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# Artificial Propagation and Induction of Triploidy in Largemouth Bass *Micropterus salmoides* and Ploidy Discrimination using Erythrocyte Length

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## Abstract

**We describe an artificial propagation procedure and simple ploidy discrimination techniques using erythrocyte major axis length for largemouth bass *Micropterus salmoides*. Hormonal treatments of 5 mg/kg of carp pituitary and 50 μg/kg of leutinizing hormone-releasing hormone (LHRH) produced viable gametes in 21–24 h, and triploidy was induced using a pressure treatment of 563 kg/cm² on embryos for a 1-min duration exactly 5 min following fertilization. We produced about 500 fingerling triploids and about 500 diploid controls, and verified genetic status of a subset of each group using flow cytometry. Erythrocyte length was measured for 10 known diploid and 10 known triploid individuals. Remaining fish were internally microtagged with group-specific tags and mixed to test the model. We developed ploidy discrimination intervals based on the 99% confidence limits of mean erythrocyte length (MEL, $N$ = 25 erythrocytes) for individual fish, which were 14.43–16.66 μm for triploids, and 10.23–13.62 μm for diploids. Logistic regression provided the discrimination model: Ploidy status (±) = −196.16 + 13.97 × MEL, with positive (+) outcomes considered triploid. Both discrimination techniques were 100% effective at differentiating ploidy of the 22 microtagged largemouth bass recollected from the mixed population. We did not observe a significant change in erythrocyte length as fish size increased, indicating that erythrocyte length is an accurate predictor of ploidy for all sizes of largemouth bass.**

The largemouth bass *Micropterus salmoides* is an economically important species. Due to tradeoffs between somatic and gonadal energy partitioning, the growth potential of largemouth bass can be limited by reproductive requirements. During reproductive development, energy for somatic growth and maintenance can be reallocated to reproductive output, resulting in reduced growth after sexual maturation (Allen and Stanley 1978). Such slow growth can be counterproductive to the objectives of fisheries managers and the aquaculture industry. One solution would be to produce nonreproducing largemouth bass that invest less energy into reproductive development and output. If reproduction can be eliminated, the resulting energy surplus could supplement growth (Thorgaard and Allen 1987).

The two techniques that are currently available to mass-produce sterile fish are application of strong doses of steroid hormones and chromosomal manipulations (Stanley 1981; Donaldson and Hunter 1982; Purdom 1983; Thorgaard 1983; Yamazaki 1983; Dunham 1990; Ihssen et al. 1990; Strüssmann et al. 1993). Chromosome manipulation techniques, such as triploidy induction, appear especially suitable because of the public's acceptance of the final product over hormone-treated fish (Brown and Roberts 1982; Refstie 1982; Bye and Lincoln 1986; Strüssmann et al. 1993). In previous studies, the induction of polyploidy has proven an effective method of producing permanently sterile fish with reduced gonad development (Purdom 1976; Wolters et al. 1982; Cassani and Caton 1986; Parsons and Meals 1997). Increasing

the number of chromosome sets in fish can yield faster growth and larger ultimate size (Valenti 1975; Purdom 1976; Wolters et al. 1982; Chrisman et al. 1983) and can have beneficial applications to fisheries management and aquaculture.

Applying high pressure to fertilized eggs has successfully produced largemouth bass triploids (Garrett et al. 1992). However, Garrett et al. (1992) pressure-treated limited numbers of fertilized eggs, and only 61 individuals from six different treatments (1–28 individuals per treatment) were analyzed for ploidy status. Also, largemouth bass are difficult to propagate artificially. Most hatchery propagation of black bass relies on natural or semi-natural reproduction in which male and female fish engage in nesting behavior in ponds or spawning tanks (Snow 1975). Whereas effective production of triploid largemouth bass requires the ability to obtain viable gametes on demand, propagation techniques need to be advanced.

Methods for mechanical induction of triploidy are not always 100% effective (Harrell et al. 1998). Hence, induction success of treatments must be verified to determine the proportion of triploids obtained. Accepted technologies for detecting polyploidy include cytological karotyping (Thorgaard 1983), staining nucleolar-organizing regions (Phillips et al. 1986), particle size analysis that measures erythrocyte cell or nuclear volumes (Thorgaard 1983; Johnson et al. 1984; Wattendorf 1986; Cassani 1990), and flow cytometry analysis (Allen 1983; Johnson et al. 1984). While these techniques generally provide accurate verification of polyploidy (Harrell et al. 1998), the high cost of equipment and associated expertise make them unavailable to many producers and managers.

Microscopic measurements of erythrocyte dimensions have been used to verify triploidy in some fish species (Garcia-Abiado et al. 1999). The sizes of blood and other cells correlate with DNA cellular content, which in fish is primarily related to ploidy level (Fange 1992). This technique is less complicated than other techniques and can be performed with minimal equipment and training. The reliability of this technique for differentiating diploids and triploids has varied among fish species, ranging from 70.8% in rainbow trout *Oncorhynchus mykiss* (Tambets et al. 1991) to 95–100% in landlocked Atlantic salmon *Salmo salar* (Benfey et al. 1984). In this manuscript, we evaluate the use of erythrocyte length measurements, by comparison with flow cytometry analysis, for differentiating diploid and triploid largemouth bass. In this preliminary study, we developed two discrimination techniques using erythrocyte cell length to assist in ploidy determination, and validated both techniques' performance using internally-tagged diploid and triploid largemouth bass.

## Materials and Methods

### *Triploid Production*

Broodstock were collected from Lucchetti Reservoir, Puerto Rico. Males and females were sorted based on degree of reproductive development, and only fish with free-flowing gametes or advanced gonadal development were transported to the hatchery facilities at Caribe Fisheries in Lajas, Puerto Rico. Fish with naturally free-flowing gametes were immediately spawned. Largemouth bass with advanced gonadal development but without free-flowing gametes were artificially induced to release gametes using hormone injections. Both males and females were injected with 5 mg/kg carp pituitary extract in the body muscle tissue between the second dorsal fin and the lateral line, and with 50 μg/kg leutinizing hormone-releasing hormone (LHRH) in the dorsal lymphatic node. Injected fish were held in concrete tanks for spawning on the following day.

Eggs were stripped into a dry container, and milt was stripped from males and collected via pipette. Milt was mixed with about 10 mL of 0.3% NaCl irrigation to in-

crease the volume, and then poured over the eggs. Fertilization was considered to be instantaneous, although 1 min of fertilization time was allowed before dividing the eggs into control and treatment groups. Eggs from the treatment group were placed into a mesh basket, and the basket was inserted into a water-filled pressure chamber. At 5 min post-fertilization, eggs were subjected to 563 kg/cm$^2$ (8,000 p.s.i.) for 1 min (Garrett et al. 1992). No treatment was given to control eggs. Eggs from both groups were placed on incubation mats within concrete hatching tanks.

Hatching began within 48 h, and estimated egg mortality was about 40%. Swim-up and first feeding followed about 3 d post-hatching, and fry were fed live brine shrimp *Artemia gracilis* twice daily to satiation before being moved to a natural prey base (*Poeciliidae*) in grow-out ponds. When juveniles were large enough (at least 37 mm total length), they were tagged with binary coded wire tags to differentiate treated ($N$ = 477) and control fish ($N$ = 487). These fish were then combined and released into Lucchetti Reservoir to be recaptured later for validation of the discrimination technique. A subset of each group was retained for cytometric determination of ploidy and development of the erythrocyte measurement discrimination technique (age of fingerling = 53 d).

### *Verification of Ploidy*

Ploidy levels of a subset of control and treated individuals were assayed using flow cytometric analyses. Erythrocyte cells were collected from individual fish (length range = 34–58 mm total length), RNAase was added to prevent RNA staining, and the DNA was stained with propidium iodide. Propidium iodide intercalates into double stranded nucleic acids and provides a fluorescent signature. Whereas fluorescence of the cells is directly related to DNA content, triploids tend to be about 50% brighter than diploids. For each sample verified using flow cytometry, 10,000 measurements of erythrocyte fluorescence were taken. For more detailed methodology and explanation of flow cytometry, see Kerby and Harrell (1990).

We used 10 triploid individuals and 10 diploid individuals from the groups verified by flow cytometry to develop the discrimination protocol for using erythrocyte major axis length to differentiate ploidy level. Sample fish ranged in size from 17 to 25 mm total length. No fixative agents were used, although a small amount of 0.7% sodium chloride irrigation was used to dilute the samples. Blood samples were collected from each fish by severing the caudal peduncle, and blood smears were prepared by placing a small drop of diluted blood on a glass microscope slide and covering with a cover glass. For each fish, cell lengths were measured for 25 randomly-selected erythrocytes under 1,000× magnification (including 10× eyepiece) with oil immersion using an ocular micrometer fitted inside the eyepiece of a compound microscope.

For each blood sample, we calculated the mean erythrocyte length and the 99% confidence interval for the mean (Cimino 1973). For each ploidy group, we used the lowest and highest confidence limits of mean erythrocyte length from the 10 individuals to establish the bounds of the discrimination intervals. Discrimination intervals are explained further in the results.

In addition to the discrimination interval technique, we created a prediction model using logistic regression. Mean erythrocyte length was the independent variable, and ploidy status was the dependent variable. The resulting equation calculated the logit value—the logarithmic of the probability that an erythrocyte mean length was triploid divided by the probability it was not. The logistic function model was designed to provide a positive (triploid) or negative (diploid) response to erythrocyte mean length based on the sign of the output number.

After developing the original discrimination intervals and prediction model with

TABLE 1. *Stocking and ploidy statistics of control and pressure-treated largemouth bass stocked in Lucchetti Reservoir on 5 May 2000. All lengths are measured in total length. Ploidy was determined using flow cytometry.*

| Treatment group | Number stocked | Mean length (mm) | Standard error of mean (mm) | Minimum length (mm) | Maximum length (mm) | Number verified | Percent triploid[a] |
|---|---|---|---|---|---|---|---|
| Control | 487 | 46.7 | 1.30 | 37 | 88 | 21 | 0 |
| Treated | 477 | 63.3 | 1.41 | 42 | 99 | 23 | 100 |

[a] Based on flow cytometry of 21 control and 23 treated largemouth bass.

known ploidy fish, we periodically recollected tagged bass from Lucchetti Reservoir using electrofishing. All tagged largemouth bass were measured for total length (TL, mm) and weighed (g), then transported alive to the lab where erythrocyte length was used to determine ploidy. Slides were prepared using the same methods used to develop the discrimination intervals, and 100 erythrocytes were measured for each fish. We did not remove and analyze the tag to determine the treatment group of each recapture until after ploidy was determined using the blood smear technique. This served as a blind test of the verification procedures.

We used two-tailed *t*-tests with a significance level of 0.05 to test for differences in erythrocyte size distributions between groups. Mean coefficient of variation (CV) was calculated from CV values of individual blood samples and provided a comparison of the technique's sensitivity to sample sizes for diploid and triploid samples. All statistics were calculated using SigmaStat 2.03© computer software.

## Results

### *Propagation and Induction*

The use of a single treatment of carp pituitary extract and LHRH produced free-flowing gametes in more than half of the largemouth bass injected. Peak gamete production usually occurred within 21–24 h following injection at water temperatures ranging 23–28 C. Water temperature during spawning, induction, and hatching ranged 23–25 C. Male largemouth bass produced a very low volume of milt, which was most effectively collected using a pipette. The quality and viability of hormone-induced eggs did not appear to be as high as that of natural-induced eggs, which appeared to exhibit slightly higher hatching success.

We successfully produced over 500 control and 500 pressure-treated fingerling largemouth bass from four female and five male largemouth bass brood fish. Cytometric verification determined that 100% of a sample ($N$ = 23) of the pressure-treated group were triploids and 100% of a sample ($N$ = 21) of the control group were diploids. Both groups were stocked into Lucchetti Reservoir in May 2000 (Table 1).

### *Erythrocyte Length*

Similar to red blood cells of other fish, largemouth bass erythrocytes are oval and elliptical disks with a compact nucleus. Major axis lengths of triploid largemouth bass erythrocytes were significantly larger ($P < 0.001$) than the corresponding lengths of diploid largemouth bass red blood cells. Combined erythrocyte lengths of the original 10 triploid largemouth bass displayed a mean of 15.64 μm (SE = 0.06 μm), and individual means ranged 15.00–16.10 μm (SE ranged 0.13–0.26 μm, mean CV = 5.79%). The uppermost and lowermost 99% confidence limits for the individual means were used to produce the triploid discrimination interval, 14.43–16.66 μm.

Combined erythrocyte lengths of the original 10 diploid largemouth bass displayed a mean of 11.82 μm (SE = 0.07 μm), and individual means ranged 10.76–

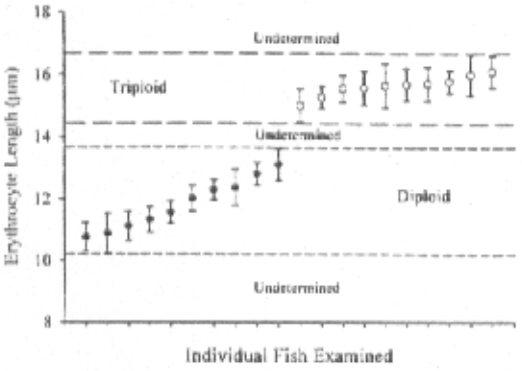


FIGURE 1. *Largemouth bass ploidy discrimination technique developed from known-status diploid (N = 10) and triploid (N = 10) fish. Circles represent mean erythrocyte lengths for diploid (solid) and triploid (open) individuals, and error bars represent the 99% confidence interval for the mean. New samples coinciding with the overall confidence interval of either ploidy group would be designated as that ploidy status. Samples outside of the discrimination intervals would be considered undetermined and should be verified using a different technique.*

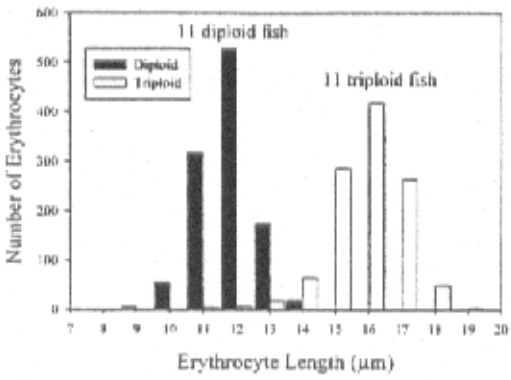


FIGURE 2. *Frequency distributions of erythrocyte cell lengths (N = 100 per individual) from 22 microtagged largemouth bass collected in Lucchetti Reservoir.*

13.10 (SE ranged 0.12–0.23 µm, mean CV = 6.97%). The uppermost and lowermost 99% confidence limits for the individual means were used to produce the diploid discrimination interval, which was 10.23–13.62 µm.

Using these discrimination intervals (Fig. 1), samples of unknown ploidy with mean erythrocyte major axis lengths between 14.43 and 16.66 µm are designated triploid, and mean lengths between 10.23 and 13.62 µm are designated diploid. Unknown samples not coinciding with either the diploid or triploid discrimination intervals would be considered "undetermined."

Logistic regression of erythrocyte length and ploidy status provided the prediction model:

$$\text{Ploidy status } (=) = a + b \times \text{MEL}$$

where ploidy status (the logit prediction statistic) is given as either a negative (diploid) or positive (triploid) integer, and MEL is the mean erythrocyte length for a given fish sample. Coefficients *a* and *b* were estimated as −196.159 and 13.974, respectively.

### *Verification Using Erythrocytes*

We collected 22 microtagged largemouth bass from Lucchetti Reservoir during a 6-mo period following stocking. Erythrocyte length distributions of all microtagged fish displayed two distinct modes (Fig. 2). Means of erythrocyte measurements from individual fish were plotted against the discrimination intervals, and the technique accurately determined ploidy for all 22 recaptures (Fig. 3), with no fish considered "un-

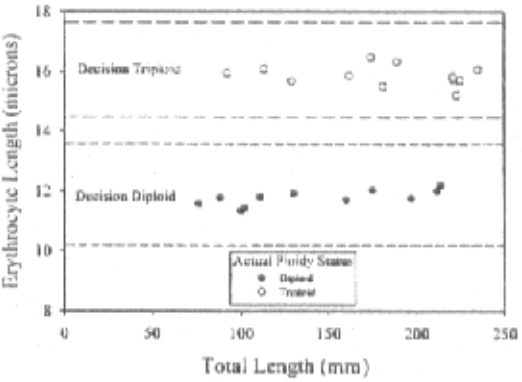


FIGURE 3. *Mean erythrocyte length (based on 100 erythrocytes per individual), erythrocyte discrimination intervals (short dash – diploid; long dash — triploid), and actual ploidy status of 22 microtagged largemouth bass recollected from Lucchetti Reservoir, Puerto Rico. Erythrocyte cell length is plotted against individual fish total length. Standard errors (error bars) of mean cell lengths ranged 0.05–0.18 µm.*

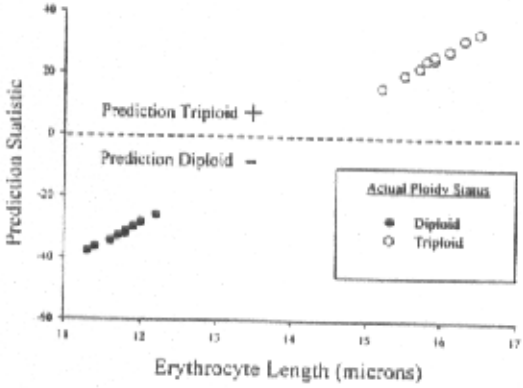


FIGURE 4. *Logistic regression model predictions of ploidy status using mean erythrocyte length (based on 100 erythrocytes per individual). Actual ploidy status of the 22 microtagged largemouth bass is given.*

determined." Binary wire microtags verified that the actual treatment group of each recaptured largemouth bass matched the erythrocyte technique's classification, indicating that the discrimination interval technique was 100% successful during the testing phase. The logistic regression equation also correctly predicted ploidy status for all 22 microtagged fish as well, providing a 100% success rate (Fig. 4).

There were no obvious changes in diploid or triploid erythrocyte major axis length as the fish grew (Fig. 3). The initial mean erythrocyte lengths for diploid and triploid largemouth bass (11.82 and 15.64 μm, respectively) were determined from fish about 20 mm total length. Recaptured largemouth bass ranged in size from 88 to 235 mm total length, and associated mean erythrocyte lengths and standard errors fell well within the technique's discrimination intervals for both diploid and triploid individuals.

## Discussion

We were able to successfully spawn largemouth bass on demand in Puerto Rico during their natural spawning season. Natural-induced eggs appeared to be more viable, but they were not always available since the female must be collected immediately before or during the spawning event. A combination of carp pituitary and LHRH injections was successful at inducing ovulation in developing females and provides the opportunity for greater control in the propagation process. The reduction in viability associated with hormones can be offset by the increase in available broodstock (and the available number of eggs) afforded through greater control using the hormones.

The approach described by Garrett et al. (1992) was successful at producing triploid largemouth bass in Puerto Rico, despite higher water temperatures during our induction procedure than during their procedures. In their study, induction was performed at 18 C, and a 1-min treatment of 8000 p.s.i. at 5-min post-fertilization yielded 100% triploidy for all fish verified. Whereas metabolic rate increases with temperature, the duration of the required post-fertilization time interval is expected to decrease as temperature increases. However, we achieved 100% induction success at temperatures ranging 23–25 C using the same post-fertilization time interval proposed by Garret et al. (1992). Whereas we only verified about 4% of the pressure-treated group, it is possible that some of the 500 pressure-treated fish were not triploids, although no diploids were collected in subsequent sampling.

Our results with triploid largemouth bass are consistent with the findings that the incorporation of a triploid genome in fish causes a significant increase in erythrocyte cell and nucleus measurements (Benfey and Sutterlin 1984; Fange 1992; Boron 1994; Garcia-Abiado et al. 1999). Measurements of cell major axis lengths and calculation of their respective mean were able to correctly classify 100% of diploid and triploid fish using the erythrocyte discrimination intervals and the logistic regression model. Whereas erythrocyte size did not appear related to largemouth bass length, these techniques will be appropriate to verify triploidy for all sizes of fish.

We tested the technique using 100 mea-

surements per fish, but sample size could be lowered depending on the desired level of accuracy. Because mean CV was similar between diploid and triploid fish, sample size requirements should be similar for each ploidy group. The overlap in cell size was minimal, but triploids tended to have a small proportion (about 2%) of erythrocytes within the primary size distribution (10–13 μm) of diploid erythrocytes. The largest diploid erythrocyte observed was 15 μm, but this size group represented only 0.09% of the entire distribution. Whereas much of the triploid distribution was 15 μm or larger, the presence of these larger cells in high numbers could alone be used to differentiate triploid and diploid largemouth bass.

These techniques were not designed to differentiate mosaics, tetraploids, or other genetic arrangements. Thus, in situations where samples may contain other variations in chromosome number, we recommend using more precise techniques. However, it is reasonable to assume that further refinement of the erythrocyte length technique using other chromosomal variations could expand its applicability to differentiate haploid and tetraploid individuals as well, given the proportional change in cell size associated with chromosome number.

We have shown measurement of erythrocyte major axis length to be a viable, practical alternative for distinguishing ploidy levels in largemouth bass. Blood samples can be easily removed from small fish by severing the caudal vein with a sharp scalpel or from biopsy of larger fish without harm. Samples can either be examined immediately or stained for examination at a later date (Cimino 1973). The simplicity of techniques and equipment make it ideal for fish managers and culturists alike, as both start-up cost and training are minimal. Measurements of cell lengths of 100 erythrocytes and calculation of the mean can be performed in about 25 min, making it a realistic alternative for small-scale studies or where immediate verification of individual fish is required. Reducing the number of measurements per sample can decrease the processing time, but accuracy may decrease as well.

Although other techniques such as flow cytometry and use of a Coulter counter can measure cells with greater speed and accuracy, the cost of equipment can be prohibitively high (up to US$33,000 for a Coulter unit). Some organizations perform analyses on a contract or per sample basis, but cost can range US$75–$120/h excluding sample preparation, shipping, and potential loss of fish during transit. These costs could be restrictive to small-scale aquaculture operations and research projects, especially if only a small number of samples are required.

Our results indicated that the erythrocyte technique was 100% effective during the testing phase of the study. Although we only evaluated 22 individuals, the magnitude in difference in erythrocyte cell length between ploidy types suggests that misclassifications are unlikely. However, it is best to err on the side of caution when triploid largemouth bass are to be introduced into waters where diploid largemouth bass do not exist or are of different genetic composition. To avoid possible reproduction of misclassified diploids in these circumstances, we recommend flow cytometry or using a Coulter counter to verify ploidy until additional data are available to confirm the technique's accuracy. For other applications, erythrocyte length measurement appears to be a valuable technique for distinguishing between diploid and triploid largemouth bass.

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