FLOW CYTOMETRIC AND CHROMOSOMAL EVIDENCE FOR THE PRODUCTION OF VIABLE OFFSPRING BY TRIPLOID RAINBOW TROUT MALES (*Oncorhynchus mykiss*).

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SUMMARY

Ova collected from diploid female trout were inseminated with sperm from triploid males, diploid males or with a mixture of both (2nX3n, 2nX2n and 2nX2n+3n crosses). Survival from fertilization to hatching was 0.57% in 2nX3n crosses, 10.7% in 2nX2n crosses, and 10.9% in 2nX2n+3n crosses, and it was affected by low egg quality. Survival from hatching to 4 months was 10%, 65% and 44% for the 2nX3n, 2nX2n and 2nX2n+3n crosses ($P<0.05$). Hatching embryos in 2nX3n crosses exhibited morphological abnormalities, although a few juveniles were obtained and one of them was a sexually maturing precocious male.

Ploidy of parents and offspring was examined by flow cytometry and by image analysis of modified Azure A-stained tissue smears. Flow cytometry on red blood cells and image analysis of erythrocytes and hepatocytes revealed a hypertriploid genome in all 2nX3n offspring and in 12.5% of 2nX2n+3n progeny. Metaphase plates analyzed in gill epithelia from these individuals revealed aneuploid figures and multiple levels of ploidy. These data provide the first evidence of limited functional fertility of triploid trout males.

INTRODUCTION

Induction of triploidy by physical or chemical treatment of fertilized eggs has proven to be a valuable tool in the production of sterile fish, although the degree of reproductive disruption depends on sex and species. In general, gametogenesis and sexual maturation are severely disrupted in triploid females, while triploid males display secondary sex characters and courtship behavior, develop an endocrine profile similar to diploid males, and undergo aneuploid spermatogenesis (Benfey et al., 1986). However, no direct experimental evidence on the generation of viable progeny by triploid males in salmonids has been provided to date.

We are currently investigating the performance of triploid rainbow trout produced by heat shock applied to freshly inseminated eggs. Triploid and diploid (control) siblings are being raised throughout three sexual cycles under identical conditions. During the first spawning season, a number of males in the triploid group displayed secondary sex characters such as dark coloration of the skin and modification of body shape. The objective of the work presented here was to assess the reproductive potential of these individuals and to characterize their fertility and the ploidy of their offspring.

MATERIALS AND METHODS

Six triploid males produced from crosses of RTJ and RTL rainbow trout strains (19.8 months old, 1.85 kg mean body weight) and six diploid males (same age, 1.9 kg mean body weight) were injected with 25 μg/kg LHRH to induce spermiation. Forty eight hours after

injection, they were anesthetized and necropsied to obtain blood and liver samples and morphometric parameters. A sample of sperm from each male was evaluated for motility and cell density. Approximately 1 ml of sperm from each male was used to inseminate eggs obtained from diploid females. The following crosses were conducted: 2nX2n (diploid X diploid), 2nX3n (diploid female X triploid male) and 2nX2n + 3n (diploid female X 1:1 volume mixture of sperm from triploid and diploid males). Fertilized eggs were stocked in indoor 15 l covered fiberglass tanks (T = 13.6 ± 0.8 °C), photoperiod adjusted to natural light cycles, and daily mortalities recorded. At 4 months of age, all surviving progeny were anesthetized, and blood and liver imprints collected and fixed in 10% phosphate-buffered formalin.

Ploidy was determined by flow cytometry on red blood cells and Quantitative DNA analysis of blood and liver smears. Blood collection methodology and flow cytometry procedures followed those of Thorgaard et al. (1982). A CAS-200 Image Analyzer was used for the determination of average DNA content (pg) on Azure A-stained, formalin-fixed blood and liver smears (Teplitz et al., 1990). Several individuals among the offspring were karyotyped (Thorgaard and Disney, 1990).

RESULTS

BROODSTOCK

Significant differences in gonadal weight and gonadosomatic index were detected between diploid and triploid males (lower in triploids). Semen collected from triploid males had a significantly lower cell density, including two males with no detectable sperm cells. No motility was observed in any triploid sperm, but all observations on sperm motility were conducted 48 hrs after collection of samples.

SURVIVAL OF PROGENY

The survival of eggs and embryos from incubation to hatching was low in all crosses including 2nX2n matings. By day 10 postfertilization, survival was 49.2% ± 13 for 2nX3n, 52.3% ± 14 for 2nX2n, and 55.6% ± 21 for 2nX2n + 3n matings (mean ± s.e.m.). Hatching success was 0.57% ± 0.5, 10.72% ± 6 and 10.89% ± 5 for the 2nX3n, 2nX2n and 2nX2n + 3n crosses, with only one cross in the 2nX3n group producing hatchable fry (3.4%). Dark pigmentation and the beginning of exogenous feeding were delayed in 2nX3n group compared to 2nX2n and 2nX2n + 3n groups. Survival from hatching to four months was 10%, 65.5% ± 2 and 44.4% ± 2 for the 2nX3n, 2nX2n and 2nX2n + 3n groups. A single 2nX3n cross produced 4 month-old juveniles. One of the surviving juveniles from this cross was a precocious male, as revealed by histological analysis of the gonad.

FLOW CYTOMETRY AND IMAGE ANALYSIS

Of the 43 animals analyzed (Table 1), flow cytometry of red blood cells revealed the presence of a triploid or near-triploid genome in the 6 triploid parents, the single 4 month-old 2nX3n offspring and in 2/14 (12.5%) of the 2nX2n + 3n progeny (Figure 1). Average DNA values observed for parents and offspring by image analysis of blood and liver smears are summarized in Table 1. Multiple comparison of means revealed the presence of three significantly different ($P < 0.0001$) levels of DNA content equal to the diploid (diploid parents, 2nX2n offspring and 87.5% of progeny from 2nX2n + 3n crosses), triploid (triploid parents) and aneuploid hypertriploid (2nX3n offspring and 12.5% of 2nX2n + 3n progeny) ploidy values. Image analysis data suggested the presence of mosaicism in blood smears collected from the three hypertriploid offspring.

CHROMOSOME ANALYSIS

Metaphase plates obtained from gill epithelia of the three hypertriploid individuals revealed the presence of aneuploid figures with a variable number of chromosomes (mean fundamental number = 147 ± 22, mean ± s.d., n = 7), but were in general close to the triploid karyotype (3n = 90, FN = 156). Grossly different levels of ploidy within a single individual were found in the three hypertriploid fish examined.

DISCUSSION

Triploid males used in this study displayed secondary sex characters and 4 out of 6 were able to produce low-density semen, although they had a significantly lower gonadosomatic index than diploid males. In spite of the high mortality observed in all matings from the beginning of the incubation period, which possibly relates to poor egg quality, this study provides the first evidence of potential limited fertility in triploid trout males, reinforced by the finding of one precocious male among the 2nX3n offspring. Such marginal fertility should be considered in the evaluation of the reproductive capacity of triploid salmonids, particularly if they are to be released into the wild.

Hypertriploid progeny obtained in 2nX3n and 2nX2n + 3n crosses showed mosaicism within a tissue (blood and gill epithelia). In both cases, mosaicism seems to appear concomitantly with aneuploidy. The bidirectional, wide histograms recorded by image analysis of blood imprints, together with the metaphase plates observed in gill epithelia of 2nX3n and 2nX2n + 3n hypertriploid offspring suggest a non-disjunctional origin for these aneuploid cells (Pérez Carrasco et al., unpublished).

The presence of a hypertriploid genome in offspring from triploid males suggests that fertilization by near-diploid spermatozoa took place. The mechanism by which diploid sperm could be produced in triploid testis is unknown. The formation of multipolar spindles during mitosis and the preferential elimination of chromosomes during spermatogenesis are plausible processes that may yield near-diploid gametes in a triploid gonad, and that have been described in other polyploids (Pera, 1975, Ohtani, 1993).

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PLOIDY GROUP	n	AVERAGE DNA (pg)		
		BLOOD	LIVER	POOLED
2n PARENTS	9	5.46±0.209	5.28±0.159	5.37±0.203
3n PARENTS	6	8.43±0.186	8.19±0.397	8.32±0.314
OFFSPRING	(28)			
2nX2n	11	5.49±0.298	5.68±0.192	5.60±0.238
2nX2n+3n	14	5.44±0.319	5.72±0.147	5.59±0.279
	2	8.83±0.401	8.82±0.249	8.83±0.333
2nX3n	1	8.32±0.495	8.19±0.44	8.25±0.472

Table 1. Average DNA values observed on blood and liver Azure A-stained smears. n, number of individuals. Data are means ± standard deviations.

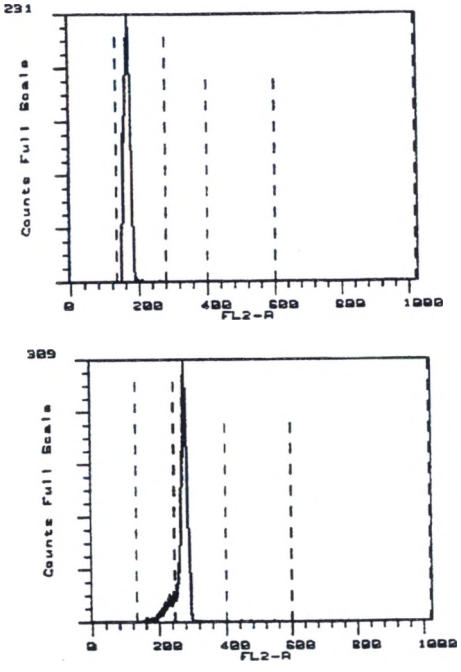

Figure 1. Flow cytometric histograms of diploid 4 month-old offspring resulting from a 2nX2n cross (top) and near-triploid 4 month-old offspring resulting from a 2nX3n cross (bottom).