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Research Article

Optimisation of UV Treatment Duration to Induce Haploid Androgenesis in the Nile tilapia (*Oreochromis niloticus* L.)

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Abstract: The optimum UV duration times of eggs were examined in order to develop a simple and safe method for inducing androgenetic development in the Nile tilapia, *Oreochromis niloticus*. The yield of androgenetic haploid *O. niloticus* to the pigmentation stage was 18.53 ± 5.3% (relative to controls) with an optimal UV irradiation dose of 540 Jm^{-2} (at 150 μWcm^{-2}) for 6 min. Most embryos developing after fertilisation with normal spermatozoa showed abnormal morphology and a haploid number of chromosomes (n = 22). The success of oocyte denucleation was also assessed by using the recessive "blond" skin pigmentation character.

Key Words: Androgenesis, chromosome manipulations, Nile tilapia, *Oreochromis niloticus*

Nil Tilapyasında (*Oreochromis niloticus* L.) Haploid Androgenesis Üretimi İçin UV Muamele Süresinin Optimizasyonu

Özet: Nil tilapyasında, *Oreochromis niloticus*, androgenetik gelişmeyi sağlamak için, basit ve güvenli metot geliştirmek amacıyla, yumurtaların optimum UV ışınlarına maruz bırakılma süreleri incelendi. Optimum UV irradizasyon dozu olan 540 Jm^{-2} de (150 μWcm^{-2} de) 6 dakika muamele, pigmentasyon safhasında % 18,53 ± 5,3 (kontrol grubuna oranla) androgenetik haploid *O. niloticus* üretmiştir. Normal spermatozoa ile döllenmeden sonra gelişen birçok embriyo abnormal morfoloji ve haploid kromozom sayısı göstermiştir. Yumurtaların çekirdekten mahrumiyetleri resesif "sarı" deri pigmentasyon karakteri kullanılarak da değerlendirilmiştir.

Anahtar Sözcükler: Androgenesis, kromozom manipulasyonları, Nil tilapyası, *Oreochromis niloticus*

Introduction

Androgenetic development (all paternal inheritance) in fish can be triggered by fertilising gamma, X-rays or UV irradiated eggs with normal spermatozoa, but the resultant embryos show inviable abnormality due to haploidy.

Viable diploid androgens can be produced by doubling the paternal chromosome set through suppression of the first cleavage using physical shocks such as temperature and pressure or by fertilisation of inactivated eggs with diploid spermatozoa from tetraploid males.

The applicable potential of androgenesis involves the rapid establishment of inbred lines for breeding programmes and research purposes (1,2), sex control using anticipated super male (YY) in the male heterogametic species (3,4), production of a nucleo-cytoplasmic hybrid between different species and recovery of genotypes from cryopreserved sperm, particularly for those which are facing extinction or the threat of contamination by hybridisation (1,5-7).

Haploid androgenesis has been induced using ^{60}Co in the loach (*Misgurnus anguillicaudatus*) (1), flounder (*Pleuronectes flesus*) (2), masu salmon (*Oncorhynchus masou*) (8), rainbow trout (*Oncorhynchus mykiss*) (9) and brook trout (*Salvelinus fontinalis*) (10). Briedis and Elinson (11) induced haploid androgenetics in fertilised frog (*Rana pipiens*) eggs using pressure and deuterium oxide (D_2O) to inhibit male pronucleus movement by the disruptive effects of microtubule-specific agents on pronuclear movement.

Studies with amphibians (12,13) showed that the transparency of the amphibian egg and the fact that the egg pronucleus is oriented toward the animal pole after fertilisation facilitated treatments with UV. However, the opacity of some fish eggs and the failure of the egg nucleus to demonstrate any particular orientation before or after fertilisation may present problems owing to the poor penetrance of UV (3,6). Despite these disadvantages, UV light has been successfully used in the irradiation of eggs from the white sturgeon (*Acipenser*

transmontanus) (14), common carp (*Cyprinus corpio)* (15,16), Nile tilapia (4), loach *Misgurnus anguillicaudatus* (17) and African catfish (*Clarias gariepinus*) (18). Furthermore, UV irradiation results in no residual fragments, in contrast to gamma irradiation, and it is easy to use anywhere, inexpensive and safer to apply (1,3,4,19,20). Therefore, optimisation of the intensity and the duration of irradiation is the first step for the successful production of haploid androgens. In the present study, we examined the optimum UV duration time of eggs in order to develop a simple and safe method for inducing androgenetic development in the Nile tilapia.

Materials and Methods

Origin of fish stock and their maintenance

The *O. niloticus* brood stock used for this study were descended from an electrophoretically tested, pure stock of the Tilapia Reference Collection maintained at the Institute of Aquaculture, University of Stirling, Scotland (21). The origin of blond fish is described by Scott et al. (22), McAndrew et al. (23) and Hussain (24).

All fish were reared in recirculating freshwater systems. Lighting in all the systems was adjusted by an automatic timer to 12 h light and 12 h dark. The water temperature was maintained at 28 ± 1ºC. Individual female broodstock were kept in partitioned glass tanks of 120 cm X 44 cm X 30 cm. All tanks were aerated by airstones coupled to a low-pressure blower unit. All fish were fed with commercial trout feed (Trouw Aquaculture Nutrition, Russhive, UK) three times a day *ad libitum*.

Fish breeding, stripping and fertilisation of eggs

Under aquarium conditions, mature females of *O. niloticus* spawn at approximately 2-6 week intervals. Females which are ready to spawn have a swollen urogenital papilla and show pre-spawning behaviour such as nest building and cleaning. After anaesthetising the female, the 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 in a clean, sterile Petri dish (100 mm in diameter) and were washed carefully with water from the recirculating system several times until ovarian fluid and any blood were removed. Then the eggs were sub-divided into a number of batches, as the experimental design required. Milt was also stripped from males in a similar way to egg collection using a glass capillary tube to collect the milt, which was then put into a clean 1.5 ml microtube and stored at 4 ºC until use. Milt contaminated with water and urine was rejected.

Eggs were fertilised *in vitro* by mixing the milt with "dry" eggs and then 10-20 ml of aquarium water was added. The fertilised eggs were left in the Petri dish for 2-30 min for water hardening, washed, and transferred to a recirculated system for further development.

The embryos in each batch were checked and counted at four development stages: morula 6-8 h after fertilisation (a.f.); pigmentation 45-50 h a.f; hatching 80-90 h a.f. and yolk sac resorption 9-11 days a.f. Survival was calculated as (Number of embryos surviving at a given development stage / total number of eggs) x 100.

UV irradiation of eggs

UV irradiation of eggs was carried out according to Myers et al. (4). A 254 nm UV lamp (Ultra-Violet Products, San Gabriel, California) mounted on a camera copy stand was used for irradiation. UV treatments were standardised by placing 4 ml of unfertilised eggs (150-250 eggs) in a vial with enough filtered water to bring the total volume of eggs and water to 14-15 ml. The eggs in water were then poured into a glass Petri dish (75 mm in diameter) which was then placed on a stirrer. The distance between the lamp and Petri dish was adjusted to provide a dose of 150 $\mu W/cm^{-2}$ using a radiometer (Ultra-Violet Products, San Gabriel, California).

All treated and untreated batches of eggs were incubated identically in a recirculated system.

Determination of ploidy

Fish metaphase chromosome spreads were prepared from newly hatched or one-day-old post-hatched larvae according to the original procedures described by Kligerman and Bloom (25), Chourrout and Itskovich (26) and Chourrout (20).

Experimental design for optimisation of UV duration time

Optimisation of UV duration time was carried out by irradiating six batches of eggs with UV light for 2, 4, 6, 8, 10 or 12 min and fertilising with sperm from blond tilapia males. This colour pattern was first reported by Scott et al. (22) and can be used as a visual marker to indicate the successful production of the haploid androgenetic fish because of a recessive "blond" skin pigmentation marker. A portion of the eggs was retained

as a control group and fertilised with sperm from the same blond male. Four different females and one blond male were used for this experiment.

Statistical analyses

Since the egg quality of each spawn varied greatly within and between females, the survival of each treatment was always calculated relative to the survival of their corresponding diploid control group. When the survival rate of the control group was less than 30%, that particular batch of eggs was not included (4). The data from the results of morula stages were transformed to arc-sine for statistical analyses and normality was tested by the Anderson-Darling Normality test and a test for homogeneity of variance was applied (27). Only the results of the morula stages were tested by one-way ANOVA since they were normally distributed. The other non-parametric data for pigmentation, hatching and yolk sac resorption stages, which included many zero values, were transformed to square root and tested by the Kruskal-Wallis Test (27,28). The results were presented as mean and standard error of mean (± SE). All statistical analyses were performed by Minitab 9.2 software.

Results

The effect of UV exposure for 2, 4, 6, 8, 10 and 12 min on the percentage of morula, the percentage of pigmented and unpigmented embryo and the percentage of abnormal embryos in the presumptive haploid "blond" androgenetic Nile tilapia are presented in the Table and Figure 1. All measurements are in relation to untreated controls.

At the morula stage, all the levels of fertilisation of the treatment groups and controls were quite similar and there were no significant differences between them ($P > 0.05$). As seen in Figure 1A, the fertilisation levels decreased with increased UV exposure time.

At the pigmentation stage, the survival of average pigmented (38.29 ± 1.18%) and unpigmented embryos (39.56 ± 0.97%), were not significantly different in the control group indicating that the females used in these experiments were heterozygous for the blond locus (Figure 1B). For 2 and 4 min UV duration, pigmented embryos were observed at a survival rate of 5.51 ± 1.93% and 0.22 ± 0.22%, respectively. Although the highest survival of unpigmented embryos (46.03 ± 13.6%) was obtained for 2 min UV duration, these treatments yielded pigmented embryos showing only partial success with oocyte denucleation. A 6 min UV exposure time with a survival rate of 18.53 ± 5.3 % provided the best survival amongst the treatments giving only blond embryos. The survival rates of the treatment groups declined with increasing UV exposure time.

At the hatching stage, there were no significant differences between normal developed pigmented and unpigmented embryos with a survival rate of 32.14 ± 1.08% and 35.48 ± 2.10%, respectively, in the control group ($P > 0.05$) (Figure 1C). In the 2 min treatment group, survival rates of 1.01 ± 1.01%, 1.52 ± 1.52% and 3.24 ± 2.24% were observed in normally developed pigmented and unpigmented embryos and abnormal embryos, respectively. Only abnormal embryos were produced in 4, 6 and 8 min treatments while 10 and 12 min UV duration did not result in any hatched embryos. There were no significant differences between the treatments in terms of abnormality ($P > 0.05$). None of the UV treated embryos, including pigmented embryos, in the 4, 6, 8, 10 and 12 min treatments survived for more than a few days post-hatching.

Analysis of some of the embryos by karyological examination (Figure 2) showed a typical single set of chromosomes (n = 22) (29).

Discussion

The present study indicates that UV irradiation successfully inactivated the nuclear DNA in Nile tilapia eggs. The yields of viable denucleated eggs to the pigmentation stage varied between 4.76 and 30.52% (relative to the controls) with a mean of 18.53 ± 5.3% for 6 min UV duration with a total dose of 540 Jm^{-2}. The yield is comparable with that of 22.9 ± 1.6% in the Nile tilapia (4), 22% in the loach (30) and 22.5 ± 2.8% in the muskellungen (*Esox masquinongy*) (31). In the common carp, an optimal dose of 2500 Jm^{-2} produced 53.9% surviving haploids at hatching as well as a few biparental diploids (16). Bongers et al. (18) were able to produce higher numbers of androgenetic haploids (81% to hatching, relative to control) in African catfish using an optimum UV dose of 1250 Jm^{-2}. Arai et al. (30) successfully produced 22% hatched androgenetic haploids in loach with a dose of 750 Jm^{-2}. The yield of haploid androgenetic muskellunge was 22.5 ± 2.8% with optimal UV irradiation doses of 620-1320 Jm^{-2} (31). Marengoni and Onoue (32) obtained survival rates of

Table. The effect of UV exposure (at 150 μWcm^{-2}) for 2, 4, 6, 8, 10 and 12 min on the morula, pigmentation and hatching of presumptive "blond" androgenetic haploid Nile tilapia, *O. niloticus*. % Relative control data are in parentheses. Common superscripts in the same column signify means which are not significantly different. *: Females were heterozygous for blond gene, R: Relative to controls.

		Pigmentation stage		Hatching stage			
UV dose (min)	Experiment no.	Morula embryos	Unpigmented embryos	Pigmented embryos	Normal developed pigmented embryos	Normal developed unpigmented embryos	Abnormal embryos
0	1*	98.02	37.62	39.60	30.20	30.69	0.99
	2*	92.26	40.07	35.35	33.67	34.01	1.35
	3*	90.94	38.51	37.54	34.30	36.57	1.29
	4*	91.12	42.06	40.65	30.37	40.65	2.80
	Mean	93.08 ± 1.67^{a}	39.56 ± 0.97^{b}	38.29 ± 1.18^{c}	32.14 ± 1.08^{b}	35.48 ± 2.10^{b}	1.61 ± 0.41^{a}
2	1*	96.66 (98.61)	63.59 (82.35)	6.28 (8.13)	2.51 (4.06)	3.77 (6.09)	5.86 (9.47)
	2*	70.91 (76.86)	33.33 (44.20)	6.67 (8.84)	0.00	0.00	2.17 (3.51)
	3*	88.44 (97.26)	12.54 (16.49)	0.32 (0.43)	0.00	0.00	0.00
	4*	87.50 (96.03)	33.98 (41.08)	3.85 (4.65)	0.00	0.00	0.00
	Mean	85.88 ± 5.40	35.86 ± 10.50	4.28 ± 1.50	0.63 ± 0.63	0.94 ± 0.94	2.00 ± 1.38
	Mean (R)	92.19 ± 5.14^{a}	46.03 ± 13.6^{b}	5.51 ± 1.93^{b}	1.01 ± 1.01^{a}	1.52 ± 1.52^{a}	3.24 ± 2.24^{a}
4	1*	93.31 (95.19)	11.48 (14.87)	0.00	0.00	0.00	1.44 (2.32)
	2*	78.41 (84.99)	20.60 (27.31)	0.66 (0.88)	0.00	0.00	1.66 (2.41)
	3*	80.91 (89.00)	4.37 (5.75)	0.00	0.00	0.00	0.00
	4*	87.50 (96.03)	19.68 (23.80)	0.00	0.00	0.00	0.00
	Mean	85.03 ± 3.36	14.03 ± 3.82	0.17 ± 0.17	0.00	0.00	0.78 ± 0.45
	Mean (R)	91.30 ± 2.62^{a}	17.93 ± 4.83^{ab}	0.22 ± 0.22^{a}	0.00	0.00	1.18 ± 0.69^{a}
6	1*	92.55 (94.42)	14.36 (18.60)	0.00	0.00	0.00	0.00
	2*	82.74 (89.68)	23.02 (30.52)	0.00	0.00	0.00	1.80 (2.61)
	3*	82.24 (90.43)	3.62 (4.76)	0.00	0.00	0.00	0.00
	4*	89.73 (98.48)	16.73 (20.23)	0.00	0.00	0.00	0.00
	Mean	86.82 ± 2.56	14.43 ± 4.04	0.00	0.00	0.00	0.45 ± 0.45
	Mean (R)	93.25 ± 2.02^{a}	18.53 ± 5.3^{ab}	0.00	0.00	0.00	0.65 ± 0.65^{a}
8	1*	81.10 (82.74)	7.20 (9.32)	0.00	0.00	0.00	0.00
	2*	72.32 (78.39)	19.72 (26.15)	0.00	0.00	0.00	1.04 (1.50)
	3*	79.83 (87.78)	4.04 (5.31)	0.00	0.00	0.00	0.00
	4*	87.96 (96.53)	14.38 (17.39)	0.00	0.00	0.00	0.00
	Mean	80.30 ± 3.20	11.34 ± 3.53	0.00	0.00	0.00	0.26 ± 0.26
	Mean (R)	86.36 ± 3.89^{a}	14.54 ± 4.62^{a}	0.00	0.00	0.00	0.38 ± 0.38^{a}
10	1*	68.96 (70.35)	4.51 (5.84)	0.00	0.00	0.00	0.00
	2*	79.33 (85.98)	3.39 (4.49)	0.00	0.00	0.00	0.00
	3*	65.58 (72.11)	1.31 (1.72)	0.00	0.00	0.00	0.00
	4*	81.93 (89.91)	9.35 (11.30)	0.00	0.00	0.00	0.00
	Mean	76.45 ± 5.93	4.64 ± 1.70	0.00	0.00	0.00	0.00
	Mean (R)	79.59 ± 4.91^{a}	5.83 ± 2.01^{a}	0.00	0.00	0.00	0.00
12	1*	68.12 (69.50)	3.18 (4.12)	0.00	0.00	0.00	0.00
	2*	74.83 (81.11)	5.86 (7.77)	0.00	0.00	0.00	0.00
	3*	31.10 (34.20)	0.79 (1.04)	0.00	0.00	0.00	0.00
	4*	86.40 (94.82)	2.94 (3.56)	0.00	0.00	0.00	0.00
	Mean	65.11 ± 11.96	3.19 ± 1.04	0.00	0.00	0.00	0.00
	Mean (R)	69.91 ± 12.98^{a}	4.12 ± 1.38^{a}	0.00	0.00	0.00	0.00

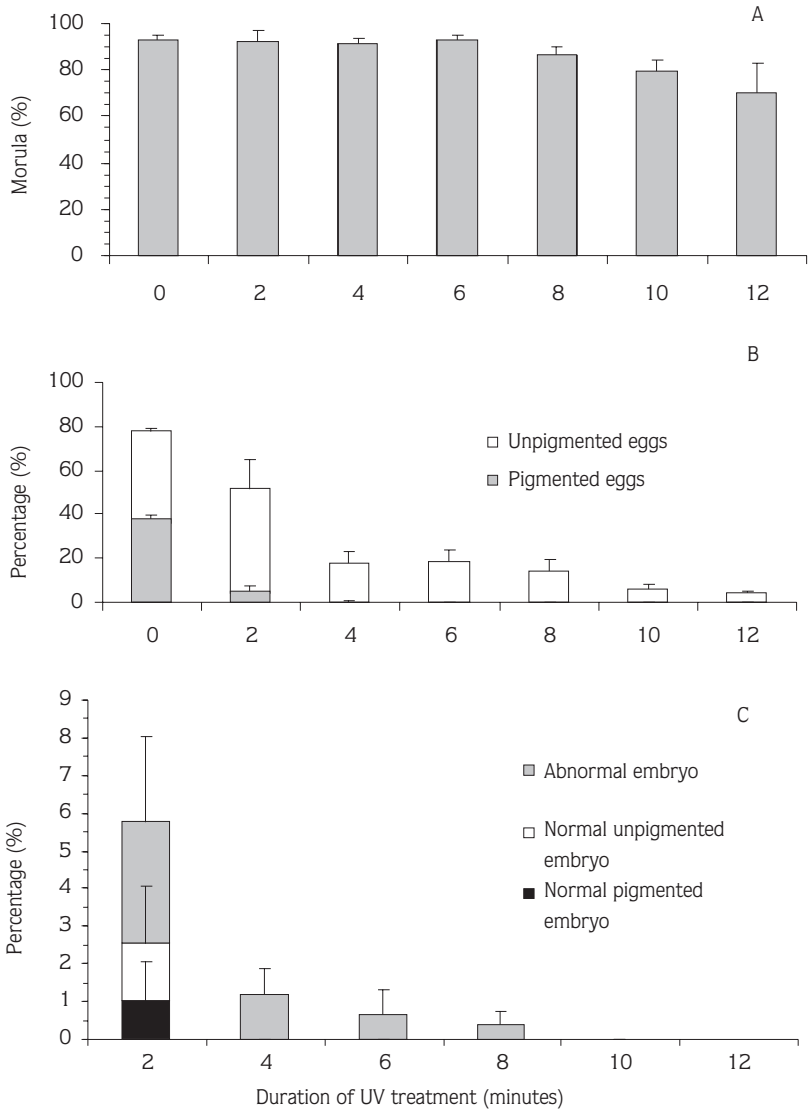

Figure 1. Percentage of embryos observed at morula (A), pigmentation (B) and hatching (C) of presumptive "blond" androgenetic haploid Nile tilapia, *O. niloticus*, (% relative to the diploid control) subjected to 150 μWcm^{-2} intensity.

57.6% and 55.8%, and 57.1% and 56.0% (relative to controls) in androgenetic haploid *O. aureus* and *O. niloticus*, respectively, at total UV doses of 594 and 693 Jm^{-2}, respectively.

By using the recessive "blond" skin pigmentation character in spermatozoa, it was possible to assess whether oocyte denucleation was successful. All haploid embryos showed non-pigmentation under optimal UV irradiation of 5-8 min whereas 2 min and 4 min UV irradiation produced some pigmented embryos. The blond colour variant was also used successfully by Myers et al. (4) in the production of androgenetic haploid tilapia and they also observed some pigmented embryos in the same UV irradiation treatments as the present study. Pigmented embryos were observed in the 2 and 4 min UV treatments and blond embryos showed aberrant development. Therefore, to ensure that host eggs are totally denucleated, the UV treatment should be at least 5 min to 8 min at 150 μWcm^{-2} or a total dose of between 450 Jm^{-2} and 720 Jm^{-2}. Myers et al. (4) reported that variable sensitivity to UV irradiation from species to species could be explained by differences in the thickness, composition and optical qualities of egg chorion, egg size and shape, and the relative position of the female pronucleus.

It was concluded that for successful haploid induction, the UV duration time has to be at least 5 min which will ensure total degradation of the egg nucleus.

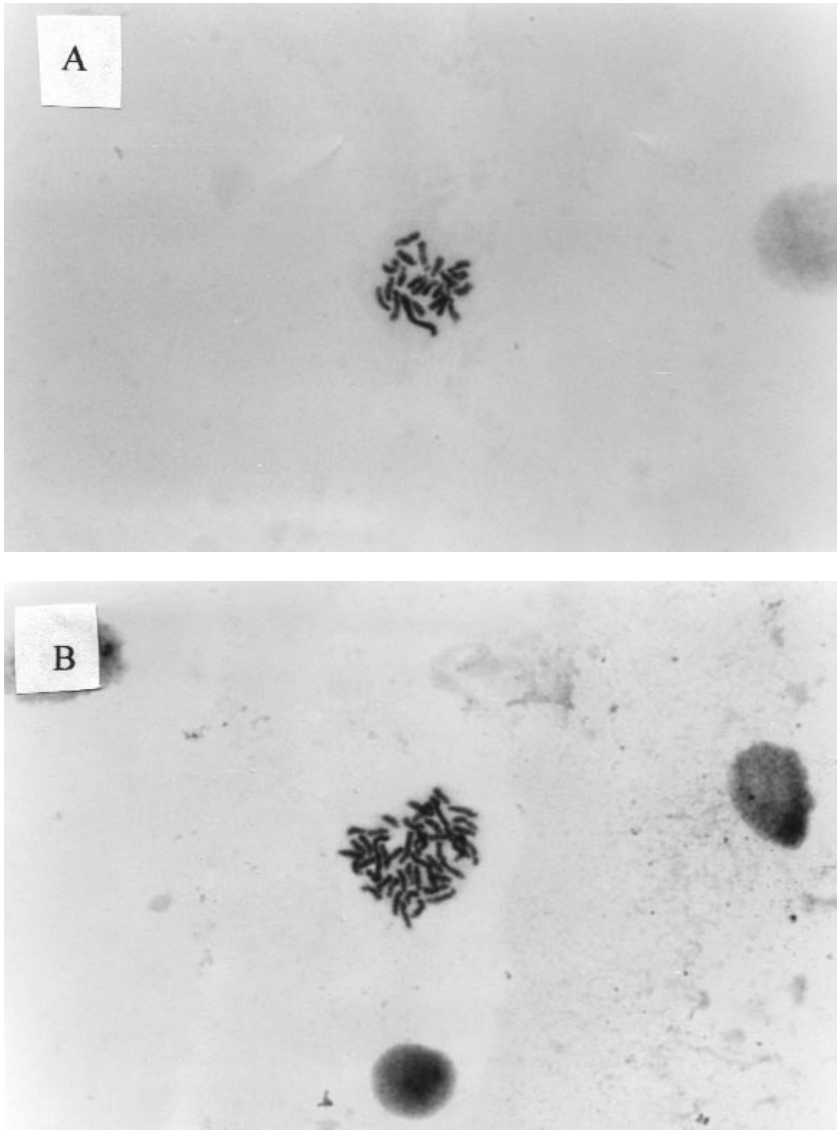

Figure 2. Metaphase chromosome spread of A. androgenetic haploid (N=22) and B. diploid (N=44) embryos of *O. niloticus*.

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