

A New Method for Early Ploidy Evaluation of Grass Carp Larvae

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Abstract.—A method is presented for identifying ploidy in newly hatched grass carp (*Ctenopharyngodon idella*) by using disaggregated whole tissue prepared as a single-cell suspension in nuclear isolation medium (NIM). The reduced cell density of triploid fishes compared to

that of diploids can be demonstrated by analyzing the cell suspension with a Coulter counter. The method is rapid, requiring about 1–3 min to prepare and analyze a sample.

Existing methods for ploidy analysis of fish larvae are generally expensive (i.e., flow cytometry) or time-consuming, or involve a certain degree of microtechnique not always available to the fish culturist (Johnson et al. 1984; Rivlin et al. 1985;

Johnstone and Lincoln 1986; Phillips et al. 1986). Most states that permit the use of grass carp (*Ctenopharyngodon idella*) for aquatic weed control now require that these fish be triploid. Certain methods for triploid induction generally result in a high percentage of triploid offspring, but 100% success is rarely attained (Cassani and Caton 1986). Some producers find that production of less than 90% triploids is unacceptable for economic reasons, because each fish must be checked for triploidy before stocking. The producer risks wasting pond space and other resources on fry that may be discarded if the triploidy percentage is unacceptable.

Most producers of triploid grass carp use a Coulter counter to determine the ploidy of erythrocytes from fingerling fish. Grass carp grown in Florida must be about 27 d old to have erythrocytes of appropriate maturity and in sufficient volume for processing with a Coulter counter (Wattendorf 1986). I describe here a method for determining the ploidy of grass carp larvae by means of Coulter counter analysis of individual whole larvae up to 30 h old.

A Coulter counter is in principle an electronic particle counter and sizer; Wattendorf (1986) described the physics underlying the mechanics of particle counting and sizing in this system. The method described herein was modified from one involving a flow cytometer to evaluate the ploidy of disaggregated whole tissue derived from individual grass carp larvae (Kerby and Harrell 1990). Flow cytometry is similar to Coulter counter analysis in that cells or particles (nuclei) are individually analyzed. However, flow cytometry for ploidy evaluation is usually based on the amount of fluorescence from a particle (i.e., cell nucleus) stained with a DNA-specific dye. The time required to run a preset number of nuclei through the flow cytometer is somewhat indicative of the cell density of an organism if a single individual is sampled in a preset volume of diluent. The time necessary to acquire 20,000 nuclei at a constant flow rate in the flow cytometer from single-larva samples was consistently 60 s for tetraploid grass carp, compared with about 26 s for diploids (Cassani et al. 1990). Other researchers have found that triploid fish have a reduced cell density (fewer cells per fish) compared to that of diploids of the same species (Swarup 1959; Small and Benfey 1987; Kerby and Harrell 1990).

I theorized that reduced cell density in triploids could be demonstrated with a Coulter counter on samples prepared as single-cell (nucleus) suspen-

sions. To accomplish this, grass carp larvae were individually placed in round-bottomed, polystyrene tubes (12 × 75 mm) containing 1 mL of nuclear isolation medium (NIM; Kerby and Harrell 1990). (A liter of NIM stock solution was composed of 12 mL Nonidet P-40 [a detergent lysing agent used as an aid in disaggregating whole tissue] and 988 mL Isoton II [an electrolyte diluent].) Larvae were placed one by one in NIM over 8–10 min; I assumed that the potential number of new cells produced in this time by the last larva killed was insignificant compared to the number of cells at the beginning of the period. Samples were kept overnight at 4–5°C for processing the next day. A single-cell (nucleus) suspension was prepared from a larva by repeated trituration (two to four times) with a 23-gauge needle on a 1-mL syringe. This method allows for dissolution of the cell membrane while leaving the cell nucleus intact. A fluorescent dye (50 mg propidium iodide/L) was also added to the samples for flow cytometric analysis as a check on the accuracy of the Coulter counter. The addition of a dye is not necessary for Coulter counter analysis because the Coulter counter is only meant to count and size the particles. The methods used for flow cytometric determination of ploidy were the same as those described by Cassani et al. (1990).

After disaggregation, the sample was transferred to a plastic cup (Accuvette) used for holding most types of Coulter counter samples. The sample vial was rinsed with 1 mL Isoton II into the Accuvette, and the total volume brought to 10 mL. The samples were measured on a Coulter counter (model ZBI) with the following settings: amplitude, 0.25; aperture, 0.25; lower threshold, 15; and upper threshold, 80. The Coulter counter was set at the 500- μ L sample size, and a 100- μ m aperture tube was used.

Polyploidy was induced by means of hydrostatic pressure (as detailed by Cassani and Caton 1986) for all individuals tested. The triploid larvae tested resulted from a cross of a single female with two males; the single tetraploid individual resulted from a separate cross and a different female. For larvae that were 4–5 h old, three counts were made on each of 20 diploid and 15 triploid larvae, and five counts were made on one tetraploid larva; for 30-h-old larvae, three counts were made on 20 diploid and 20 triploid larvae. The counts were then averaged for each ploidy within an age, and Duncan's multiple-range test was used to determine significant differences between means. All means were found to be significantly different

TABLE 1.—Cell counts derived from a Coulter counter for grass carp larvae of different ages and ploidies. Counts are for 500- μ L portions prepared by disaggregating whole individual larvae in 1 mL of nuclear isolation medium. All means are significantly different (Duncan's multiple-range test, $P < 0.01$). C.I. = confidence interval.

Sample	Num-ber of larvae tested	Total number of counts	Number of cells	
			Mean	95% C.I.
4–5-h-old larvae				
Diploid	20	60	27,375	26,718–28,031
Triploid	15	45	19,975	19,418–20,531
Tetraploid	1	5	12,620	12,189–13,050
30-h-old larvae				
Diploid	20	60	52,755	50,852–54,657
Triploid	20	60	36,627	35,543–37,710

($P < 0.01$) for both the 4–5- and 30-h-old larvae (Table 1). Differences in cell (nucleus) density between ploidies for 4–5-h-old larvae were 27.0 and 36.8% for mean diploid–triploid and triploid–tetraploid samples, respectively.

Tissue quantity and differentiation in larvae older than 30–48 h make disaggregation more difficult and may require filtering with 70- μ m-mesh nylon cloth. Higher particle densities resulting from larger individuals cause the Coulter counter aperture tube to clog more frequently. A partially obstructed aperture can result in a particle count biased on the high side because the constriction forces particles to move through the aperture faster than normal. Therefore, it is important that the aperture be cleared or checked before each run.

Distinguishing diploid from triploid individuals based on nuclear size as is done with erythrocyte samples can also be accomplished with this method by viewing the pulse amplitude on the Coulter counter oscilloscope. The larger the particle, the greater the electrical resistance, and the higher the pulse will be. To test this theory, an additional 40 samples (4–5-h-old larvae; about 50% diploid and 50% triploid) were prepared as described, pre-checked for ploidy with a flow cytometer, and marked so that the operator could not see the result. The samples were arranged in a random sequence of diploid and triploid fish. After a few minutes of practice at distinguishing known diploid and triploid samples prepared in the same manner, each of two researchers was 100% accurate in identifying the 40 unknown diploid and triploid samples simply by observing the pulse height on the oscilloscope and relating relative differences to particle size. Particle size differences

of diploid and triploid erythrocyte nuclei are more easily distinguished than differences in samples derived from whole tissue (in this case the whole organism) due to the uniformity of erythrocyte nuclei. However, we were highly accurate in identifying diploid and triploid samples prepared as single-cell (nucleus) suspensions from a whole larva based only on differences in nucleus size. The addition of a channelizer to the Coulter counter system would greatly enhance this method. The channelizer further analyzes the sample by recording each particle in one of 1,000 channels each representing a discrete size range; the result is a particle volume frequency curve. The position of the volume frequency curve along the X axis should allow an even more accurate segregation of diploid and triploid samples.

A number of variables other than ploidy can affect particle (cell or nucleus) density. If the sample volume is altered, proportional variations in particle counts will result. Also, egg and larval incubation temperatures will cause the cell (nucleus) count to vary. Grass carp larvae 4–5 h old maintained at a relatively high temperature will have considerably more cells (due to faster growth) than larvae maintained at a lower temperature. The results for this study (Table 1) are based on ambient hatchery water temperatures of 25–26°C. Differences in Coulter counter settings (particularly the threshold settings and aperture tube size) will cause different results from those in Table 1. However, differences in the counts as related to cell density or nucleus size between diploid and triploid samples are large enough for accurate segregation as long as the methods involved in preparation and analysis are consistent, even if somewhat different from the ones presented here.

I have presented a new method for accurately determining ploidy of newly hatched grass carp larvae by means of Coulter counter analysis based on reduced cell (nucleus) density and larger nuclear size in triploid individuals. The method is relatively rapid: about 1–3 min is required to prepare and test a single sample (excluding overnight storage in NIM). The time necessary to prepare chromosome spreads from individual larvae can be quite variable and depends on the skill of the individual. Because most producers of triploid grass carp already have a Coulter counter, the only costs would be for the lysing agent, needles, and syringes. The method is considered preferable to chromosome enumeration on a gross scale because many thousands of nuclei are objectively and rapidly analyzed.

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