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Suppression of Meiotic and Mitotic Cell Divisions in Nile tilapia, *Oreochromis niloticus* L., and Induction of Triploids and Two Types of Gynogenetic Diploids

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Abstract

Studies were conducted to interfere with the normal functioning of spindle apparatus during meiotic and mitotic cell divisions in Nile tilapia, *Oreochromis niloticus* L., eggs using both temperature and hydrostatic pressure shock treatments, therefore, leading to the induction of triploids and two types of gynogenetic (meiotic and mitotic) diploids.

Introduction

Despite the worldwide popularity of tilapia in aquaculture, the main drawback in all existing commercial strains is their precocious maturation in tropical and subtropical pond conditions. This leads to prolific breeding and overcrowding in grow-out systems, resulting in undesirable stunting and low yields of harvestable size fish. There are other problems associated with the commercial tilapia species, and many existing stocks are of poor genetic material due to 1) inbreeding of stocks through poor broodstock management, and 2) introgression of genes from other less desirable feral tilapia species. Genetic research of cultured fish has a short history compared to that of crops and domestic ani-mals (Pullin and Capili 1988). Until recently some attention has been given to genetic approaches to solve the aforementioned problems in tilapia.

Production of triploid tilapia progeny by genome manipulation techniques using a variety of methods has attracted considerable attention (Valenti 1975; Chourrout and Itskovich 1983; Don and Avtalion 1986, 1988a; Penman et al.

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1987; Pandian and Varadaraj 1987, 1988; Hussain et al. 1991). The potential of the commercial application of these techniques to replace hybridization and use of hormones in tilapia culture is obvious, but the recent nature of this work and the lack of follow-up trials makes this unlikely in the near future. Triploid individuals are expected to be functionally sterile because of a failure of homologous chromosomes to synapse correctly during the first meiotic division and therefore to be of benefit in aquaculture, since maturation processes often have profound and, ultimately, limiting effects on growth.

In addition to these developments in tilapia genetic research, meiotic gynogenetics have been produced in many fish species which are not completely homozygous (Chourrout 1987; Ihssen et al. 1990). The first mitotic gynogenetics in fish was produced by Streisinger et al. (1981) in zebra fish. The technique has since been applied to other fish (Chourrout 1984; Onozato 1984; Naruse et al. 1985; Ijiri 1987; Nagy 1987; Taniguchi et al. 1988; Gouidie et al. 1991; Han et al. 1991; Komen et al. 1991; Quillet et al. 1991). Although meiotic gynogenesis in tilapia has been induced by many authors (Chourrout and Itskovich 1983; Penman et al. 1987; Don and Avtalion 1988b, 1988c; Varadaraj and Pandian 1989; Pandian and Varadaraj 1990; Varadaraj 1990; Marian and Pandian 1992; Pandian 1994), only few published works have reported induction of mitotic gynogenetics in the fish (Mair et al. 1987; Hussain et al. 1993). The first generation of mitotic gynogenetics have limitations for direct use in culture, but they are valuable as potential and completely homozygous broodstock to produce a second generation of clonal lines.

The present study was conducted to compare temperature (heat/cold) and pressure shock treatments for the suppression of meiotic and mitotic cell divisions of fertilized eggs, leading to the induction of triploids and two types of gynogenetic (meiotic and mitotic) diploids in a single species (*Oreochromis niloticus* L.).

Materials and Methods

Source of Broodstock

The *O. niloticus* broodstock used in this study came from the Tilapia Reference Collection maintained at the Institute of Aquaculture, University of Stirling, Scotland. Broodstock maintenance, breeding of fish, and egg incubation were as described by Hussain et al. (1991).

Induction of Triploidy

Eggs from female fish were fertilized with fresh sperm then divided into four equal batches. Three of the batches were exposed separately

to hydrostatic pressure (8,000 p.s.i. for 2 minutes at 9 minutes after fertilization [a.f.]), heat (41°C for 3.5 minutes at 5 minutes a.f.) and cold (9°C for 30 minutes at 7 minutes a.f.) shocks (Hussain et al. 1991). The fourth batch of eggs was untreated and acted as control.

Induction of Meiotic and Mitotic Gynogenesis

The meiotic gynogenetic individuals were produced by exposing eggs fertilized with UV-irradiated milt (with a UV dose of 300-310 $\mu W\ cm^{-2}$ for 2 minutes with a sperm concentration of 2.5 x $10^7\ ml^{-1}$ in 2.05 ml of modified Cortland's solution, Hussain et al. 1993) to the optimal second polar body retaining pressure and heat shock treatments (Hussain et al. 1991). The techniques to interfere with the first mitosis to produce the mitotic gynogenetic diploids using both late pressure (shocks of 8,000-10,000 p.s.i. by 500 p.s.i. steps; durations of 1-4 minutes by 1-minute increments applied 25-65 minutes a.f. by 2.5-minute increments) and heat (shocks of 40-42°C by 1°C steps; durations of 2.5-5.5 minutes by 1-minute increments applied 20-55 minutes a.f. by 2.5-minute increments) shock treatments (Hussain et al. 1993). All treatment batches of eggs except UV control and normal control were exposed to elevated shock treatments.

Determination of Ploidy

The ploidy of all treatment and control batches was determined by chromosome preparation from a sub-sample of newly hatched or 1-d old larvae (Hussain et al. 1991). The ploidy rate was assessed by karyotypic analysis of several (>3) karyotypes per individual and 10-15 individuals per treatment. The haploid, diploid and triploid metaphases in *O. niloticus* were composed of one (n = 22 including one large marker chromosome), two (2n = 44 including two marker chromosomes) and three (3n = 66 including three marker chromosomes) sets of chromosomes (Fig. 1a-c). Aneuploid metaphase (hyperhaploid or hypodiploid) was composed of more than 22 and less than 44 chromosomes in this species (Fig. 1d).

Electrophoretic Analysis

The starch gel electrophoresis of biopsid tissues of all the broodstock and progeny was used to ascertain their genotypes in gynogenetic experiments as described by McAndrew and Majumdar (1983). One known highly polymorphic locus, *ADA** (adenosine deaminase, ADA. E.C. No. 3.5.4.4.) with three alleles (**135, *121* and **113*) was selected as marker.

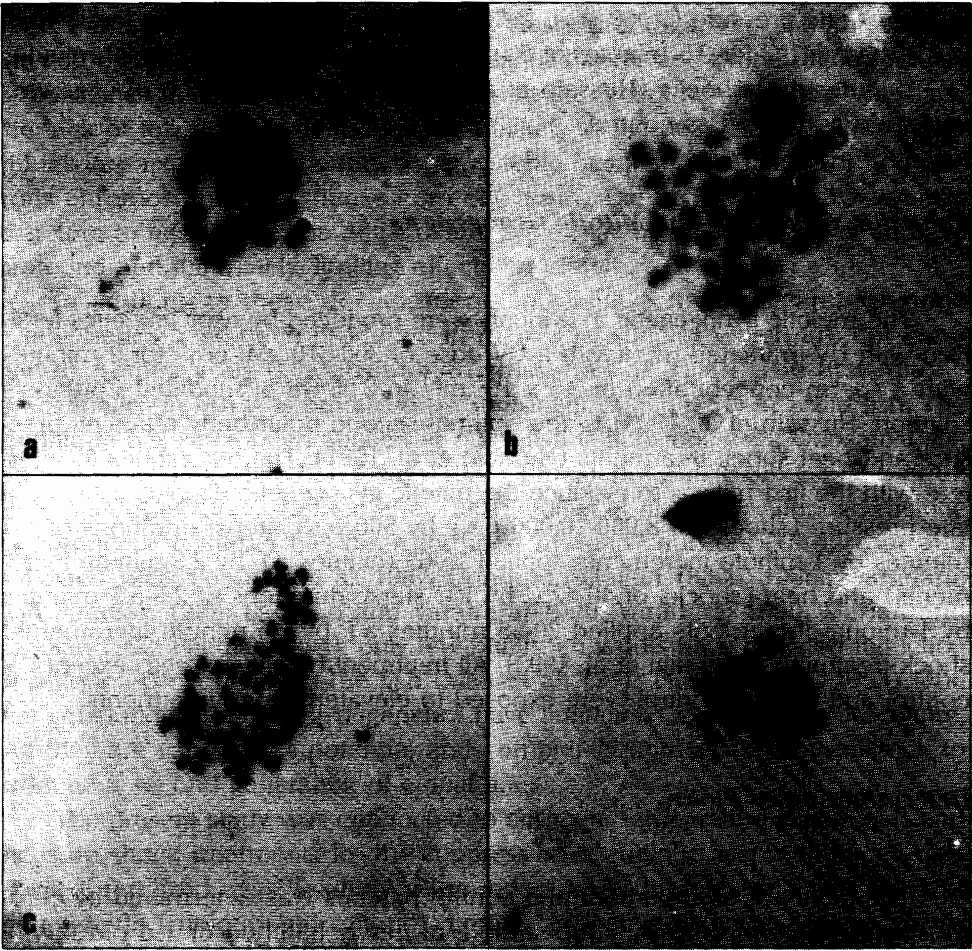

Fig. 1 a-d. Metaphase chromosomes of *O. niloticus*; a. haploid spread (n = 22 including one large marker chromosome), b. diploid spread (2n = 44 including two large marker chromosomes), c. triploid spread (3n = 66 including three large marker chromosomes), d. aneuploid spread (number above 22 and below 44 including two large marker chromosomes).

Results

Hussain et al. (1991) described the results of a study to identify treatment optima in inducing triploidy by exposing newly fertilized eggs of *O. niloticus* to altered intensities, durations and timings of application of hydrostatic pressure, heat and cold shocks. It is more important to identify any inter-individual difference in response to particular treatments; and this was the objective of this study where the apparently preferred agent optima were applied in a directly comparable manner to the eggs of eight different females (Fig. 2). As expected, all animals karyologically analyzed (10-15 per treatment group) were made triploid by the optimized pressure (8,000 p.s.i., 2 minutes duration applied 9 minutes a.f.), heat (41±0.2°C, 3.5 minutes duration applied 5 minutes a.f.) and cold (9±0.2°C, 30 minutes duration applied 7 minutes a.f.) shocks and therefore the

difference between agents related to variability in survival. Mean triploid yields following pressure shock (84.8±3.4%, N=8) were significantly greater ($P<0.05$) than those seen after cold shocking (37.2±11.6%, N=8). Cold shock survivals showed a lot of inter-female difference despite the uniformly high control and pressure shock survivals. Although in most instances, individual yield values were greater after exposure to pressure than after exposure to heat treatments, mean yields following heat shock (70.3±5.5%, N=8) were not significantly different ($P>0.05$) from those following pressure shocks.

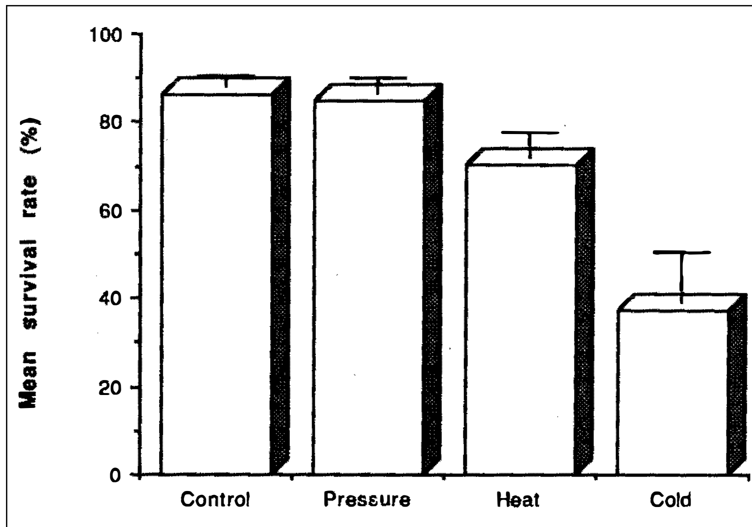

Fig. 2. The exposure and effect of optimal pressure, heat and cold shocks on the recently fertilized eggs of *O. niloticus* for the induction of triploidy by the interference of meiotic events of cell division.

The averaged results, showing the effect at different times after fertilization (a.f.) of applying heat and pressure shocks on the subsequent relative survival and production of putative mitotic gynogenetics at hatching (HAT) and yolk sac resorption (YSR) are presented in Fig. 3 and 4, respectively. The optimal parameters for heat shock were 41°C for 3.5 minutes at 27.5-30 minutes a.f., and that for pressure was 9,000 p.s.i. for 2 minutes at 28°C at 40-50 minutes a.f. where the pressure level was higher than that required for meiotic events (8,000 p.s.i.). Such narrow windows for successful suppression of mitotic events were revealed by karyological analysis of all mitotic gynogenetic survivors (i.e., normal-looking diploids). On the other hand, a high frequency of haploid or haploid/diploid mosaic was observed in either side of the 27.5-30 minutes a.f. for heat shock and in either side of 40-50 minutes a.f. for pressure shock.

Isozyme analysis of putative mitotic gynogenetic survivors at *ADA** locus confirmed complete homozygosity, therefore, restoration of diploidy occurred by inhibition of first mitosis. In contrast, only heterozygotes appeared in all samples of meiotic gynogenetic progeny providing evidence of 100% recombination confirmed that diploidization was caused by the retention of the second polar body.

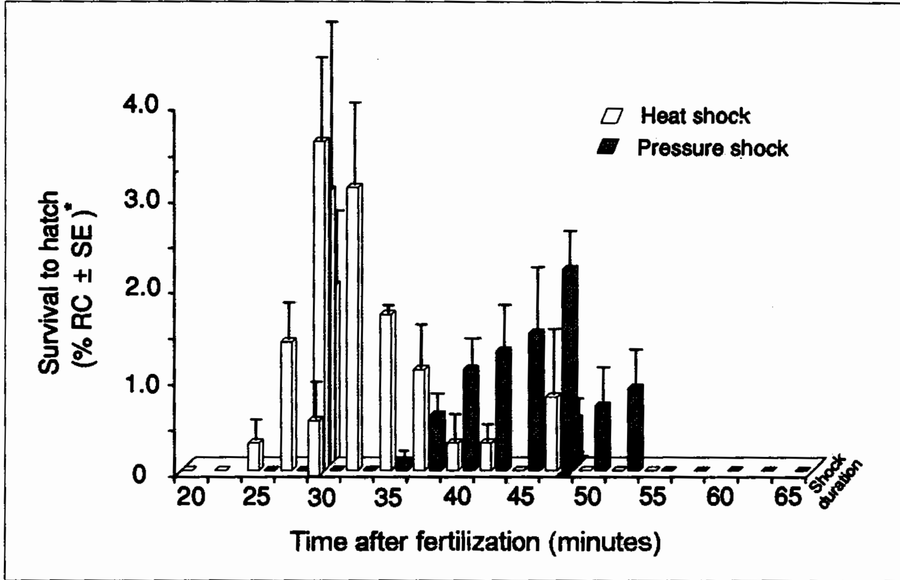

Fig. 3. The application of heat (41°C x 3.5 minutes) and pressure (9,000 p.s.i. x 2 minutes) shocks at different times after fertilization to *O. niloticus* eggs on the subsequent survival and incidence of putative mitotic gynogenetic diploids at hatching. *RC relative to control survival rates. The results of heat and pressure shock durations at 30 and 47.5 minutes a.f., respectively, are presented sequentially from front to back on the "Z" axes.

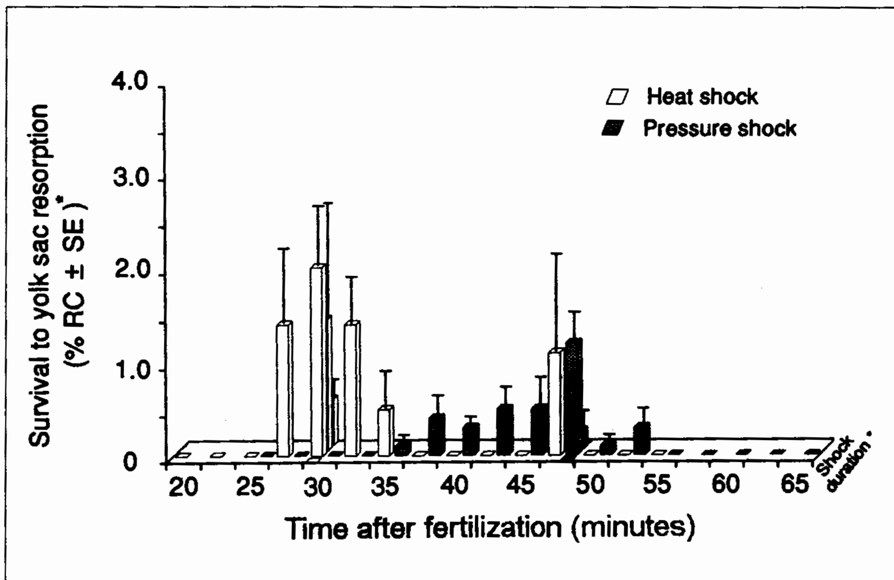

Fig. 4. The application of heat (41°C x 3.5 minutes) and pressure (9,000 p.s.i. x 2 minutes) shocks at different times after fertilization to *O. niloticus* eggs on the subsequent survival and incidence of putative mitotic gynogenetic diploids at yolk sac resorption. *RC relative to control survival rate. The results of heat and pressure shock durations at 30 and 47.5 minutes a.f., respectively, are presented sequentially from front to back on the "Z" axes.

Discussion

In the present and previous studies (Hussain et al. 1991, 1993), a series of trials were attempted to identify treatment of optima, using both temperature (heat/cold) and pressure shocks, for ploidy manipulation in a single species (*O. niloticus*); and perturbation of meiotic and mitotic cell divisions of fertilized eggs was used as experimental model. The optima of hydrostatic pressure shock treatments used for the induction of 100% triploidy is probably the first work in *O. niloticus*. The results of optimal heat shock treatments are essentially close to those of Chourrout and Itskovich (1983) but are slightly different from those of Don and Avtalion (1986, 1988a). The results of optimal cold shock treatments are comprehensive and entirely different from the previous works (Valenti 1975; Don and Avtalion 1988a).

The extent of inter-individual response and variation in triploid yields was carefully monitored in the present study with comparative trials following the exposure of eggs from different females to optimal pressure, heat and cold shocks, where pressure had been shown to have certain advantages over heat and cold in inducing triploidy. The cold shock in the present study, which was effective as an optimum for the eggs of one female, was observed to be less ideal, even lethal, for those of another, therefore resulting in high inter-female variations. Heat shock showed lower inter-individual variation than cold, but on average were not as great as pressure in optimizing triploid yields. A similar situation following temperature shock for the induction of triploids, in the use of heat to produce triploid Atlantic salmon, has been reported where pressure shock was the preferred method of triploidization because of extreme variability in response to the same heat shock treatment of eggs from different females (Benfey and Sutterlin 1984; Benfey et al. 1988; Johnstone 1989). The present results demonstrate that since heat shocks narrow the window of opportunity for triplodization, making timing of application of shock more critical, and inter-individual response to cold shocks is apparently greater, the pressure treatments come closest to this ideal for maximum interference with meiotic events of *O. niloticus* eggs.

Like salmonids and other fish, production of genetically sterile tilapia by aforementioned techniques might be of benefit in aquaculture. The impact of reproductive sterility in mixed sex culture of *Oreochromis* spp. would be useful for improving production by preventing sexual maturation particularly in ponds. The use of sterile fish should be the technique of choice if there is a risk of gene introgression of farmed stocks into native wild stocks.

In this study, it is clearly revealed that the pressure level for the successful inhibition of mitotic events was higher (9,000 p.s.i.) than required for meiotic events (8,000 p.s.i.), but the effective duration was the same (2 minutes). The result is similar to that reported for ayu by Taniguchi et al. (1988) where pressure higher than that required for meiotic shocks was necessary to disrupt the mitotic spindle. The result of heat shock application was essentially similar to

that of Mair et al. (1987). This study would suggest that their range of 25-35 minutes a.f. may be too wide, mosaicism at the extremes serving to narrow the effective treatment "window."

Closer inspection of optimization of the present results shows that the timing of heat shock for interference of mitotic events is earlier than that of pressure shock, because the higher temperature advances the rate of all biological processes. This will make the timing of shock more critical, as the width of the effective window will be narrower than for corresponding pressure shocks. Some earlier studies showed that pressure shocks often result in improved survival compared to temperature shocks. This was the case for meiotic gynogenetics produced in this study where, at the selected optimum, the overall survival for pressure was 24.0%$\pm$7.5, N=10 and that for heat was 13.6%+4.5, N=10 at YSR. Pressure and heat were found equally effective in inhibiting mitotic processes. No significant differences in survival were observed at YSR for the mitotic gynogenetics of pressure (1.2%$\pm$0.3, N=5) and heat (2.0$\pm$0.6, N=5). However, it might be argued that pressure gives a wider window for successful induction of mitotic gynogenetics and therefore less risk of mosaicism.

The gynogenetic diploid produced by suppression of first cleavage of eggs is considered more useful than the meiotic type for the fixation and establishment of a new race of fish in aquaculture. Purdom and Lincoln (1973) pointed out that producing inbred lines by the conventional methods of sib-mating requires the maintenance of several lines with close inbreeding for up to 20 generations. But gynogenesis, especially by inhibiting first mitotic division, could dramatically shorten the time required to produce completely homozygous progeny in the first generation, and an "inbred line" or "clone" in the second.

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