Theor Appl Genet (1995) 90: 205–210

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Induction of diploid androgenetic and mitotic gynogenetic Nile tilapia (*Oreochromis niloticus* L.)

Received: 12 April 1994 / Accepted: 20 May 1994

Abstract Androgenesis is a potentially valuable technique for recovering fish from gene banks composed of cryopreserved sperm, developing inbred lines, and analyzing patterns of inheritance. The procedure for producing diploid organisms whose nuclear DNA is wholly of paternal origin is dependent on: (1) the denucleation of "host" eggs, and (2) the inhibition of the first mitotic division in order to double the haploid sperm chromosome complement following fertilization of host eggs. Denucleation of tilapia (*Oreochromis niloticus* L.) eggs was carried out using UV irradiation. Treatment durations of 5–8 min (total dose of 450–720 J/m^2) produced acceptable yields of viable denucleated eggs [22.9±1.6% (±SE) of controls] as estimated by the survival of haploid androgenetic tilapia to 48 h post-fertilization. Successful mitotic inhibition was accomplished using a heat-shock of 42.5°C for 3–4 min, applied at 2.5-min intervals from 22.5 to 30 min post-fertilization (mpf). The mean survival of androgenetic diploid fish to yolk-sac absorption for treatment groups varied from 0.4% to 5.3%, relative to the controls. Differences in the suceptibility of eggs from different females to UV irradiation were a significant factor in the overall yield of androgenetic diploids. Paternal effects did not significantly influence the androgenetic yield, suggesting that individual males would not be selected against. For comparative purposes mitotic gynogenetic "mitogyne" diploids were produced from UV-irradiated sperm. Mean survival to yolk-sac absorption varied from 0.5% to 10.64%, relative to controls. Similar optima for androgenetic and gynogenetic induction were found in the period 25–27.5 mpf (minutes post-fertilization). Induction treatments would appear to be operating on the same developmental events in both these techniques, and the results suggest that the UV irradiations used do relatively little damage to the eggs beyond nuclear inactivation. The results indicate that the production of androgenetic *O. niloticus* is possible on a consistent basis and that the application of this technique may be useful in quantitative and conservation genetics.

Key words Androgenesis · Gynogenesis · Tilapia
Gene bank · Chromosome manipulation

Communicated by C. Smith

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Introduction

The induction of androgenetic and gynogenetic fish represents a valuable addition to classical genetic methods. The creation of uniparental fish offers a method of producing high levels of inbreeding, potentially 100% in a single generation, and monosex populations, either directly or through the creation unique genotypes, such as "YY" or "WW". Furthermore, androgenesis may be critical to the development of cryopreserved gene banks for fish species. The oocytes of most finfish species are too large and yolky to be satisfactorily cryopreserved; however, spermatozoa and dissociated blastula cells have been successfully frozen and recovered (McAndrew et al. 1993). Repeated backcrossing to cryopreserved sperm could be used to recover stocks, but the process would be prolonged over several generations (never achieving 100% recovery) and require the cryopreservation of considerable quantitites of sperm. Thus, it would be valuable to develop androgenetic procedures for fish species whereby the cryopreserved sperm is the sole contributor to the nuclear DNA of any offspring.

Androgenetic organisms have been observed to occur spontaneously (Yamazaki 1983). This primarily occurs where the oocyte is overripe or where interspecific or intergeneric crosses have been made and apparently the pronuclei do not fuse. Induced androgenesis requires that the nuclear DNA content of the oocyte be inactivated, usually

through some form of ionizing radiation. Gamma [^{60}Co] and X-ray irradiation are most commonly used because of their high penetration (Purdom 1969; Arai et al. 1979; Parsons and Thorgaard 1985). Alternatively, ultraviolet light (UV) has been used extensively with amphibian eggs (Gurdon 1960; Gillespie and Armstrong 1980) and with fish, but on a much more limited scale with the latter (Kowtal 1987; Bongers et al. 1994) due to its low penetration. UV radiation is seen to have a number of potential benefits. The low penetrance of UV irradiation reduces potential health risks to the users 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). These chromosome fragments may be large enough to succesfully replicate in the resulting "presumptive" androgenetic individual. Preliminary studies have indicated that [Co^{60}] gamma irradiation is not an efficient method of irradiating tilapia eggs, partially due to the logistical and biological difficulties of transporting gametes to a reactor site (Myers et al. 1993). Finally, the relative cost and availablility of UV irradiation equipment relative to Gamma (^{60}Co) and X-ray facilities would make it widely available to scientific and commercial users.

The nuclear inactivation process produces a "host" egg, a developmental vehicle for the sperm DNA. Normally, sperm would contain a single chromosome complement: for viable (diploid) androgenetic production this would then have to be doubled through the inhibition of the first mitosis. Chemical, thermal, and hydrostatic pressure treatments have all been shown to be effective to some degree in preventing the first mitotic division without interfering with DNA replication. It is possible through a combination of egg denucleation and mitotic inhibition to recover organisms whose nuclear DNA comes solely from haploid sperm, sperm that potentially has been cryopreserved for an extended period.

The project described here was initiated to develop androgenetic techniques for tilapia species, initially the Nile tilapia *Oreochromis niloticus* L. Tilapia are an ecologically and economically important group of fishes that are cultured worldwide; however, the introduction of cultured species and transfer of species have meant the loss or degradation of indigenous tilapia stocks through competition and hybridization. The development of cryopreserved gene banks could provide a way of safeguarding these genetic resources, in addition to in vivo techniques, before there is further degradation. Androgenesis could allow the recovery of cryopreserved genetic material in the form of diploid organisms. These techniques may also be useful in the development of inbred lines for husbandry purposes and laboratory studies.

Materials and methods

Experimental animals

Broodstock *O. niloticus* L. of an Egyptian strain (McAndrew and Majumdar 1983) were obtained from the University of Stirling's Institute of Aquaculture Tilapia Reference Collection. This strain contains a recessive "blond" skin pigmentation character (Scott et al. 1987) that was used as a visual marker to indicate the successful production of the androgenetic organisms. Homozygous "blond" males were crossed with homozygous wild type or wild type × red heterozygous females (McAndrew et al. 1988) for androgenetic induction. Broodstock were analyzed for electrophoretic variation at the *ADA** locus (adenosine deaminase, ADA, E.C. No. 3.5.4.4), which has three known alleles (**131*, **121*, and **113*), using methods described by McAndrew and Majumdar (1983). Parents for both androgenesis and gynogenesis induction were selected on the basis of variation at the *ADA** locus, such that the paternal genotype was different from the maternal. Gametes were acquired from broodstock using standard hatchery strip-spawning techniques. Individual treatment groups within a spawn contained between 100 and 250 eggs.

Oocyte denucleation

Oocyte denucleation was accomplished using a 254-nm UV lamp (Ultra-Violet Products, San Gabriel, Calif.) mounted on a camera copy stand. UV treatments were standardized by placing 4 ml of unfertilized eggs in a vial with enough water to bring the total volume of eggs and water to 14 ml. The eggs and water were then poured into a glass petri dish (75 mm in diameter) and the dish placed on top of an "egg whirler" (constructed by D. Milroy and B. Howie, Institute of Aquaculture, Stirling). UV intensity was monitored using a radiometer (Ultra-Violet Products, San Gabriel, Calif.) and the distance between the lamp and the top of the petri dish adjusted to provide a dose of 150 $\mu W/cm^2$. The duration of exposure to the UV light was varied from 2 to 10 min.

Mitochondrial DNA (mtDNA) was extracted from groups of eggs irradiated for 0, 2, 6, or 12 min using standard methods (Chapman and Powers 1984). Aliquots of mtDNA were then digested with ultaviolet endonuclease (UVE). This enzyme primarily removes UV-induced pyrimidine dimers from DNA. The digested DNA was extracted with phenol and chloroform (once each), precipitated with ethanol, resuspended, and run on 0.8% alkaline agarose gel. The gel was then neutralized and the DNA transferred onto a charged nylon membrane by vacuum blotting (Pharmacia LKB). The membrane was then probed using purified *O. niloticus* mtDNA labelled with [^{32}P] by random priming (Boehringer, Mannheim). Autoradiographs were prepared from the filters following washing.

Sperm irradiation

Sperm collection and preparation for irradiation was carried out according to procedures in Hussain et al. (1993). Sperm were subjected to an irradiation dose of 300–310 $\mu W/cm^2$ for 2 min (360–372 J/m^2).

Mitotic inhibition

Mitotic inhibition to "diploidize" haploid androgenetic zygotes was accomplished using a heat-shock treatment modified from Hussain et al. (1993). Eggs were transferred to hatching jars in an incubation system two min after fertilization to ensure normal development prior to treatment. Incubation temperature was maintained at 28.0±0.5°C throughout the study. Eggs were poured directly into plastic tea strainers immersed in a temperature-controlled water bath at 22.5–37.5 min post-fertilization (mpf). The intensity of the temperature shock was varied from 41°–42.5°C for a 3- to 6-min duration. Following treatment, the eggs were immediately returned to the incubator.

Haploid gynogenetic eggs were treated using the optimal heat-shock protocol derived from the androgenesis induction study. A temperature shock of 42.5°C for 4 min was applied 20–30 mpf as described for androgenesis.

A portion of the androgenetic and gynogenetic haploid zygotes were retained as UV-treated controls and not subjected to the heat

shock. These eggs and untreated control eggs were observed for the initiation of the first cleavage under a dissecting microscope to determine any changes in the developmental rates of haploid zygotes. Eggs were removed from the jars at 5-min intervals for observation. The UV-treated controls were further reared to assess the success of the irradiation procedures.

The success of the oocyte denucleation was assessed on the basis of the presence (wild type) or absence (blond) of melanophores in 36-h post-fertilization embryos. The blond embryos, presumptive androgenetic haploids, were also analyzed via metaphase preparations. Karyotypes were obtained from 48-h-old embryos placed in 0.05% colchicine (Sigma Chemical) for 4–5 h. Subsequent procedures followed Kligerman and Bloom (1977). At least five karyotypes were counted per individual, and 10 individuals were sampled from each group. Denucleated eggs which were subsequently treated for mitotic inhibition and the control group were scored for survival and the presence or absence of pigmentation at 36 h post-fertilization and at the time of yolk-sac absorption (10–11 days).

Further verification of presumptive androgenetic and mitotic gynogenetic "mitogyne" fish was provided via starch-gel electrophoresis. Fish, 30 mm or larger, were anaesthetized, and a small portion of the caudal fin tissue sampled to ascertain their genotype at the *ADA** locus.

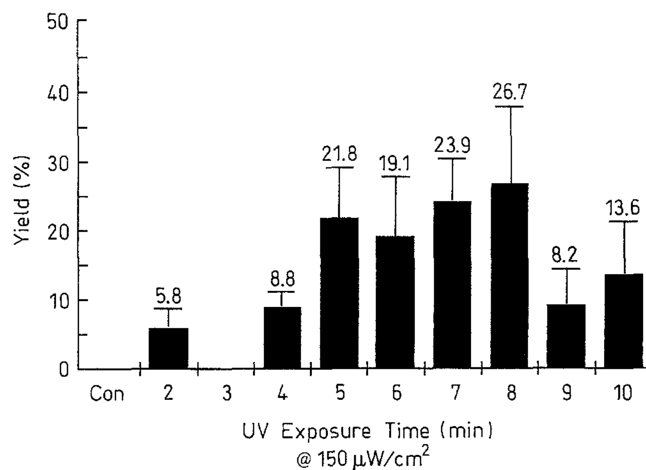

Fig. 1 Average yield (%) of presumptive "blond" androgenetic haploid Nile tilapia *Oreochromis niloticus* subjected to UV-irradiation treatments. Treatment durations varied from 2 to 10 min at a light intensity of 150 $\mu W/cm^2$, total dose 180–900 J/m^2. Yields are based on the average of 8 spawns with 100–200 eggs per treatment/spawn

Statistical analysis

The egg quality of each spawn varied greatly within and between females. For this reason differences in gamete quality among spawns were corrected by dividing the survival of the treatment groups by the survival of their corresponding control group.

Statistical analyses of UV control experiments, the percentage of blond embryos produced, and androgenesis induction (the survival of treated blond embryos to yolk-sac absorption) were performed using regression analysis for exposure duration and one-way ANOVA (GLM) for female tag number and male tag number, all as fixed factors. Additionally, multiple regression analysis was used with female and male tag number as a dummy variable to remove parental differences from the analysis of survival. Similarly, the survival of presumptive gynogenetic fish to yolk-sac absorption was analyzed via one-way ANOVA (GLM) using time of treatment as a fixed factor. In all of the statistical analyses significance was set at the $P< 0.05$ level.

Results

Oocyte Denucleation

Where the quality of eggs obtained was good (control survival to 36 h post-fertilization>30%), the success of the denucleation procedure was directly dependent on the duration of the UV irradiation. Comparisons of androgenetic haploid yield (Fig. 1) indicated that an 8-min treatment using 150 $\mu W/cm^2$, a total dose of 720 J/m^2, provided the best survival to 36 h post-fertilization, 27.0±10.7%(SE). Regression analysis indicated that treatment duration was a significant factor using a linear model ($P< 0.012$, df=41). The 2- and 4-min treatment did contain some pigmented embryos, 11.0% and 4.0% relative to the controls respectively. None of the UV-treated embryos, including the pigmented embryos, in the 4-, 6-, 8-, and 10-min treatments survived beyond the 3 days post-hatching, presumably because they were haploid, aneuploid, or diploid but had sustained considerable damage during the UV treatment.

The specific female used did have a significant effect on the percentage of androgenetic haploids produced ($P< 0.001$, df=6). Similarily, the inclusion of female tag number as a dummy variable with treatment duration in a multiple regression produced a significant regression ($P< 0.0191$, df=8).

Analysis of some of the embryos (n=374) that survived beyond the 48-h stage by karyological examination showed the expected haploid number for *O. niloticus*, n=22 (Majumdar and McAndrew 1986). No chromosome fragments were identified in any of the metaphase preparations.

Presumptive gynogenetic haploid groups were not directly analyzed to establish their haploid nature as they were produced by standard methods (Hussain et al. 1993). Embryos from these groups did exhibit aberrant development (enlarged pericardial sac, small head, and deformed spine) commonly associated with haploidy. Additionally, none of the embryos survived more that a few days post-hatching.

The autoradiographs of UVE-digested mtDNA revealed no differences between the control and UV-irradiated eggs. Both groups showed slight digestion of the mtDNA, probably due to a low level of non-specific DNase activity in the UVE preparation (R. Waters, personal communciation). Positive controls (purified mtDNA irradiated directly with a 254-nm lamp) revealed extensive digestion of the mtDNA. These results suggest that the mtDNA in eggs given up to twice the optimal dose for denucleation had sustained no significant damage.

Mitotic inhibition

The intensity, duration, and time of application of the heat shocks strongly influenced the production of diploid androgens (Fig. 2). Shock intensities below 42.5°C failed to produce any androgenetic diploid fish. The survival of an-

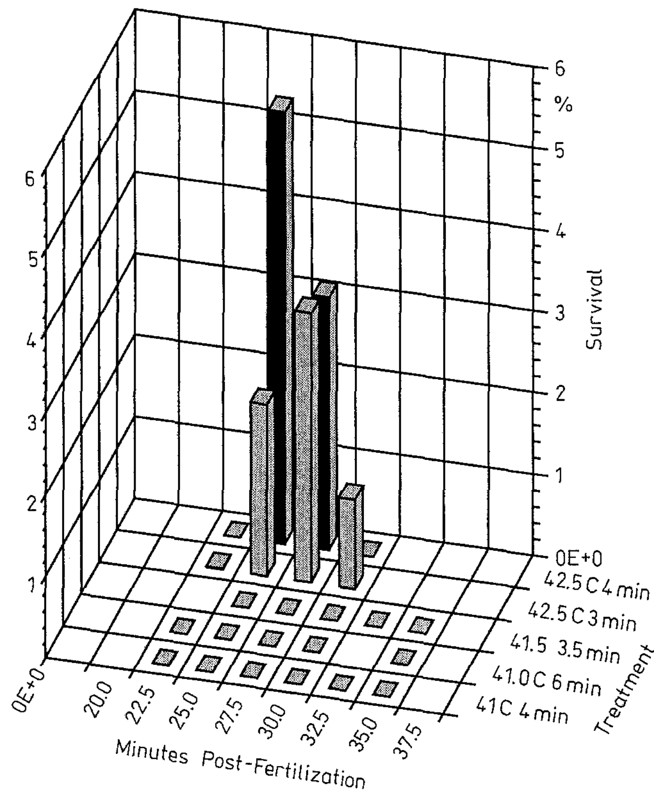

Fig. 2 Average survivals (%), relative to controls, of presumptive "blond" androgenetic Nile tilapia *O. niloticus* embryos to yolk-sac absorption (8 days post-fertilization) subjected to various thermal shocks to inhibit the first mitotic division. Averages for each thermal shock are based on 4–15 spawns, and treatments within each shock consist of 150–300 eggs/spawn

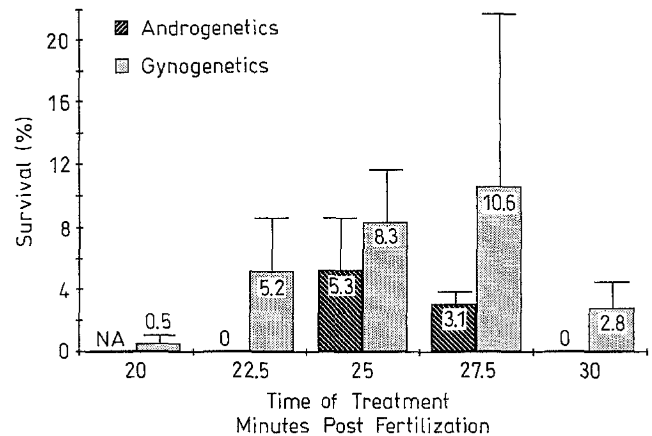

Fig. 3 Average survivals (%), relative to controls, of diploid androgenetic and mitogyne Nile tilapia *O. niloticus* to yolk sac absorption. All eggs were subjected to a heat-shock induction treatment of 42.5°C for 4 min applied at 2.5-min intervals 20–30 min post-fertilization. Averages are based on 4–15 spawns, and treatments with each shock consist of 150–300 eggs/spawn

drogenetic diploid tilapia to yolk-sac absorption varied from 1.1±1.1% to 5.3±3.3% in those treatments that exhibited any survival. A heat-shock treatment of 42.5±0.1°C for 4 min applied 25 mpf at 28°C ambient temperature appears to be the most effective (5.3±3.3%). Variation within treatment protocols (between different females) resulted in the one-way ANOVA for induction treatment being nonsignificant (P< 0.807, df=7), while the ANOVA for specific female effect was significant (P< 0.026, df=7), but not the multiple regression using both factors (P< 0.523, df=53). The male used in each cross did not have a significant effect (P< 0.179, df=7) on the production of androgenetic diploids, as determined via one-way ANOVA. The repeatability of the treatments also varied. For example, the 42.5°C-4-min duration treatment at 25 mpf produced the highest average survival, 5.3±3.3% (2.3±1.4% unadjusted for control survivals), but fish were only produced in 3 of the 7 spawns (42.9%), while the same treatment applied at 27.5 mpf produced fish in 5 of the 6 spawns (83.3%) with an average survival of 3.1±0.2% (1.3±0.8% unadjusted for controls survivals).

The average survival of mitotic gynogens treated from 20–30 mpf varied from 0.5±0.5% to 10.6±5.6%, relative to controls. Variation between treatment times was nonsignificant (P< 0.265) due to the high variation between spawns. Nevertheless, a similar pattern of treatment optima was found for mitogyne and androgenetic treatment (Fig. 3).

The developmental timetable of haploid androgenetic and mitogyne eggs was similarly altered relative to diploid control eggs. The onset of first cleavage was delayed by 12.2±0.6 and 14.3±1.7 min in the gynogenetic and androgenetic eggs, respectively, relative to diploid cleavage, which occurred at 87.4±0.6 min at 28°C.

Analysis of presumptive "blond" androgens and gynogens via electrophoresis verified that only the paternal or maternal *ADA** genotype was present, respectively. The combination of skin coloration and electrophoretic variation would strongly suggest that the progeny were indeed androgenetic.

Discussion

UV irradiation was effective in disrupting nuclear DNA in tilapia eggs, as has been observed in UV-irradiation experiments with spermatozoa (Chourrout 1984, Don and Avtalion 1988). Pigmented embryos were found in the 2- and 4-min UV treatments, and the "blond" embryos in these groups tended to exhibit aberrant development. Given the need to ensure that "host" eggs are denucleated it would be necessary to provide a UV treatment of at least 5 min at 150 $\mu W/cm^2$ (450 J/m^2) and up to 10 min (900 J/m^2). Higher UV doses have been needed to produce androgenetic haploid common carp, *Cyprinus carpio* L., with an optimum UV dose of 2500 J/m^2 yielding 53.9% androgenetic haploids relative to controls (Bongers et al. 1994). In the only other study using UV as a denucleation treatment no lamp intensity was given, and the treatment duration was 40 min (Kowtal 1987) for the white sturgeon, *Acipenser transmontanus*, eggs. Differences in the thickness,

composition, and optic qualities of egg chorions, egg size and shape, and the relative position of the female pronucleus prior to fertilization from species to species make it difficult to compare egg irradiation treatments. UV irradiation treatments for tilapia spermatozoa show a similarly wide variation in intensity, 200–2750 J/m^2 (see review by Mair 1993), which may be due, in part, to variation in sperm concentrations in such experiments.

Although it can be inferred from the survival and pigmentation data that *O. niloticus* eggs can be effectively denucleated, the UVE analysis confirms that the mtDNA, a component of the egg that might be expected to be more susceptible to UV damage, is apparently not affected by doses of UV that are more than adequate to denucleate the egg. The reasons for this are undetermined but may be due to the relative positions of the female pronucleus and the mitochondria in the egg or the level or protection offered to the respective DNA molecules by surrounding membranes, etc. The lack of damage to the mtDNA is reassuring when considering the regeneration of nuclear genomes or the creation of clonal lines via androgenesis.

It was evident that female differences in susceptibility to UV treatment exist. The mechanism for these differences is currently unknown. It may become necessary to select broodstock suitable for producing "host" eggs under a specific UV exposure protocol. For these reasons the UV-irradiation protocol described in this study may not necessarily be effective, a priori, with other species, even other tilapia species.

Diploid androgenetic and gynogenetic tilapia were successfully produced using a heat-shock protocol, 42.5°C for 3 or 4 min applied 22.5–30 mpf. The treatment temperature is significantly higher than that reported for inducing mitogynes in *O. niloticus*, 41.0±0.1°C for 3.5 min at 27.5 mpf (Hussain et al. 1993), and *O. aureus*, 41.1±0.1°C for 3.5 min at 20–35 mpf (Mair 1993). Although the time of treatment application is similar in all the studies, suggesting that the same mitotic processes are being affected, there are considerable differences in the intensity of the treatment. The yields of diploid mitogynes in previous studies, 1–3%, are far lower than the average survival to yolk-sac absorption, 10.6%, for the best mitogyne treatment, 42.5°C for 4 min applied 27.5 mpf, in this study. Further work is necessary to establish whether the higher treatment temperature in this study is universally more effective at inhibiting mitotic events in tilapia, or whether specific interactions are responsible. In general, individual female, strain, or species effects may influence the time and intensity of mitotic inhibition treatments, and no single treatment may be optimal; instead, a range of treatments may be needed to guarantee an acceptable level of success in any one cross (Johnstone 1993).

The delay in cleavage time for haploid androgenetic and gynogenetic eggs is presently unexplainable, but probably related to one of the various mechanisms that control the mitotic processes (Hartwell and Weinhert 1989). It may be difficult to directly transfer methodologies derived for diploid eggs to haploid eggs, and vice versa. For example, the difference between induction optima for diploid androgenetics and mitogynes, 25–27.5 mpf, and tetraploid tilapia, 22.5 mpf (Myers and McAndrew, unpublished), may be explained by this difference in development. In either case, the optima occur at approximately 30% of the first cleavage interval which corresponds with the formation of the asters following fusion of the pronuclei (Garcia 1991).

The survival of androgenetics to yolk-sac absorption in the "best" treatment group, 5.3%, may be sufficient for use in gene banking programs. The absence of any significant paternal effect influencing androgenetic diploid production would suggest that the androgenesis procedure would not select for or against any male in a gene banking programme, although there would be selection against deleterious recessive genes. Furthermore, given the significant maternal influence on androgenetic diploid induction levels, if females selected for "host" egg production were used the yields could be improved considerably. Low survival levels for induced androgenetic fish are, however, common to all studies. In diploid androgenetic rainbow trout, *Oncorhynchus mykiss*, the average survival to yolk-sac absorption varied from 7.2% to 9.5% for several strains of trout (Scheerer et al. 1986). Similar values were obtained for the induction of androgenetic diploid common carp, 8.1–9.3% (Bongers et al. 1994). May et al. (1988) produced androgenetic brook trout, *Salvelinus fontinalis*, but at what were described as very low levels of survival beyond hatch.

The production of androgenetic fish using sperm as the only DNA source is the preliminary step in developing a procedure for recovering cryopreserved stocks. To date, a total of over 100 viable androgenetic diploids have been produced. These fish are being studied to evaluate the potentially deleterious impact of the homozygous genome on their growth and fertility. Additionally, because of the heterogametic nature of male *O. niloticus* androgens would be either "YY" males or "XX" females and any F_1 progeny would be "XY" males. Some type of backcrossing scheme or hormonal sex-reversal would be needed to return the population to a normal XY/XX (50:50) system. Furthermore, the influence of the non-nuclear components of the host egg on the subsequent development of androgens must be evaluated, especially mtDNA.

Acknowledgements The UVE preparation was a gift from Dr. R. Water, School of Biological Sciences, University of Wales, Swansea. The authors would also like to thank Ms. E. Mooney for her assistance with the project. This project and funding for JM and SP was supported through funds provided by the Overseas Development Administration Grant R4700. In addition, DJP and PB were supported by the ASEAN-EC Aquaculture Development and Coordination Programme and YB by the British Council.

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