RAPID FLOW CYTOMETRY METHOD FOR TRIPLOIDY DETERMINATION IN TURBOT
(Scophthalmus maximus L.)

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ABSTRACT

Accurate methods for assessing triploidy are important because treatments designed to induce poliploidy are not 100% and offspring from treated eggs are usually a mixture of triploids and diploids.

The most common method is kariotype preparation and chromosome counting. Flow cytometry was more recently applied to triploidy determination and usually carried out using erythrocytes.

This paper describes a rapid and simple method for triploidy determination from the larval stage of turbot (*Scophthalmus maximus* L.) by flow cytometry.

High-quality histograms were obtained from turbot larvae and the very good reproducibility provided unambiguous discrimination of diploids and triploids even in early stages of development.

Keywords: flow cytometry, larval stage, triploidy, turbot.

I. INTRODUCTION

Production of sterile fish has become a major concern in aquaculture, because as sterility in commercial fish stocks has the potential to increase production yields, as metabolic energy which would otherwise be used for gonadal development is redirected to somatic growth (Nagy, 1987). Another advantage is the avoidance of reproduction in farmed species which become sexually mature before reaching an economic size.

Triploids are generally expected to be sterile due to the likely failure of chromosomes to pair correctly during meiosis. (Benfey and Sutterlin, 1984). Triploidy can be induced by disruption of developmental events post-fertilization, to prevent extrusion of the second polar body, thus retaining a diploid maternal set within the ovum. A variety of techniques has been developed to induce triploidy in fish (for review see Thorgaard and Allen, 1987; Thorgaard, 1992; Tave, 1993).

Accurate methods for assessing triploidy are important because treatments designed to induce poliploidy are seldom 100% effective (Allen and Stanley, 1979;
Chourrout, 1980; Thorgaard et al., 1981) and offspring from treated eggs are usually a mixture of triploids and diploids.

The most common method is karyotype preparation and chromosome counting (Chourrout and Hape, 1986). Other current methods involve microscope cytophotometry (Gervai et al., 1980) or indirect evaluation from erythrocyte nucleus diameters or volume (Chourrout et al., 1986; Child and Watkins, 1994). All these methods are tedious and time-consuming, and determination is made from a limited number of cells.

Flow cytometry was more recently applied to triploidy determination and usually carried out using erythrocytes (Allen, 1983; Crozier and Moffett, 1989). This is only possible when well-grown fish are used, but early knowledge of triploidy induction results can be advantageous.

The present research describes a rapid and simple method for triploidy determination from the larval stage of turbot (Scopthalmus maximus L.) by flow cytometry.

II. MATERIAL AND METHODS.

Reproductively mature fish were obtained from the Centro Oceanográfico de Santander, where a domesticated turbot stock is maintained.

Eggs and milt were stripped from females and males and fertilization was carried out in plastic basins. The result of fecundation were divided into experimental batches. Triploidy was induced by exposing eggs to heat shock.

Treatments were administered by transferring batches as required to a temperature regulated water bath equipped with mechanical water circulation to assist temperature equilibration. In each experiment one batch was not subjected to heat shock and was used as the control group.

After treatment, eggs were immediately transferred to incubation tanks in the laboratory and reared at room temperature until hatching. Tanks were checked frequently and dead eggs removed.
On day 5 or 6 post-fertilization (at the moment of hatching), samples of 20 larvae were collected in order to determine the ploidy level. The method used was based on the measurement of nuclear DNA content by flow cytometry.

The procedure used in the sample preparations was previously developed in human tissues by Vindeløv (1983).

Larvae of each sample were gently disrupted into 1 ml of citrate buffer (pH 7.6) and 500 μl of the mixture were then centrifuged for 10 minutes at 1,200 revolutions/min. The supernatant was discarded and the pellet was resuspended in 300 μl of a solution with NP-40, spermine and trypsin during 10 minutes at room temperature. A second solution with trypsin inhibitor and Rnasa was added for 10 minutes. At last, nucleus were stained for 10 minutes using a solution of the propidium iodide and spermine light protection.

These preparations were then analysed with a Cytorom absolute (Ortho diagnostic systems). The apparent ploidy of each sample was calculated from the relative proportion of triploid cells to the total number analyzed by the curve-fitting program ModFit (Verity Software House).

### III. RESULTS AND DISCUSSION

Analysis of larval samples yielded data from a population of approximately 2,000 larvae. The calculation of apparent ploidy assumes that each larva, diploid and triploid, makes an approximately equal contribution of cells to the overall pool of cells, a reasonable assumption as the larval size is very similar.

To check this, a known sample of 5 individuals (3 diploids and 2 triploids) was analyzed by flow cytometry (FCM). The results (Figure 1A), indicated that there was a good agreement between the frequency of each cell population (58.77 and 41.23% respectively) and the number of individuals belonging to the different classes present in the sample.

Flow cytometry was carried out for 100 samples, of which 65 were shown to be mixtures of triploid and diploid individuals.
The least squares curve-fitting methods of ModFit produced estimates of mean relative fluorescence (relative DNA content), coefficients of variation (CV) of fitted curves, and relative number of events in each population.

Examples of histograms from diploid and triploid of turbot larvae and their statistical values are shown in Figures 2A and 2B respectively. In these samples only one cell population was found at channels 51.56 and 75.51.

Two cell populations, well differentiated, were evident from FCM analysis of some treated samples (Fig. 1B). Channel values observed for peak means (76.72 for triploid, 51.18 for diploid) correspond to a fluorescence ratio of 1.5, identical to the ploidy ratio (3 \( \div 2 \)). This calculation was possible as the ordinate (channel number) is a sufficiently linear representation of DNA content (Allen, 1983).

The mean model channel number of the diploid cell populations examined was 52.06 ± 0.68, while that of the triploids was 78.05 ± 0.27, giving a DNA ratio 1.488 ± 0.016, close to the expected 2:3 ratio of diploids-triploids, confirming the latter group as triploid.

One of the most important criteria for determining if a histogram is possible to evaluate for ploidy status is CV of the mean DNA content cell population. Its value corresponds to the ability to resolve two different DNA content populations in the same sample. Histograms with values of 8% are generally acceptable to most research (Allen, 1983; Johnson et al., 1987; Crozier and Moffett, 1989; Allen and Bushek, 1992). In our work the CV range of variation was from 3.17 to 5.15% in diploid cell populations and from 1.48 to 4.33% in triploid cell populations.

In conclusion, cytofluorometry proved to be a fast procedure for evaluating polyploidy. High-quality histograms were obtained from turbot larvae and the very good reproductibility provided unambiguous discrimination of diploids and triploids even in early stages of development.

REFERENCES


Figure 1. Histograms obtained from samples analysis larvae turbot: A, known mixture, B, unknown mixture.
Figure 2. Typical histograms of only one cell population: A, diploid, B, triploid individuals.