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# Problems associated with tetraploid induction and survival in grass carp, *Ctenopharyngodon idella*

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ABSTRACT

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Heat and hydrostatic pressure shocks were used to induce tetraploidy in fertilized eggs of grass carp. Estimates of percent tetraploidy at 1 day post-hatch ranged from 25 to 100 (average 62.5) for pressure treatments of 9000 and 10 000 psi, initiated 36, 39 and 42 min after fertilization at 21°C. These optimal treatment times were generally 16–20 min before cleavage and suggested karyokinesis inhibition. Tetraploidy was also induced in multiple-cell zygotes pressure shocked (9000 and 10 000 psi) at 65, 66 and 70 min after fertilization. Heat shocks of 42°C for 1.25 and 1.5 min induced tetraploidy ranging from 0 to 100% over a broad range of treatment times (33–60 min after fertilization). A series of parallel 1-min heat shock treatments failed to produce tetraploids. Overall, percent hatch was quite variable, but was directly related to tetraploid conversion for most treatments. No tetraploid larvae survived longer than 50 days of age. The occurrence of aneuploids (hypotetraploids) and 2N–4N mosaics was considered at least partly responsible for the mortality.

## INTRODUCTION

Chromosome set manipulation in fish has received considerable attention in recent years. Induced gynogenesis has facilitated the production of pure (isogenic) lines and monosex populations (Stanley and Sneed, 1974; Purdom, 1983; Shelton, 1986). Triploids potentially grow faster, are functionally sterile and are generally produced through second polar body retention (Allen et al., 1986; Krasznai and Marian, 1986; Lincoln and Bye, 1987). Tetraploid induction in finfishes is a relatively recent form of genetic manipulation, the objective of which is to ultimately produce triploids by interploid

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matings of 2N and 4N broodstock, thus circumventing the necessity for shocking eggs to effect retention of the second polar body.

Chourrout et al. (1986) successfully crossed 2N and 4N rainbow trout and produced a high percentage (>93%) of triploid offspring. The growth of triploid salmonids produced in this way was faster than that of triploids resulting from polar body retention (Myers et al., 1986). However, the production of viable autotetraploids in other species of fish has been generally unsuccessful (Myers, 1986; Myers et al., 1986; Curtis et al., 1989).

Thermal or pressure shock treatments used to induce tetraploids are usually applied at a time during the first mitosis that corresponds to either karyokinesis (chromatid separation; early shock) or cytokinesis (cleavage and division of the cytoplasm; late shock). Shock treatments timed for inhibition of karyokinesis resulted in better survival, at least in salmonids (Myers et al., 1986). The type of shock treatment can also affect the "quality" and quantity of induced polyploids by acting on different cell mechanisms. Chourrout (1986) suggested that heat shocks can affect processes that control disjunction and migration of all chromosomes (centriole reproduction), producing a complete effect (polyploid) or no effect (diploid); in contrast, suboptimal pressure shock has a more gradual effect, possibly impairing spindle fiber formation and resulting in only partial disjunction, as evidenced by the occurrence of aneuploids or mosaics.

Natural reproduction is undesirable for grass carp (*Ctenopharyngodon idella*) used primarily for aquatic weed control in the United States. Functionally sterile triploid grass carp are now routinely used in many areas of the United States and methods for inducing triploidy in grass carp by retention of the second polar body have been well documented (Cassani and Caton, 1985, 1986; Thompson et al., 1987). However, an interploid cross involving a tetraploid parent ($2N \times 4N$) would theoretically result in high-percentage triploid induction with considerably less handling and expense than that associated with conventional methods. Our objective was to describe an efficient method of producing tetraploid grass carp.

## MATERIALS AND METHODS

Grass carp were spawned as described by Cassani and Caton (1986), from early April to early June. Both heat shock and hydrostatic pressure treatments were evaluated for efficiency of tetraploid induction. After stripping and fertilization (egg/sperm activation), eggs were held in a 70–80-l container of water maintained at $21 \pm 0.3$°C. A recirculating pump gently rolled the eggs to facilitate normal water hardening. Generally, 400–800 ml of eggs were used for heat shock and 400 ml for pressure treatments; the number of eggs varied greatly depending on when the treatment was initiated. The volume of grass

carp eggs increases greatly during the water hardening process: at 21 °C, there were 51 eggs/ml 30 min after fertilization but only 26 eggs/ml 60 min after fertilization.

Fertilized eggs were heat-shocked in saran baskets immersed into 25 l of water heated with a thermostatically controlled heater-circulator. Fertilized eggs to be pressure-treated were placed in a cylinder and piston-type pressure chamber with a volume of 605 ml. Chamber temperature was maintained at or near ambient by immersing it in the same water in which the females were held. The chamber and treatment method were described by Cassani and Caton (1986).

Eggs were shocked using either heat or pressure methods at 3-min intervals, starting 30 min after fertilization and continuing until 60 min. The time to first cleavage was variable depending on egg ripeness and temperature, but generally started within 60 min after fertilization at 21 °C. Karyokinesis was expected to occur within this 30-min treatment period. Pressure treatments were replicated at 63, 65 and 70 min after fertilization and performed once at 75, 80, 85 and 90 min after fertilization.

Eggs were heat-shocked at 42 °C for durations of 1.0, 1.25 and 1.5 min at the times described above. This temperature is very close to the upper lethal threshold, and we assumed that it would provide the most effective shock. Hydrostatic pressure was evaluated at three levels (8000, 9000 and 10 000 psi) maintained for 70 s. Preliminary trials indicated that longer durations at pressure ranges of 8000–10 000 psi resulted in unacceptably low survival ($< 1\%$) of eggs.

Percent hatch was estimated by examining 100–200 randomly collected eggs at 10× magnification 12 h after fertilization. The blastodisc of zygotes not hatching had completely disintegrated 10–12 h after fertilization, allowing a reasonably accurate estimate of percent hatch. Our estimates of percent hatch are conservative, since slightly abnormal zygotes, as compared to the controls, were not counted.

The heat shock treatment of 42 °C for 1 min and the pressure treatment at 9000 psi were applied to eggs from the same female, as were those for treatments at 42 °C for 1.5 min and at 10 000 psi. Eggs for the remaining treatments (42 °C for 1.25 min and 8000 psi) were obtained from different females. Generally, two or more males were used to fertilize a batch of eggs. All treatment (shock) intervals were replicated at least twice, but due to 0% hatch in some replicates, apparently resulting from interfemale differences in egg ripeness among other factors, only the best data were presented with respect to tetraploid conversion.

All ploidy estimates were made using flow-cytometric analysis. With this method, DNA was quantified indirectly by staining cellular DNA with a DNA intercolating fluorescent dye, followed by excitation with an argon laser. A Becton Dickinson FACScan® flow cytometer was used to quantify and characterize the fluorescence over a range of 256 channels. Laser excitation was

at 488 nm and the wavelength for emission was 585 nm with propidium iodide. The specific methodology was modified from a protocol designed by Hinshaw et al. (1986). Specifically, newly hatched whole larvae were individually placed in round-bottomed, polystyrene tubes (12 mm×75 mm) containing 1 ml of nuclear isolation medium; a liter of this stock solution contained 12 ml Nonidet P-40, 10 mg ribonuclease A, 50 mg propidium iodide fluorescent dye, and enough Isoton® II to achieve 1 l of solution[1]. The samples were held in the dark for 16–20 h at 4–5°C. A single cell suspension was prepared from larval tissue by repeated trituration, using a 1-ml syringe and a 23-g needle. Some samples were filtered through a 70-$\mu$m mesh nylon screen. However, filtering was found to be unnecessary when only a single larva was used per sample. The cytometer was operated in the linear mode and the detector and amplifier settings were 550 and 1.31, respectively, for all analyses. From 8 to 10 larvae per treatment were sampled for flow-cytometric analysis.

When tetraploid induction in a specific treatment was high (i.e., >50%), larvae were isolated in nursery ponds or reared in concrete tanks for post-shock estimates of percent survival and tetraploidy.

## RESULTS

Tetraploidy was induced with heat shock over a wide range of treatment times (Fig. 1). Heat shock for 1 min resulted in relatively high survival but produced only low percentages of 2N–4N mosaics. Longer heat treatments (1.25 and 1.5 min) caused lower survival to hatch at all treatment times, but an estimated 100% tetraploid larvae resulted at certain treatment times and durations (39, 51 and 54 min) (Fig. 1). A slight trend toward better survival was evident at the early (36–42 min) and late (51–60 min) treatments, possibly corresponding to the timing of karyokinesis and cytokinesis, respectively.

Diploid–tetraploid mosaics occurred at most heat shock treatment times and durations and is in contrast to Chourrout's (1986) hypothesis that heat shock affects cellular mechanisms that produce polyploids completely or inhibit the process altogether. Variations in survival and tetraploid conversion between durations were somewhat inconsistent and may have been due to inherent differences (i.e., genetic or egg ripeness) between females. Heat shock techniques were also inconsistent when used for triploid induction in grass carp (Cassani and Caton, 1986).

Results for treatments involving hydrostatic pressure indicated that pressures above 8000 psi produced the best combinations of survival and percent tetraploidy (Fig. 2). At 9000 and 10 000 psi, tetraploidy associated with better survival was consistently produced at treatment times of 36, 39 and 42

[1]Reference to trade names or manufacturer does not imply Government endorsement of commercial products.

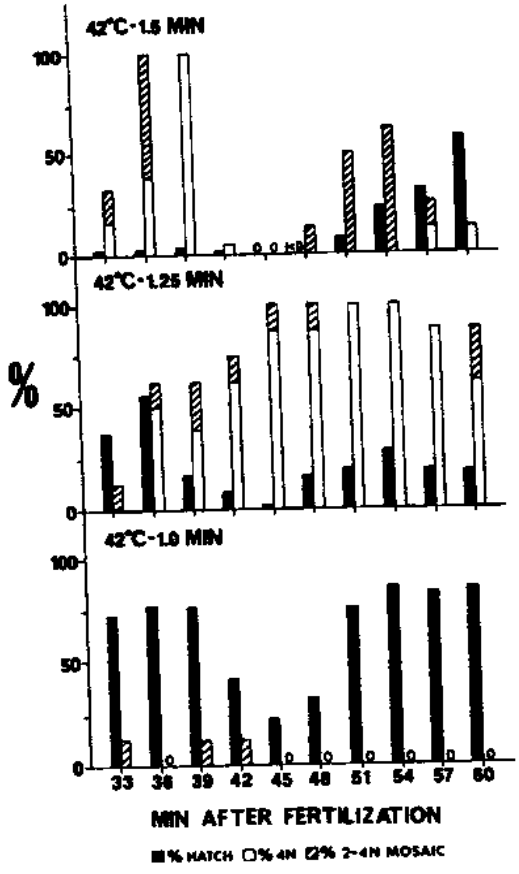


Fig. 1. Percent tetraploid, 2N–4N mosaics and estimated percent hatch for heat shock treatments at various treatment intervals. Diploids resulted when the combined percentage of 4N and 2N–4N mosaics did not equal 100.

min. These treatment times probably correspond to the period of karyokinesis. Tetraploid induction levels ranged from 25 to 100% (average 62.5%) during this period with acceptable survival (Fig. 2). No tetraploids were detected from pressure treatments made during or just before the first cleavage (i.e., 54–63 min post-fertilization). However, tetraploids were produced at 65, 66 and 70 min after fertilization at 10 000 psi. The number of blastomeres for developing zygotes at treatment times of 65, 66 and 70 min ranged from 4 to at least 32. Multiple cell divisions at this stage (i.e., up to 70 min after fertilization) appeared to be susceptible to either karyokinetic or cytokinetic inhibition but the shock effect was apparently incomplete as evidenced by the occurrence of 2N–4N mosaics (Fig. 2). Treatments of 10 000 psi at 75, 80, 85 and 90 min after fertilization resulted in 100% mortality before hatching.

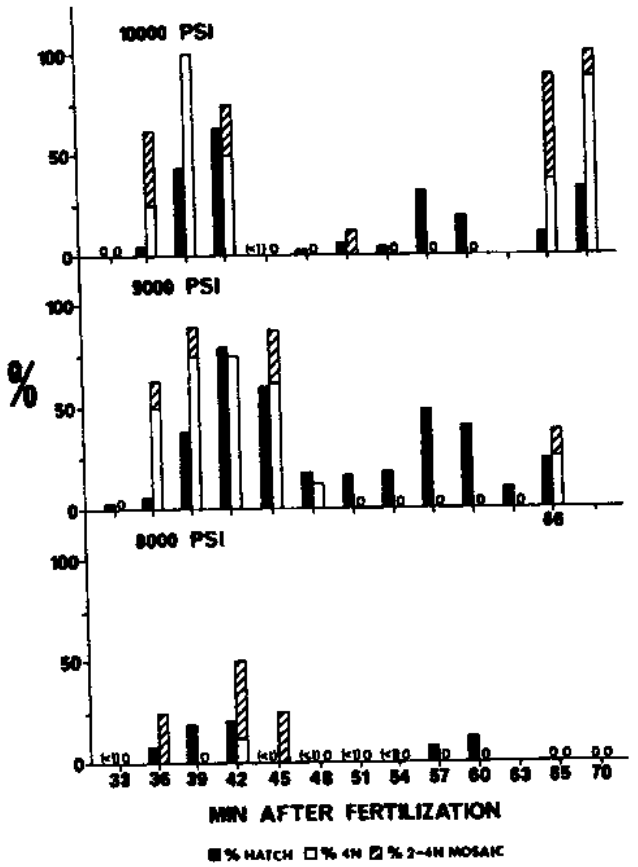


Fig. 2. Percent tetraploid, 2N–4N mosaics and estimated percent hatch for pressure shock treatments at various treatment intervals. Diploids resulted when the combined percentage of 4N and 2N–4N mosaics did not equal 100.

As with heat shock, some 2N–4N mosaics were produced by most pressure treatments having survivors.

The only types of mosaics detected in our study were of the 2N–4N type, with considerable variation in the percentage of 4N cells. Diploid–tetraploid mosaics with predominantly 2N cells were by far the most common form. However, mosaics with almost equal proportions of 2N and 4N cells, as well as some with predominantly 4N cells, were also encountered. Diploid samples often have a small secondary peak in the tetraploid range as a result of cell cycling (i.e., replication of chromosomes before cytokinesis) or double particles (i.e., coincidence). This secondary peak in diploids represented 8.9–16.4% of the 20 000 cells analyzed per sample. Consequently, a sample was not considered mosaic unless the percentage of 4N cells exceeded 22.4, the lowest percentage of 4N cells in any sample designated a mosaic.

Evaluation of ploidy by means of a Coulter Counter or flow cytometer on peripheral blood from pond-reared fingerlings indicated no surviving tetraploids or mosaics from either heat or pressure treatments. Less than 1% of the survivors were triploid but spontaneous triploidy occurs infrequently as evidenced by the presence of triploids in the controls of other experiments. Larvae resulting from treatments yielding high percentage tetraploids and reared in tanks, exhibited high mortality after 30–40 days (Fig. 3). Gross examination of tetraploid fry under magnification revealed extensive anomalies of the swim bladder and various deformities of the head.

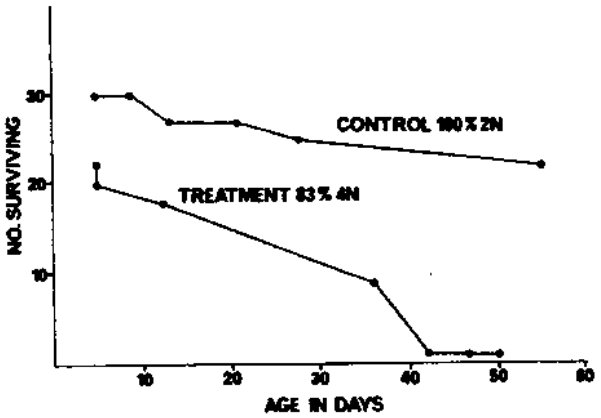


Fig. 3. Mortality curves for a typically high percentage tetraploid treatment group and a control group.

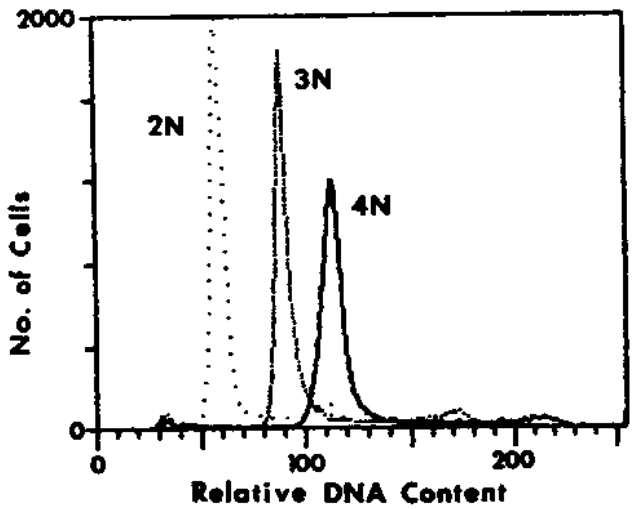


Fig. 4. Typical diploid (2N), triploid (3N) and tetraploid (4N) histograms derived from samples of individual grass carp larvae and flow cytometric analysis.

TABLE 1

Kolmogorov–Smirnov two-sample statistics for comparison of histograms. The histograms compared are typical of those encountered using the same methodology and are illustrated in Fig. 4

| Histograms compared | $D$ | $D/S(n)$ |
|---|---|---|
| 2N with 3N | 0.77 | 77.02 |
| 2N with 4N | 0.83 | 82.62 |
| 3N with 4N | 0.68 | 67.90 |

$D$ = The greatest difference between the two summation curves.
$D/S(n)$ = A value indicative of the similarity of the curves compared.

Visual inspection of typical 4N histograms ss in Fig. 4, indicated the possibility of hypotetraploidy because many histograms were somewhat short of the expected channel number (i.e, twice the 2N level). The use of a Kolmogorov–Smirnov two-sample test for the analysis of histograms was used to compare typical 2N with 3N, 2N with 4N, and 3N with 4N histograms (Young, 1977). $D$ and $D/S(n)$ values (Table 1) for dual comparisons of 2N–3N, 2N–4N and 3N–4N histograms were derived from histograms similar to those shown in Fig. 4. Theoretically, both $D$ and $D/S(n)$ for the 2N–3N and 3N–4N comparisons should be identical. However, this statistic suggests that the 3N–4N comparison is more similar than the 2N–3N as is visually evident in Fig. 4, further substantiating the theory that most of the tetraploids produced were aneuploid hypotetraploids (individuals having fewer chromosomes than the expected number).

## DISCUSSION

The subvitality of tetraploid grass carp may be due to a number of problems associated with incomplete polyploid conversion. One such problem is the frequency of 2N–4N mosaics associated with both heat and pressure treatments. Mosaics have been reported as an unwanted result of polyploid induction in other species of fish. Various combinations of 2N, 3N and 4N cells have been reported in mosaic individuals for several species of salmonids, especially where chemical agents such as colchicine or cytochalasin B were used (Refstie et al., 1977; Allen and Stanley, 1979; Smith and Lemoine, 1979; Refstie, 1981). Channel catfish mosaics containing combinations of 2N–5N cells were reported by Bidwell et al. (1985) to occur frequently from heat-shocked eggs especially at higher temperatures. However, the characterization of mosaics by chromosome enumeration or by nuclear volume or size is difficult and inconsistent. Flow cytometry is preferable because large numbers of cells from the entire organism can be rapidly and objectively analyzed.

Aneuploidy appears to be another problem that could be linked to poor

survival. An examination of tetraploid histograms produced from flow-cytometer data for both heat- and pressure-shocked larvae, revealed that many tetraploid histograms were relatively flat with a broad base and apparent subpeaks, which might indicate a wide range in chromosome number (DNA content) or an aneuploid condition. This same phenomenon was observed with tetraploid hybrid grass carp (grass carp×bighead carp, *Hypophthalmichthys nobilis*) where histograms from suspected tetraploids derived from flow cytometry of erythrocyte samples were relatively broad with subpeaks (Allen and Stanley, 1983). Diploid histograms from untreated "control" larvae contrasted with tetraploid histograms in having relatively narrow peaks (Fig. 4). The fact that most 4N histograms for both shock methods were somewhat less than twice the mean diploid channel number also suggests that hypotetraploids were being produced. Tetraploid histograms from flow-cytometric analysis for hybrid grass carp were only 1.78 times the average for diploids and were thought to be a result of aneuploidy (Allen and Stanley, 1983). A review of our data from flow-cytometric analysis showed that 4N histograms averaged 1.87 times the average value for 2N histograms ($n=30$). No similar discrepancy was evident when we compared the actual and expected values for triploids. Triploid histograms averaged 1.49 times (expect 1.5) the average value for diploid histograms ($n=30$). Allen and Stanley (1983) also considered the possibility that their 4N hybrid grass carp started as tetraploid but lost some chromosomes through subsequent cell divisions. Reduced survival has been reported for aneuploids in at least rainbow trout (Chourrout, 1986).

Another alternative explanation for poor survival in tetraploid grass carp is that tetraploid cell density is substantially reduced from that of diploid fish. The time necessary to acquire data for 20 000 cells at a constant flow rate on the flow cytometer from single larva samples was consistently 60 s for tetraploids compared with about 26 s for diploids. This same phenomenon was demonstrated in polyploid grass carp using an electronic particle counter (Coulter Counter). With this method, we found that suspected 4N samples averaged only 46% as many cells as 2N samples.

The increased DNA in triploid fish leads to increases in nuclear and cellular volume, but cell density is reduced to maintain normal organ and body size (Swarup, 1959; Hinshaw et al., 1986; Small and Benfey, 1987). No significant differences in total length of diploid and tetraploid fry of the same age were detected. The suspected reduction in cell density due to the suspected large cell size in tetraploid grass carp could have drastic effects on neurological and physiological functioning if blood, brain and retinal tissues are involved. It is suspected that visual acuity and a decrease in learning ability may result from lower cell density in brain and retinal tissue of triploid Atlantic salmon (Small and Benfey, 1987). Since tetraploid cells are probably even larger than triploids, the increased cell surface area of tetraploids may limit or inhibit cellular metabolism and thus, at least partly explain the gross anom-

alies in tetraploid grass carp larvae. Also, the potentially very large tetraploid cells may exceed the threshold for the conservation of body size discussed earlier.

Genetic deficiencies inherent to grass carp may also contribute to the lack of survival in induced tetraploids. The reduction of heterozygosity associated with a bottleneck effect due to a small founder stock in North America may account for the difficulty in producing viable tetraploids. Extensive inbreeding, resulting in decreased heterozygosity can lead to an increased probability of homozygous recessive alleles and may cause "a reduction in value for characters associated with reproduction or physiological efficiency" (Thorgaard, 1983). Utter and Folmar (1978) reported the average heterozygosity per locus (0.021) was relatively low for a domestic population of grass carp in North America. This problem is magnified when attempting to produce autotetraploids. In plants, chromosome doubling in autotetraploids has an inbreeding effect that some researchers interpret as being equivalent to 3.8 generations of conventional selfing (Bingham, 1980). Decreased heterozygosity has been attributed to decreased developmental stability in rainbow trout (Leary et al., 1983).

Heterozygosity can be increased by producing hybrid tetraploids (allotetraploids), leading to higher viability than that in autotetraploids in some, but not all, instances (Chourrout, 1984; Myers, 1986; Myers et al., 1986). Tetraploid hybrid grass carp have been incidentally produced and were apparently viable (Allen and Stanley, 1983). Further experimentation on the production of an allotetraploid grass carp is worth considering since autotetraploids appear to be completely subvital. Poor survival and growth of autotetraploids have been reported for other species of fish as well (Chourrout, 1984; Chourrout et al., 1986; Myers, 1986; Curtis et al., 1989). The identification of genetically distinct "stocks" (North American vs. Asian) may also help facilitate the production of tetraploids by increasing heterozygosity.

Consideration should also be given to the possibility that the shock treatments were of suboptimal intensity or duration. However, any increase in the maximum intensity or duration of heat or pressure shocks reported herein will very likely lead to high levels of mortality. Hydrostatic pressure was somewhat more consistent than heat shock for tetraploid conversion. However, more eggs could be shocked simultaneously with heat shock.

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