (254 nm lamp)					
	3	6	9	10	12	15
Androgenetic haploids	0	0	25	57.6	73	100
Biparental diploids	100	100	75	42.8	27	0

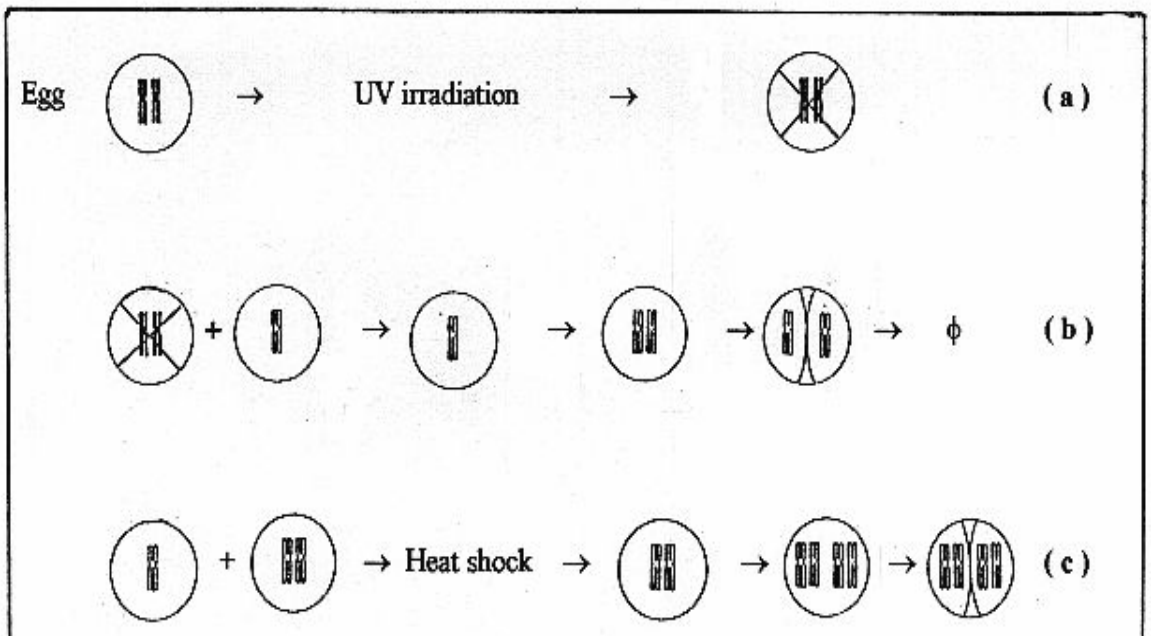
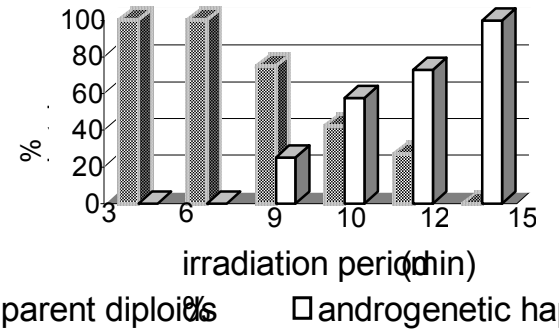
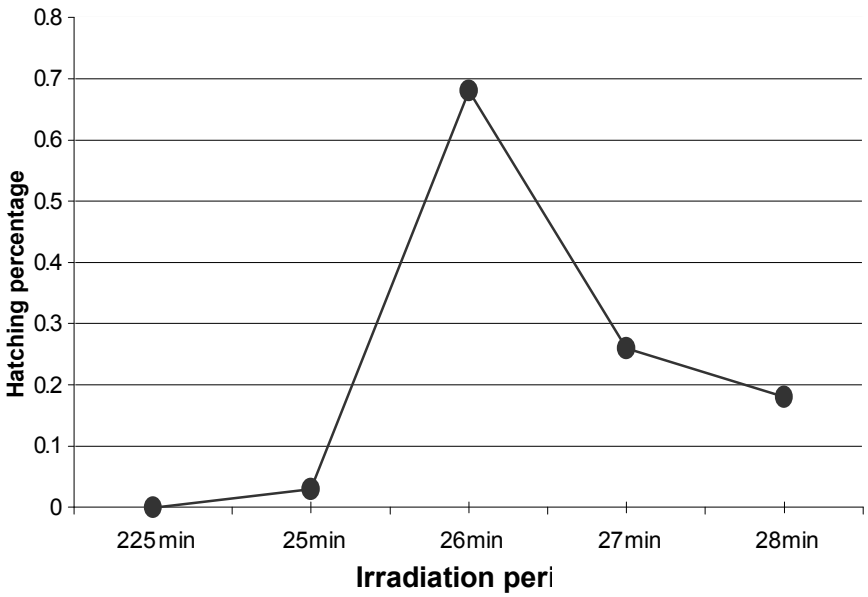


Fig (1): Induction of androgenesis in *Oreochromis niloticous*.

ϕ = Non-viable haploids.
(a): UV-irradiating eggs results in genetically inactive DNA.
(b): Fertilizing irradiated eggs with intact sperm yields haploid zygotes that start development, but die around hatching.
(c): After the initiation of haploid development, DNA is replicated. To prevent nuclear division, a heat shock is applied at the metaphase of this first mitotic division. This results in the initiation of a new cell cycle, starting (again) with DNA-replication. The zygote is now diploid and fully homozygous.



Fig(2): Relation between the UV irradiation period (min) and the hatching percent of androgenetic haploi biparental larvae of *niloticus*



Fig(3): Hatching percentages of androgenetic relation to irradiation period

الملخص العربى

إثارة التوارث الأبوى فى أسماك البلطى النيلى

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*** المعمل المركزى لبحوث الثروة السمكيه بالعباسه – وزارة الزراعة

أجريت هذه التجربه بهدف إيجاد أفضل المعاملات والظروف اللازمه لإنتاج ذكور من أسماك البلطى تركيبها الوراثى (YY) بحيث أنه عند استخدام هذه الذكور كآباء فى التزاوج مع إناث عاديه (XX) فيكون النسل الناتج بأكمله ذكور تركيبها الوراثى (XY) كإحدى الطرق المستخدمه فى إنتاج أسماك وحيدة الجنس من الذكور فقط وذلك للتغلب على ظاهرة التكاثر العشوائى الذى يحدث فى أحواض تربية أسماك البلطى.

وفى هذه الطريقه يعرض البيض الناتج من الإناث للأشعه فوق البنفسجيه وذلك لتحويل المادة الوراثية للبيض إلى مادة غير فعاله ثم يتم إخصاب هذا البيض المعامل بالأشعة بالسائل المنوى الذى سبق جمعه من الذكور وبعد عملية الإخصاب يترك البيض المخصب لعدة دقائق لإعطاء الفرصة لتضاعف المادة الوراثية الآتيه من الأب (الأسبرم) وقبل حدوث عملية الإنقسام الميتوزى الأول لتكوين الزيجوت يتم تعريض البيض المخصب لصدمه حراريه لإيقاف هذا الإنقسام الميتوزى للحصول على فرد متماثل وراثياً homogenous على كل المواقع الكروموسوميه (١٠٠% تربيه داخليه فى جيل واحد).

وتشير نتائج هذا البحث أن أنسب جرعه من الأشعه الفوق بنفسجيه والكافيه لتحويل الماده الوراثيه للأم إلى ماده غير فعاله هى تلك التى نحصل عليها عند تعريض البيض للأشعة فوق البنفسجيه (٢٥٤ نانوميتر) لمدة ١٥دقيقه على أن تكون المسافه بين البيض ومصدر الأشعة (٢٧٫٥ سم) وأن الصدمه الحراريه المناسبه هى ٤١°م لمدة ٤٫٥ دقيقه على أن تجرى هذه الصدمه الحراريه بعد مرور٢٦ دقيقه من عملية الإخصاب.