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Induction of tetraploidy in transgenic tilapia (*Oreochromis niloticus*) using physical shocks

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Abstract: The induction of tetraploidy by means of cold, heat and multiple heat shock treatments was investigated on male fish from a growth-enhanced transgenic tilapia C118 line, crossed with wild type females. After the development a new multiple heat shock protocol (two heat treatments at 41°C in 60 and 80 min. after fertilization for 5 min. per each), chromosome and X-gal *in situ* staining assays demonstrated the successful production of yolk sac tetraploid transgenic tilapia in two out of 15 embryos examined in this trial via direct correlation between higher level of ß-galactosidase expression and karyotyping in the samples verifying complete tetraploidy in this line. In the grown fish, there was evidence to suggest mosaicism or perhaps tetraploidy in at least two grown fish using the measurement of erythrocytes and karyotyping of the G1 offspring ($P<0.05$). There was, however, no evidence to indicate successful production of triploid G1 individuals following crossing of induced fish with wild type.

Keywords: Transgenic tilapia, *Oreochromis niloticus*, β-galactosidase, Tetraploidy, *lacZ* reporter gene, Sterility

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Introduction

Tilapiine species are among the most commercially important finfish in aquaculture and various species are currently being cultured in more than 100 countries (Coward & Bromage, 2000). Although basically they are non-marine warm water species, mainly indigenous to the African continent and Jordan (Fuente *et al.*, 1999), some of them tolerate brackish and marine waters to varying extent and also a variety of environmental conditions and can be easily cultured in a wide range of farming conditions. The species under study has a relatively short reproduction cycle, rapid growth and is strongly disease resistant. A fundamental problem with tilapia culture is the precocious maturation, consequently leading to stunted population, causing reduced growth. In recent years three different lines of transgenic tilapia with enhanced growth have been produced and reported at transgenic laboratory in the University of Southampton, UK (Rahman & Maclean, 1999; Rahman *et al.*, 2001a). One of these lines was subjected to a long-term growth trial and showed a 2.5 fold weight increase in 7 months over the wild type (Rahman *et al.*, 2001b). However, there is a serious concern regarding the potential impact on the natural fish population, if such transgenic fish should escape following their use in fish farms (Maclean & Laight, 2000).

One approach to solving this problem is to produce strains that are sterile, since they cannot integrate with the local gene pool. This is commonly achieved on an experimental basis using either direct or indirect triploidy methods (generated by tetraploid broodstock X diploid fish). However, the results from previous investigations have indicated that, despite the findings obtained (Hussain *et al.*, 1991), the existing methods are not reproducible and so each new generation has to be treated; nor is it possible to consistently produce 100% triploidy. Our pervious attempt was not successful to produce 100% triploid transgenic tilapia (Razak *et al.*, 1999) when followed the recommended conditions by Hussian *et al.* (1991), although a new triploid transgenic strain was created. Moreover, induced triploid organisms can revert by progressively replacing triploid cells with diploid cells (U.S. Congress Office of Technology Assessment, 1995). This could raise the question of the reliability of reversible direct triploidy in commercial aquatic organisms.

The best approach for ensuring triploidy may be regarded as indirect triploidization, a subject of great interest in aquaculture. Using this technique, a generated tetraploid can be used in the production of triploid offspring (3n) by crossing them with diploid individuals. Such triploids have superior heterozygosity than those generated mechanically (Dunham, 2004). Although theoretically this approach is a simple way to produce sterile fish, the creation of viable tetraploids is enormously difficult owing to high mortality and in some species has proved impossible (Horváth & Orbán, 1995). To our knowledge, there is only one report available regarding the production of a tetraploid broodstock, which reached sexual maturity and succeeded in producing triploid offspring (Chourrout *et al.*, 1986). In the case of tilapia, despite several attempts, there are no reports of grown tetraploids in this fish (Myers, 1986; Mair, 1988; Rahman *et al.*, 1999), although Don and Avtalion (1988) produced two 4N females which at 18 months did not exhibit any sexual behaviour.

The prime objective of this investigation was the development of viable transgenic tetraploid Nile tilapia individuals of reproductive size, which would be able to produce 100% triploid progeny. To achieve this, a number of proposed experimental conditions were tried and also the combinations of some treatments were considered.

Materials and Methods

Experiments were conducted at the transgenic laboratory at the University of Southampton, UK. The wild type eggs used in this study were crossed with sperm obtained from the G3 progeny of the C118 line growth-enhanced transgenic founder, which had previously been generated in our laboratory (Rahman *et al.*, 1997). This line is hemizygous for a transgene containing 3 copies of a growth hormone gene (OPAFPcsGH) and 34 copies of carp beta-actin *lacZ* reporter gene (Rahman *et al.*, 2000). The OPAFPcsGH construct consists of an ocean pout antifreeze gene promoter spliced to a chinook salmon growth hormone cDNA sequence with 3' and 5' flanking sequences (Du *et al.*, 1992). This construct was kindly provided to us by professor Choy Hew. *In vitro* fertilization was carried out for all experiments (Hussain *et al.*, 1991).

Approximately 150 Nile tilapia, *Oreochromis niloticus*, were maintaned in aquaria (glass tanks) with a continuous supply of well water at 28°C, which is appropriate for spawning with a photoperiodic of 13:11 hours light: dark schedule. Females were held separately due to the aggressive behaviour of males causing injury when the sexes are held together. Females with a swollen belly and redness of the genital papilla were selected for spawning. The degree of the projection of the papilla was the primary sign for ovulation. Eggs quality was judged after stripping according to appearance, and rate of fertilization was judged on a small sample to decide whether to proceed with the experiments or not.

To produce transgenic tetraploid tilapia, cold shock (CS) was initially administered as described by Don and Avtalion (1988). Next, a heat shock treatment was performed according to Mair (1988) and Myers *et al.* (1995) and termed single heat shock (SHS). The experiments also included a method described by Rahman *et al.* (1999) known as double late heat shock (DLHS). These conditions and the number of eggs are shown in Table 1. The device which has been used for any type of treatment (Cold or heat shocks) was a thermoregulated water bath.

In this study, a new condition of heat shock that combined the SHS and DLHS methods was tested and named double early heat shock (DEHS). In brief, the fertilized eggs were exposed to 41°C using thermoregulated water bath at 30 minutes after fertilization (AF) for 5 minutes and then kept in the incubator (28°C) for 15 minutes. The eggs were removed from the incubator and heat-shocked once more at 41°C, 45 minutes post fertilization, for a period of 5 minutes. Control and treatment groups with variable numbers of fertilized eggs were placed in plastic conical funnels and incubated at 28° in an upwelling flow of water following the induction. The trials along with controls were repeated in triplicate. The number of eggs and rate of hatchability were not always recorded in every case. The pre and post incubation temperature was 28°C for all the trials and the details of the treatments illustrated in Table 1.

Table 1: Parameters used for induction of tetraploidy in the C118 line of *Oreochromis niloticus* in triplicate.

Type of Shocks	Number of eggs/trial	Survival (%) 7 days AF	Treatment condition			References to the methodology used
			Temperature (°C)	Time after fertilization (min)	Shock duration (min)	
Control	A=127 B=113 C=107	44.9 77.7 41.5				
CS	A=245 B=157 C=187	inviable	11	92	60	Don & Avtalion (1988)
SHS	A=117 B=143 C=123	inviable	42.5	30	4	Mair (1988) ; Myers *et al.* (1995)
DLHS	A=303 B=243 C=425	25 21.1 18.3	41	60 & 80	5+5	Rahman *et al.* (1999)
DEHS	A=410 B=301 C=457	9.85 13.29 17.45	41	30 & 45	5+5	Present study

Identification of tetraploid individuals was undertaken for embryonic and grown fish. Before performing assays, the fish were first sacrificed by applying excess anaesthesia (Approx. 500ppm 2-phenoxyethanol). X-gal *in situ* staining assay (Rahman *et al.*, 2000) and metaphase spreads of the chromosomes were made from the embryos (Walker, 1995). Erythrocyte nuclear volume plus fertilization efficiency of sperm and chromosome number of the progeny achieved from crosses between presumptive tetraploid and wild type were also used as indicators of tetraploidy in grown fish (Razak *et al.*, 1999).

The yolk sacs of the embryos were dissected away in samples taken between 7-10 days after fertilization and then each individual was divided into fin and abdomen parts. The fin was used for chromosome spread, while the abdomen was utilized for X-gal staining. Correlation between these two assays were considered

for identification of transgenic tetraploid tilapia since these individuals can be expected to express β-galactosidase approximately two times greater than diploid transgenic fish when using histochemical in situ staining X-gal as a substrate (Rahman *et al.*, 2001a).

With regard to grown fish, 96 of the DEHS individuals were tagged with digital AVID Mini Tracker (California, USA) and coded as Til-1 to Til-96. The fish were kept until used for appropriate assays.

Chromosome preparation was performed according to the methods of Hussain and McAndrew (1994) and also Walker (1995) with some minor modifications. In short, the fins of embryos were immersed in a 0.005% colchicine solution and incubated at 28.5°C for 4-6 hours. At room temperature, the fin sections were transferred into 1.1% Na citrate for approximately 20 minutes and then immersed in a fixative of 3:1 methanol/acetic acid at 4°C. After two changes, samples were transferred into a watch glass containing 4-6 drops of 50% acetic acid and were broken up with forceps for 1 minute. Next, the cell suspension was dropped from a height of 50-70 cm onto a clean glass slide on a hot plate (50°C). Slides were air dried and stained with freshly prepared 5% Giemsa stain for 15-20 minutes. The slides were rinsed in distilled water, air-dried and after mounting with DPX, were observed by microscope (100X magnification).

Samples of abdominal muscle (Fig. 1) were fixed in freshly made fixative (2% paraformaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40, in 0.1 M sodium phosphate, pH 7.6) for 5-6 hours at 4°C, and then were washed 3 times for 5 minutes in phosphate buffered saline (PBS, pH 7.6), before being immersed in X-gal histochemical reaction mixture. The reagent comprised of 0.1 M Na phosphate (pH 7.6), 1.3 mMMgcl$_2$, 3mM $K_3Fe(CN)_6$, 3mM $K_4Fe(CN)_6$, 0.1% NP-40 and 1mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-galactosidase; Promega). The X-gal stain solution was left at 30°C overnight (MacGregor *et al.*, 1991). Upon completion of staining, tissues were washed 3 times in PBS and then analysed under a stereo microscope (either Leica MZ6, UK or Kyowa Optics, Japan).

After 18 months, 50 DEHS individuals were still surviving. All of these were sampled at the same time for the erythrocyte dimension according to the method of

Humayun *et al.* (1994). Briefly, 0.2ml blood was extracted from the caudal vein using 1ml disposable syringe, which was smeared with Cortland solution/10 mM EDTA to avoid blood clotting. Three glass-slide smears were prepared from each sample of drawn blood. After air-drying, the slides were fixed in absolute methanol for 15 minutes and stained in 10% Gimsa (BDH Co.) followed by quick rinses in distilled water and then left to dry. The major and minor axes of ten erythrocytes were randomly selected and measured from each slide using an eyepiece micrometer under 100X magnification. The formula of $a^2b/1.9$, where (a) and (b) are the minor and major axis respectively, was used for the calculation of the cell and nucleus volumes. The data was subjected to one-way analysis of variance (ANOVA) using SPSS Statistical Package version 11 and Duncan's New Multiple Range Test (DNMRT) was performed to determine the significant difference between samples.

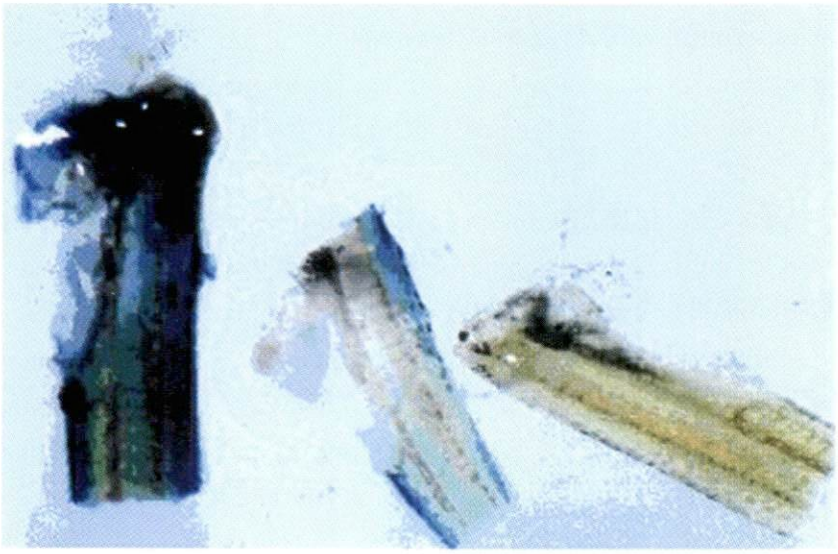

Figure 1: Comparison of ß-galactosidase expression in the heat-shock-treated individuals (DEHS trial; left), produced from crossing of the transgenic male of C118 line with the wild type female, with non-induced or hemizygous (in the middle) and non-transgenic (right) embryos (magnification 10X). Although in black and white the blue colour is not apparent in the hemizygous specimen, it is clearly visible when viewed in colour.

Results

After the shock treatments, no surviving embryos were observed beyond 7 days AF in either the CS or SHS trials. There were, however, embryos in both DLHS and DEHS treatments for further analyses. In several attempts to determine tetraploidy status, following X-gal assay, positive embryos indicated variation with respect to *lacZ* gene expression, in some cases there was only marginal discrimination between presumptive transgenic tetraploid and diploid embryos in both trials. However, it was possible to identify a correlation between the higher levels of ß-galactosidase expression and karyotyping in two out of 15 embryos examined in the DEHS trail, as both the tetraploid chromosome spread and strong blue colour precipitation was observed in the tissue (Fig. 1 and Fig. 2 bottom). A chromosomal number of 88 with 4 large easily distinguishable subtelolentric chromosomes was observed in these individuals. Through vigorous chromosome assessment of these embryos, at least 8 tetraploid spreads were distinguished in each specimen, verifying complete tetraploidy in this line. No diploid spreads were detected in these embryos, confirming occurrence of complete tetraploidy in embryonic stage for transgenic C118 tilapia. The rest of individuals were identified as either diploid (Fig. 2A top) or not conclusive.

In the case of DLHS, following the analysis of chromosomal spreads from 18 embryos, only one case of mosaisim was detected, the remaining individuals were either diploid or inconclusive. No complete tetraploids were identified.

Preliminary scoring of the samples described in section 2.4 showed that the individuals of Til-22, Til-28, Til-41 and Til-43 possessed larger erythrocytes than the rest of the sample population. Careful measurement of these samples demonstrated that the volume of erythrocytes and their nuclei were significantly ($P<0.05$) higher than those of controls, among them Til-41 with 1:2.24 ratios showed as the best candidate for tetraploidy (Table 2). The frequency distribution of nuclear measurements from 30 randomly sampled erythrocytes from Til-41, when the major axis across the nucleolus has compared with wild type, indicated that most cells were out of diploid range, despite some overlapping observed in the two cells (Fig. 3). The volume ratio of Til-22, Til-28 and Til-43 specimens, with

1.7, was relatively close to the triploidy range (1.5 ratios). The representative micrographs of the erythrocytes from Til-41 and control are presented in Fig. 4.

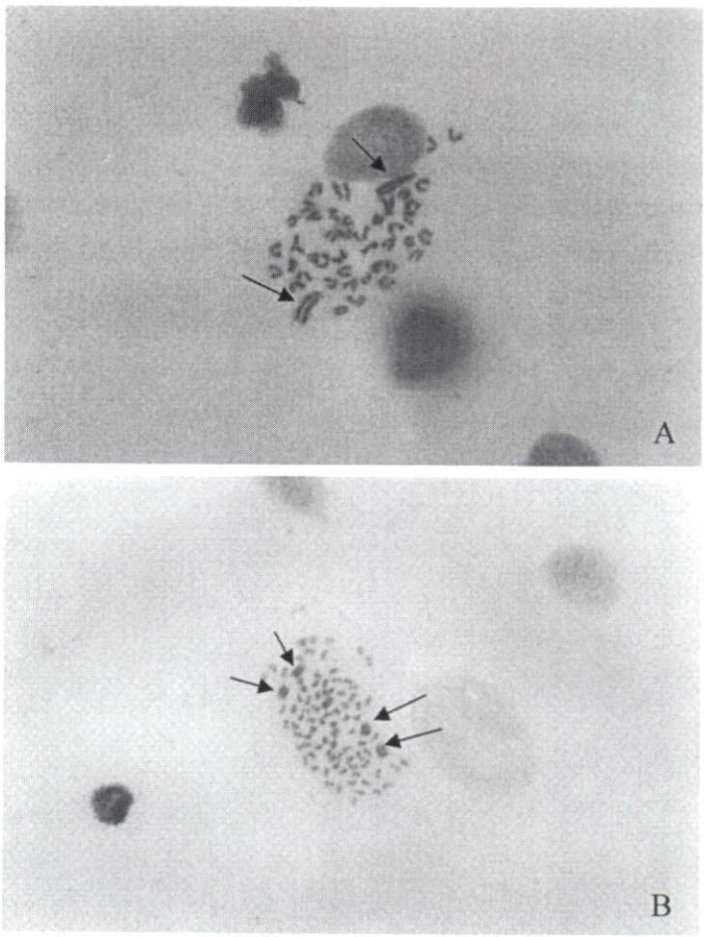

Figure 2: Metaphase spreads of *O. niloticus* in embryonic stage: Top; A diploid, Bottom; B tetraploid. Two (diploid) and Four (tetraploid) long distinguishable chromosomes are indicated by arrows (40X). Noticeably, in the diploid *Oreochromis* species, a pair large, easily distinguishable, subtelocentric chromosomes are used as markers for determination of ploidy (Majumdar & McAndrew, 1986). (Fig 2B is reproduced with permission from Maclean *et al.*, Gene 2002).

Table 2: Comparison of average cellular and nuclear volume size of DEHS-treated fish with control obtained from 30 erythrocytes for each individual.

Fish Code No.	Cellular Volume Mean ± SE (μm^3)	Nuclear Volume Mean ± SE (μm^3)	Cellular Vol. Ratio Treated-fish/Control	Nuclear Vol. Ratio Treated-fish/Control
Control*	1972±87	105±7[a]	–	–
Til-22	1882±153	172±24[c]	1.0	1.6
Til-28	2097±128	173±18[c]	1.1	1.7
Til-41	2565±189	235±21[b]	1.3	2.2
Til-43	2177±168	182±24[c]	1.1	1.7

[a-c] Values within columns with different superscripts are significantly different ($P<0.05$).
* The data was obtained from the average of three individuals.

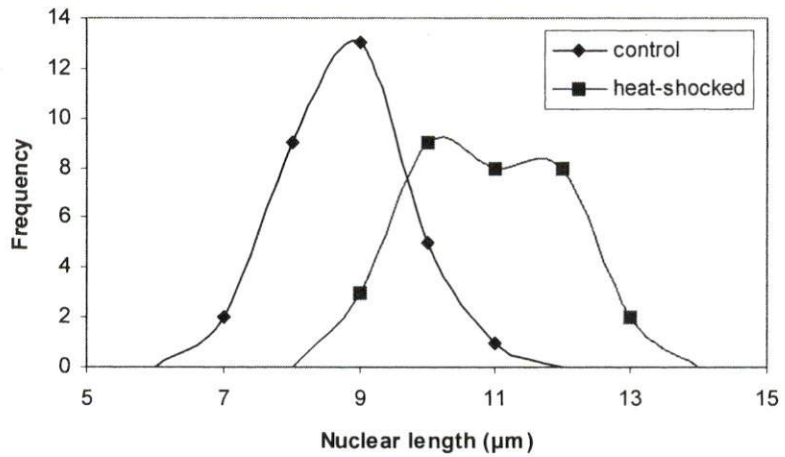

Figure 3: Frequency distribution of erythrocyte nuclear length in sample Til-41 and control (N= 30).

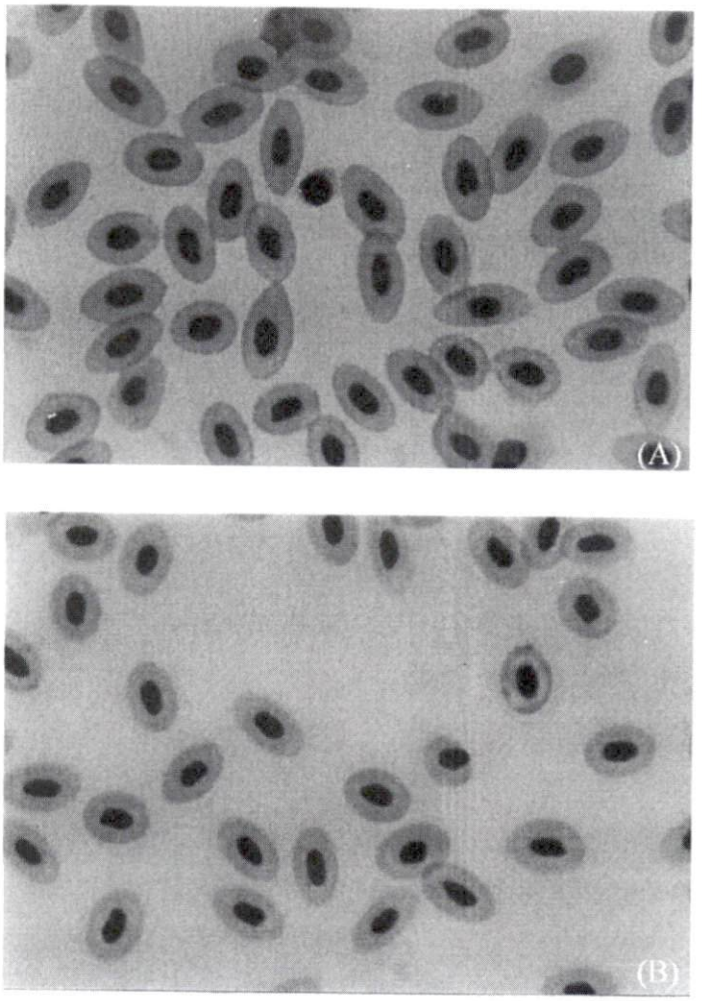

Figure 4: Effect of multiple heat-shock treatment on erythrocyte size A (top). Control red cells are peresented at the bottom B (40X).

Out of the four individuals, Til-22 and Til-28 produced sperm after LHRH (30µg/kg) induction. Crossing of both samples with wild type indicated very low rates of hatchability compared with control (Table 3). In the search for G1 triploid progeny, chromosomal spreads were prepared for ten offspring in each batch. One individual (progeny of Til-28 x diploid) demonstrated tetraploidy (Fig. 5). The rest of the embryos were identified as diploid or not conclusive. No triploid G1 embryos were detected.

Table 3: Fertilization rates in G1 embryo generated by crossing from sperm DEHS-treated samples with wild type eggs.

Fish No.	Fertilisation rates						Chromosome Spread			
	No. of eggs fertilised		No of eggs hatched		% Hatched		No.	Tetraploid	Diploid	Inconclusive
	Untreated	Treated	Untreated	Treated	Untreated	Treated				
Til-28	158	193	84	7	53.2	3.60	10	1	3	7
Til-43	183	296	104	45	56.8	15.20	10	–	2	8

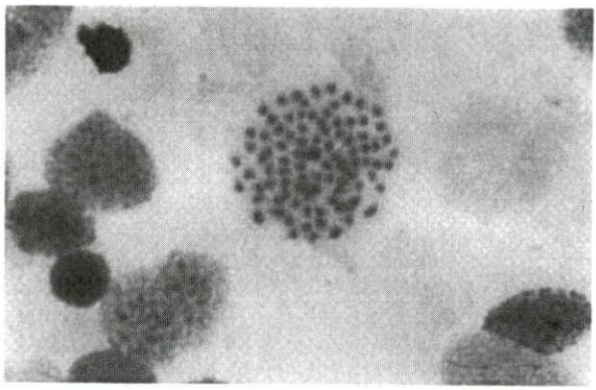

Figure 5: Methaphase spread of G1 *O. niloticus* which has been generated by crossing of Til-28 (DEHS) with female wild type (100X). The chromosome spread shown is that of a presumed tetraploid cell.

Discussion

The viability of embryos is an important criteria when searching for the optimal treatment methodology to be used for ploidy manipulation. It is generally accepted that thermal or pressure shocks are among the most effective method for induction of polyploidy (Ihssen *et al.*, 1990; Mair, 1993), although consistent results are not easy to achieve between different laboratories even when the same procedures are followed (Wolters *et al.*, 1981; Bidwell *et al.*, 1985; Dunham, 2004), apparantly owing to zygotic development AF not being synchronized (Don & Avtalion, 1988). Since the preliminary experiment on hydrostatic pressure shock induction (7500 psi for 7 min at 30 min AF) resulted in negative tetraploidy, thermal shocks were selected to induce tetraploidy in this investigation and it was thought to be more effective than other treatments reported.

CS treatment parameters were selected because of the Don and Avtalion (1988) finding, who reported successful production of tetraploidy in Nile tilapia with the same experiment. They even reported the existence of two tetraploid females to the age of 18 months, although they did not exhibit any sexual behavior. Therefore, it was initially thought this method would be appropriate for our objective.

SHS parameters were selected based on the finding that temperture below 42.5°C was ineffective for suppressing cell division in Nile tilapia (Myers *et al.*, 1995). However, at this temperature, with a shock duration time of 4 minutes, androgenic diploidy was successfully induced. The Mair (1988) findings corroborate our conclusion about the timing of shock AF in this trial. He also has shown that with the application of shock treatment for 30 min AF can more easily produce tetraploid embryos. In a number of attempts, however, there was a failure to demonstrate viable embryos in both the CS and SHS treatments owing to a high rate of mortality and embryonic deformities within a week of incubation. Because of the resulting 100% mortality rate, it can be concluded that the temperature of shocks applied in both trials were the lethal zones for this species. The lethality effect of 11°C and 42°C for Nile tilapia has been previously reported (Rahman *et*

al., 1999) when these authors performed the same experiment. The differences in results may be related to variation of individual response to a specific thermal shock treatment or to dissimilarity in the pre-incubation temperatures (28°C in our study rather than 25-26°C in trials performed by Don and Avtalion). It is known that polyploidization can be easily affected by the temperature at which the eggs are incubated before the beginning of the shock treatment because of its influence on the first cell division (Mair, 1993).

DLHS trial resulted in high survival rates but was not successful to induce tetraploidy, at least under our experimental holding condition. However, Rahman *et al.* (1999) reported approximately 80% tetraploidy in hatched fry when they used the same protocol. The reasons for these discrepancies between these two results are unclear; perhaps any tetraploid individuals in our batches were dead before assay.

The DEHS trial, which was inferred from SHS and DLHS treatments, was able to demonstrate complete tetraploidy at the fry stage of the C118 transgenic line. This has been deduced based on two criteria, namely increased reporter gene expression and karyotyping of the embryos. Although the expression of ß-galactosidase could be related to many factors, the coincidence of a strong blue colour with a tetraploid spread in the same individual leads us to conclude that such strong expression of the *LacZ* gene related to the increasing of *lacZ* copy number due to tetraploidization.

Although the erythrocyte nuclear volume size of Til-41, which showed a ratio of 2.24 against diploid fish, had provided evidence for tetraploidy, it did not produce eggs or sperm. In addition, the frequency distribution of nuclear major axes in this sample had rather broad standard error (SE) when compared with wild type fish (Fig. 3) and the existence of the overlapping observed may suggest the creation of ploidy mosaicism in this fish. This could be verified by the data, which was obtained from crossing of the two samples (Til-22 and Til-28) with the control fish. The reduced fertilization rate obtained when crossing this sperm sample with a wild (non-transgenic) female may suggest the existence of sperm with two

differing head sizes and, therefore, restriction on the ability of the diploid sperm to penetrate into the micropyle of haploid eggs (Chourrout *et al.*, 1986) or normal sperm outcompeting the larger 2n sperm to penetrate the micropyle, thus not creating haplotide progeny. Observation of sperm preparation by light microscopy also supported this interpretation, although photographs of the stained sperm proved somewhat inconclusive owing to picture quality. It is suggested to look at sperm morphology and its importance in a separate study. The instance of the tetraploid individual from G1 obtained following crossing of Til-28 with the wild type supports also the finding of ploidy mosaicism.

Mosaic individuals, having 3n, 4n, and 5n cells, due to tetraploidization, have been already reported from other species (e.g. in Atlantic salmon, Standish *et al.*, 1979; in catfish, Bidwell *et al.*, 1985; rainbow trout, Chourrout *et al.*, 1986, and in white sturgeon, Van Eenennaam *et al.*, 1996). Examples of diploid-triploid and diploid-tetraploid mosaicism have also been documented in *Carassius auratus langsdorfii* (Murayama *et al.*, 1986).

There seemed to be very little chance of producing triploid fish since the thermal shocks were provided at 30 minutes AF at which time it would be expected that the second polar body would degrade and the applied shock prevented cell division during cytokenesis (the earlier stage of mitosis such as spindle pole formation and nuclear membrane disintegration during prophase; Hussain *et al.*, 1991; Mair, 1993).

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