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EFFICIENT PRODUCTION OF TRIPLOID GRASS CARP (*CTENOPHARYNGODON IDELLA*) UTILIZING HYDROSTATIC PRESSURE

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ABSTRACT

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High percentage triploidy was induced by means of hydrostatic pressure and heat shock treatments of fertilized grass carp eggs. Estimates of triploidy exceeded 98% in 9 of 10 treatments involving hydrostatic pressures of 7000 or 8000 PSI for a duration of 1 or 2 min, applied 4 min after egg—sperm activation. Estimated hatch relative to the controls averaged 69.1%, ranging from 21.9 to 100%. The best heat shock parameters were 42°C for a duration of 1 min starting 4 min after egg—sperm activation resulting in an average of 66.7% (0—est. 100%) triploidy. Percent hatch relative to the controls averaged 72.5%, ranging from 43.9 to 100%. Delaying fertilization of ovulated grass carp eggs for up to 30 min did not have a negative effect on triploid conversion or survival compared to controls.

INTRODUCTION

The grass carp (*Ctenopharyngodon idella* Val.) has received considerable attention in North America and other areas as a biological control agent of problematic aquatic macrophytes (Shireman and Maceina, 1981; Leslie et al., 1983). The recent development of functionally sterile triploid grass carp has resulted in a more liberal approach to its introduction in areas where grass carp reproduction is unwanted. Relatively few commercial fish farms are producing certified triploid grass carp in North America. Dissemination of information dealing with triploid induction techniques by commercial farmers presently producing such fish has been limited for proprietary reasons. Triploidy has been reported in grass carp after fertilized eggs were shocked in cold water but results were inconsistent and survival was relatively low (Cassani and Caton, 1985). Hydrostatic pressure has been used successfully to induce polyploidy in salmonids and amphibians by inducing second polar body retention or first cleavage suppression in the

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fertilized egg (Dasgupta, 1962; Gillespie and Armstrong, 1979; Onozato, 1983 as cited in Arai, 1984; Benfey and Sutterlin, 1984; Chourrout, 1984). However, the amount of pressure, its duration and timing must be determined for each species.

We report here on treatment parameters utilizing hydrostatic pressure that consistently produce greater than 98% triploidy and relatively high survival in grass carp. We also report a somewhat less consistent technique for producing high percentage triploidy in grass carp by heat shock.

MATERIALS AND METHODS

Female broodfish were successively transferred from ponds to a 6000-l circular tank maintained at 22.0—24.8°C starting on 5 March and continuing at 7—10-day intervals through the first week of June. Female broodfish having a partly dilated vent and a soft distended abdomen were selected for spawning. Spawning generally took place once per week and females were acclimated to the indoor tank 3—5 days prior to spawning. Final maturation of female broodfish (age VIII—IX) was accomplished by two intramuscular injections of human chorionic gonadotropin (HCG) (440 IU/kg first injection and 1870 IU/kg second injection) 24 h apart and a third injection of carp pituitary (8.8 mg/kg) administered intraperitoneally 24 h after the second HCG injection. Ovulation occurred between 10 and 11 h after the final injection in 82% of the females injected. This hypophysation protocol is a slight modification of one reported by Bailey and Boyd (1970) and is used as a standard procedure at the Richloam Fish Hatchery (Florida Game and Fresh Water Fish Commission) and by us for the past 3 years. Males were held separately and given an intramuscular injection of LHRH (luteinizing hormone releasing hormone Des-Gly^{10}-[D-Ala^{6}] LHRH ethylamide) at 10 μg/kg 24 h prior to spawning to insure an adequate volume of sperm.

Stripping and fertilization were done according to the dry method (Rothbard, 1981) so as to administer the shock treatment at a known time after the addition of water (egg—sperm activation). The normal procedure was to gently lift the individual with the head and anterior end supported in a cloth cradle by one person, 10.25—10.50 h after the last injection. If muscle contractions ("quivering"), indicating the onset of ovulation, did not start within 10—15 s, the fish was returned to the water and tried again 30—45 min later. With this method, stripping would occur within a reasonably short period of ovulation so as to prevent over-ripening and/or the untimely expulsion of ova by the female. Four minutes after egg—sperm activation was established as a standard reference point for timing most treatments since second polar body extrusion is thought to occur approximately 5 min after egg—sperm activation in grass carp (Babrova, 1969 as cited in Stanley, 1979).

Pressure was applied to fertilized eggs by means of a two-piece pressure

chamber very similar to one described and illustrated by Dasgupta (1962). The chamber consisted of a stainless steel cylinder 40.6 cm long with an inside diameter of 45 mm and a wall thickness of 15 mm. The second piece consisted of a brass piston fitted with an O-ring 1.2 cm from the end to be inserted into the cylinder to prevent a loss of pressure and water. The piston was also drilled and fitted for direct measurement of chamber pressure with an attached pressure gauge and pressure relief valve. A second chamber was also used, identical to the one described above except that the cylinder was only 25.4 cm long. The desired egg volume was measured in a beaker at a set time after fertilization and poured into the chamber which was approximately 50% filled with water. The piston was inserted with the valve open to purge the remaining air inside the cylinder and then closed. Thrust to the piston, by means of a hydraulic press, produced the target pressure. The duration of the treatment included the time necessary to produce the target pressure with the press. Generally 1000 PSI of pressure could be generated by hand pumping a 20 000 PSI maximum capacity press at the rate of 1000 PSI in less than one second. The chamber as described was able to withstand 10 000 PSI without noticeable leaks or a drift in pressure indicated by the pressure gauge. By opening the relief valve on the press, pressure inside the cylinder was released instantaneously.

The volume of eggs used was approximately 50—60% of the total volume of the cylinder. The large and small cylinders had working or total volumes of 605 ml and 364 ml, respectively. We used 350 ml of eggs in the larger cylinder (approx. 200 000 total) and 200 ml eggs (approx. 114 000 total) in the smaller cylinder. In situations where more than 550 ml of eggs were targeted for treatment, the entire volume of eggs (usually 1200—1700 ml) was separated into as many as three groups with the second, and sometimes the third group, fertilized independently and after the first. This could always be accomplished within 30 min after ovulation with a treatment duration of 3 min or less starting 4 min after fertilization. According to Rothbard (1981), eggs may be kept without water for about 30 min before being fertilized.

Heat shocks were accomplished by immersing fertilized eggs in saran baskets into 25 l of water heated with a thermostatically controlled heater—circulator.

Percent estimates of eggs hatching were determined by examining 100—200 eggs per treatment with a dissecting microscope at 10 × magnification and compared with the controls 7—10 h after fertilization. At 22°—24°C, hatching normally starts at 26—30 h after fertilization. The blastodisc and yolk of abnormal eggs usually disintegrate completely within 10 h of fertilization, allowing for a reasonably accurate estimate of percent hatching. Estimates of percent hatch were considered conservative since not all sampled larvae viable at 10 h were counted due to slight abnormalities. The slightly abnormal appearing larvae very likely hatched but probably did not survive to the "swim up" stage. A similar method for estimating hatching rates is reported by Zonneveld (1984).

Percent estimates of triploid conversion were made by randomly extracting 10—20 newly hatched larvae per treatment and control, of which 6 to 12 survivors were used individually for chromosome preparations and ploidy determination according to the methods described by Cassani and Caton (1985).

As a check on percent estimates of ploidy made by counting chromosomes, the ploidy of several hundred to several thousand fingerlings per treatment was made by comparing erythrocyte nuclear volume of individual fish with a Coulter Counter (Benfey et al., 1984).

RESULTS AND DISCUSSION

Twelve female grass carp were spawned between 18 March and 27 May 1985. The data in Tables 1 and 2 are based on these lots. Eight additional spawnings were conducted but due to low survival (<40%) in the controls, no larvae were tested for ploidy. Early season trials accounted for the

TABLE 1

Treatment parameters and results for pressure-treated fertilized grass carp eggs. Pressure was initiated 4 min after egg—sperm activation for all treatments at an ambient temperature range of 22.0—24.8°C

Pressure (PSI)	Duration (min)	Est. % triploid	Est. % treatment hatch relative to control[bc]
3000	5:00	0	86.5
4000	5:00	33.3	100
	7:00	50.0	2.8
5000	5:00	83.3	37.1
	1:00	0	100
6000	5:00	92.3(66.6—100)[a] (n=12)	14.5(0.5—37.8)
	4:00	68.7	73.4
	4:00	83.3	75.8
	3:00	100	14.6
	1:00	66.7	99.6
7000	3:00	83.3	23.7
	3:00	100	94.7
	2:00	100	65.2
	1:00	100	94.3
8000	2:00	98.9(92.9—100) (n=7)	48.7(21.9—100)
	1:00	100	68.3
9000	1:00	100	41.7

[a]% estimates for treatments with more than one replicate are means with ranges in parentheses.
[b]% treatment hatch/% control hatch.
[c]average % hatch for all controls was 77.2 ± 8.8 SD.

TABLE 2

Treatment parameters and results for heat-shocked fertilized grass carp eggs

Treatment temp (°C)	Minutes post-fertilization	Duration (min)	Est. % triploid	Est. % treatment hatch relative to control (control)[a]
38	3:30	1:00	0	85.8 (70.5)
38	4:00	1:00	0	100 (65.5)
38	4:30	1:00	0	80.1 (70.5)
40	4:00	1:00	50.0	95.9 (61.9)
40	5:30	1:00	33.3	76.6 (56.3)
40	4:30	1:00	16.6	81.5 (74.1)
40	4:30	1:20	16.6	92.8 (74.1)
40	4:30	1:20	11.1	84.4 (88.9)
40	4:25	1:00	0	56.6 (43.1)
42	4:00	1:00	100	100 (80.6)
42	4:00	1:00	0	43.9 (84.6)
42	4:13	1:00	100	73.8 (83.1)
42	4:00	2:00	66.7	21.8 (83.5)

[a]% treatment hatch/% control hatch.

majority of situations where percent hatch was relatively low in the controls. By mid-March the majority of fish exhibited good secondary sexual characteristics as described above, but a noted increase in fertilizability and percent hatch was observed later in the season (mid-April to late May). The physiological temperature degrees (°PT) as described by Zonneveld (1984) were approximately 1100 by 1 April and 1800 by the end of May.

Overall, hydrostatic pressure proved to be a very consistent method for inducing high-percentage triploidy in grass carp. The optimal parameters of this method were considered to be 7000 or 8000 PSI for a duration of 1 or 2 min, starting 4 min after egg–sperm activation in the temperature range of 22.0—24.8°C. This combination of parameters resulted in the best compromise between triploid conversion and survival. Of the 10 treatments made at 7000 or 8000 PSI for 1 or 2 min duration, all but one (92.9%) resulted in an estimated 100% triploidy based on chromosome counts of 1-day-old larvae, and survival (% hatch) relative to the controls averaged 69.1% (Table 1). Additional ploidy determinations by means of a Coulter Counter on 354–3200 fingerlings for some treatments indicated that complete (100%) triploid conversion probably never occurred (Table 3). However, triploid conversion for fish from eggs treated at 8000 PSI for 2 min was very close to 100%, ranging from 98.3 to 99.7% for three replicates checked by Coulter Counter analysis of red blood cell nuclear volume. Because of limited pond space, larvae from several treatment replicates had to be discarded and, as a result, not all of the pre-stock ploidy estimates could be checked by means of a Coulter Counter.

TABLE 3

Pre- and post-stock estimates of percent triploidy in grass carp retained for grow-out

Pond	Treatment type	Est. % triploid[a] pre-stock	Est. % triploid[b] post-stock (no. fish tested)
R5	6000 PSI/5 min	83.0	91.7 (3200)
6	6000 PSI/5 min	97.3	99.4 (1808)
5	6000 PSI/5 min	94.1	96.0 (2469)
R4	7000 PSI/3 min	100	91.8 (1094)
R7	8000 PSI/2 min	100	99.7 (1261)
R2	8000 PSI/2 min	100	98.3 (400)
1	8000 PSI/2 min	100	99.1 (444)
R3	42 C/1 min	100	95.2 (354)

[a]Determined from chromosome counts from individual larvae (6—12/treatment).
[b]Determined by Coulter Counter (erythrocyte nuclear volume).

Estimated percent triploid for treatments involving lower pressure (3000—5000 PSI) and longer durations (3—7 min) was lower and less consistent, ranging from 0 to 83.3% (Table 1). A pressure of 6000 PSI for 1—5 min appears to be a minimum level for consistent triploid conversion over 50%. Fig. 1 demonstrates this phenomenon over a series of pressure levels at a constant duration of 1 min.

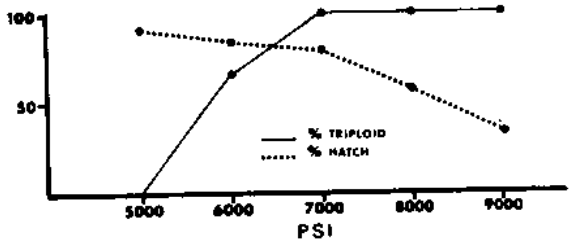

Fig. 1. Relative % triploid and % hatch from a single hatch of eggs treated 4 min post-fertilization for 1 min duration at various pressures. Mean % hatch in the controls was 85.4%.

Delaying pressure treatments until eggs have partially water hardened was lethal on one occasion. Eggs shocked at 13:55 and 14:00 min after fertilization at 5000 and 6000 PSI, respectively, for 5 min duration resulted in 100% mortality compared 51.7% hatch in the controls (Table 1). This may partially explain the failure of induced gynogenesis and the complete mortality of grass carp eggs subjected to 120 atmospheres (1763.5 PSI) for 30 min duration as reported by Stanley et al. (1975). In this case the long duration rather than the relatively low pressure was probably responsible for the poor success. Relatively long durations apparently disrupt other cellular processes necessary for survival. Optimal pressure levels for

pressure-induced polar body retention or first cleavage suppression (i.e. induced triploidy, gynogenesis and androgenesis) in salmonids ranged from 7000 to 10150, similar to the optimal range for grass carp, although shock durations were generally longer for salmonids with much later initiation times due to cooler temperatures which slow the rate of meiosis (Onozato, 1983 as cited in Arai, 1984; Benfey and Sutterlin, 1984; Chourrout, 1984; Lou and Purdom, 1984; Parsons and Thorgaard, 1985).

The occurrence of deformed larvae resulting from pressure-treated eggs was common, but rarely exceeded an estimated 10--20% of the total eggs hatching. A similar level of deformed larvae was also observed in most heat treatments. The effect of delaying fertilization and egg activation for 5—20 min after ovulation appeared to be minimal. On four of five occasions where fertilization and activation were delayed, percent hatch for treatments and controls was as high or higher than when undelayed. This method allows for a more efficient use of limited pressure chamber volume.

The best heat shock parameters tested were 42°C for 1 min duration, starting 4 min after egg—sperm activation. Average percent triploidy and percent hatch relative to the controls with these parameters were 66.7 and 72.5 respectively. A duration of 2 min at 42°C resulted in 21.8% hatching (relative to the control) compared to 43.9—100% hatching (relative to the control) at 1 min duration. Three heat shock trials at 38°C did not induce triploidy and six trials at 40°C did not result in triploid conversion exceeding an estimated 50% (Table 2). Heat shocks generally resulted in somewhat better survival than pressure treatments; however, pressure treatments were considerably more consistent with regard to high percentage (>90%) triploid conversion. The uniform effect of hydrostatic pressure on individual eggs is probably responsible for this. Also, pressure may be more effective at spindle fiber breakdown or solation as compared to heat shock resulting in more efficient second polar body retention. The cost of equipment for conducting pressure treatments is somewhat higher than for heat shock equipment but the extra cost will be offset by having to spawn fewer broodfish due to the high efficiency of pressure treatments. Also, fewer diploid fish will have to be segregated from triploid individuals when using pressure treatments, which is an important consideration where 100% triploid populations are required.

In conclusion, we report for the first time the induction of triploidy by means of hydrostatic pressure in a species of fish other than a salmonid. Utilizing hydrostatic pressure according to the optimal parameters provided here will result in a highly efficient method for production of triploid grass carp.

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