

# KARYOLOGICAL ANALYSIS AND IDENTIFICATION OF HETEROCHROMOSOMES IN EXPERIMENTAL GYNOGENETIC OFFSPRING OF RAINBOW TROUT (*Oncorhynchus mykiss*, WALBAUM)

N. Colihueque<sup>1</sup>, P. Iturra<sup>1</sup>, N. Díaz<sup>2</sup>, A. Veloso<sup>2</sup> and F. Estay<sup>2</sup>

## ABSTRACT

Experimental *Oncorhynchus mykiss* gynogenetic progenies were identified by karyotype analysis. Haploid embryos were obtained by fertilizing eggs with 10 min irradiated UV sperm. Diploid gynogenetic embryos obtained by temperature shock showed two X chromosomes, identified with Hoechst 33258/AMD fluorescent stain. Mean survival rate of diploidized embryos was 37% at hatching. Chromosome number variability in the haploid embryos suggests Robertsonian chromosome rearrangements in the maternal germ cell line, which can explain previously reported chromosome number polymorphisms in rainbow trout.

## INTRODUCTION

Gynogenesis is a mode of reproduction in which only the maternal genome is present in the offspring. Experimental gynogenesis consists of the activation of eggs with genetically inactive sperm, usually produced by UV irradiation, followed by diploidy restoration by disruption of the second meiotic division or the first cleavage by heat or pressure shock treatments. Experimental gynogenesis in fishes was first developed in 1960 (Romashov *et al.*, 1960).

Rainbow trout sperm inactivation has been obtained by UV treatments and by gamma rays. Further diploidization of the eggs by disruption of the second meiotic

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<sup>1</sup> Departamento de Biología Celular y Genética, Facultad de Medicina, Universidad de Chile, Casilla 70061, Santiago 7, Chile. Send correspondence to P.I.

<sup>2</sup> Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago 7, Chile.

division produces gynogenetic offspring with better survival at hatching (7% to 81%), in comparison with those produced by inhibition of the first cleavage (7%). In the latter, viable individuals have not been reported at advanced growth stages (Chourrout, 1980, 1982, 1984; Chourrout and Quillet, 1982; Refstie, 1983; Lou and Purdom, 1984; Purdom *et al.*, 1985; Kaastrup and Horlyck, 1987).

We are interested in the utilization of gynogenetic offspring as a model to study the chromosome number polymorphism reported in rainbow trout populations (Thorgaard, 1983; Veloso *et al.*, 1990). Chromosome attributes of gynogenetic individuals correspond to the gametes produced by the mother. In this way, it is possible to study female meiotic segregation, providing an insight to the origin of chromosome variability in this species. We are also concerned with the potential use in salmonid fish farms of diploid gynogenetic individuals (Chourrout, 1984).

In this paper we provide information on the production of gynogenetic offspring by disruption of the second meiotic division in rainbow trout. The chromosome characteristics of haploid gynogenetic offspring were analyzed. The gynogenetic condition was examined by ploidy level and the sex chromosomes identified by their morphology and fluorescent banding patterns.

## MATERIALS AND METHODS

### *Brood stocks*

Rainbow trout (*Oncorhynchus mykiss*) breeders were obtained from the Sociedad Agrícola Macul Ltda. Fish Hatchery, Santiago, Chile. Gametes were collected by the stripping method from fish previously anesthetized with 50 mg/l MS-222 (tricaine metasulfonate) for 3 min. We used semen with active sperm motility only and eggs without overripe features (Estay, 1988).

### *Treatments*

**SPERM IRRADIATION:** In each experiment, 0.5 ml of semen (one male) was mixed with 2 ml of Billard's semen diluent with 0.2% KCl (Chourrout, personal communication, 1987). Sperm suspension was placed in a Petri dish with 5 cm diameter and stirred with a magnetic shaker (60 rpm) to obtain homogeneous irradiation. A 4 W 254 nm UV lamp was fixed above this suspension. The intensity of irradiation was 5.5 mW/cm<sup>2</sup> (Thompson and Scott, 1984). During irradiation the sperm suspension was kept at 5°C.

To find appropriate UV light dosage that fully inactivates the sperm DNA, experiments were designed to detect the Hertwig effect: an increase in survival rate of fertilized eggs as a result of paternal genome inactivation (Ijiri and Egami, 1980). These experiments were performed by removing aliquots of sperm each three minutes during 24 minutes of irradiation to fertilize batches of 250 eggs.

From these experiments, egg survival was estimated at the two celled stage and at six days before hatching. Diploid or haploid condition was also determined by chromosome counting of developing embryos.

**FERTILIZATION PROCEDURE:** Eggs of each female were divided into three batches: Control (C), fertilized with normal sperm; Haploid (H), fertilized with irradiated sperm; and Gynogenetic Diploid (D), fertilized with irradiated sperm with further heat shock treatment. Each group had an average of 250 eggs, except groups D of 1989 experiments where 1700 eggs were used. The dry method was utilized to fertilize the eggs, adding 0.5 ml of irradiated sperm suspension plus 5 ml of Billard's sperm diluent, without KCl. After 5 min the eggs were washed and incubated between 10°C and 13°C. A heat shock of  $26.5 \pm 0.1^\circ\text{C}$  during 15 min (Iturra *et al.*, 1990) was applied 10 min after water activation of eggs. The whole procedure was carried out under environmental light.

### *Ploidy and survival estimations*

**PLOIDY:** This evaluation was made by analysis of chromosome metaphase plates obtained according to Iturra *et al.* (1990). Individuals with near 30 chromosomes were considered as haploids. Chromosomes were stained with Giemsa 4% pH 7.2 or acetic orcein 1%. In gynogenetic diploids the X chromosomes were identified by Hoechst 33258/AMD (Actynomicyn D) fluorescent stain. In this method the chromosomes were counterstained with 30  $\mu\text{M}$  AMD for 25 min and stained with 0.5  $\mu\text{g/ml}$  Hoechst 33258 in McIlvaine buffer for 15 min. The preparations were viewed using a Leitz microscope with filter set excitator BP 270-230 and barrier filter BP 410-580.

Chromosome number of haploid progenies of two females were determined using at least 10 metaphases in each embryo. The haploid condition was taken as a modal number. Fundamental number (NF) was checked in each metaphase plate. The chromosome nomenclature used follows Levan *et al.* (1964).

**SURVIVAL:** Embryo survival was checked at four stages of development at 11°C: two celled stage (seven and one half hours), middle gastrulation (five days), "eyed-stage" (18 days) and hatching stage (28 days). Survival at the "eyed-stage" was estimated by shocking. Survival at the first two stages was used as an estimation of fertilization rate. Random samples of 10 eggs or embryos were treated with clearing solutions (Miguez, 1980) for recognition of the first two blastomere and gastrula stages.

## RESULTS

Survival rates for the experiment on Hertwig effect are shown in Figure 1. There was a continuous decrease of survival rate but without total inviability of the embryos at high UV dosage. At low dosage (3-15 min) the lowest survival was observed at nine min, followed by an increase of viability (Hertwig effect). The fertilization rate estimated at the two-celled stage diminished 30% after 12 min of sperm irradiation. All embryos showed the haploid condition. Accordingly, 10 min irradiation was chosen as appropriate to inactivate sperm DNA.

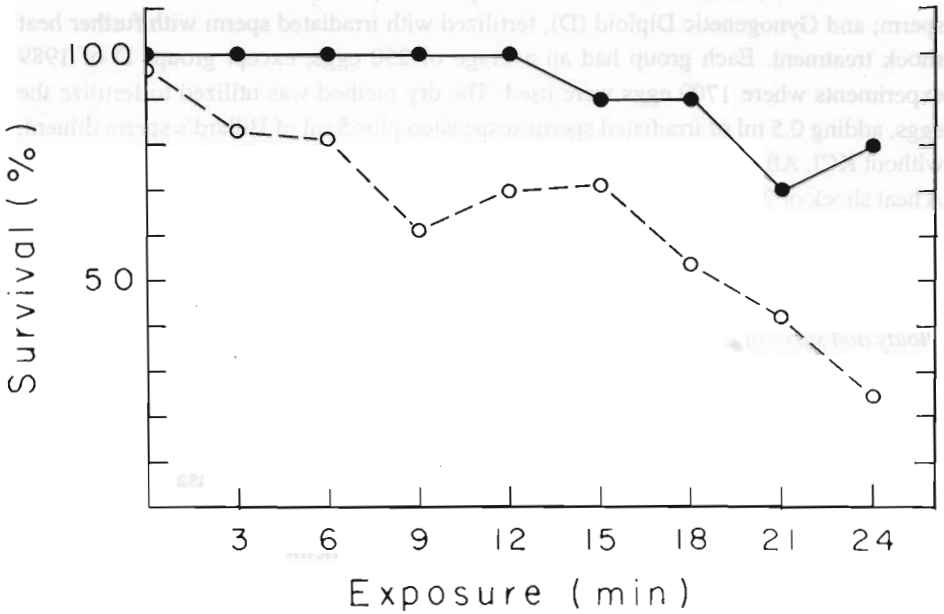


Figure 1 - Survival rates of eggs inseminated with semen exposed to different irradiation times (min). ●-●-●-●: survival rate at two celled stage (fertilization rate); o-o-o-o: survival rate six days before hatching.

Results for eight experiments of gynogenesis induction are shown in Tables I and II, corresponding to experiments performed during 1989 and 1990, respectively. From the analysis of these tables we can indicate: a) all control embryos were diploids, except in female 988 where one haploid embryos was found; control experiments presented high survival rates. At eyed and hatching stages, similar survival rates were observed. At hatching mean survival was 53% and 79% in the 1989 and 1990 experiments, respectively; b) the batches fertilized with irradiated sperm showed over 91% haploidy, and several groups presented 100% haploidy. In these groups the survival

rate decreased abruptly at hatching. Only in 1989 experiments, 4.2% survival was observed for the haploid group; c) eggs treated with heat shock showed a high and homogeneous response to diploidization (97%). The mean survival rate of these groups at hatching was similar in 1989 and 1990 experiments (37%).

Table I - Results of experimental gynogenesis in rainbow trout. 1989.

Female	Group	Ploidy (%)			Survival (%)	
		N*	n	2n	eyed-stage	hatching
A	C	12	-	100	88.1	86.3
	H	11	91	9	51.4	0.9
	D	12	-	100	77.6	64.6
M3	C	8	-	100	70	68.3
	H	9	100	-	54.3	0
	D	9	-	100	75.4	50.5
778	C	10	-	100	45	39
	H	11	100	-	46.5	14
	D	10	-	90 <sup>#</sup>	90	25
902	C	10	-	100	17.3	17.3
	H	10	100	-	35	2
	D	6	-	100	30	0
Mean	C	10	-	100	55.1	52.7
	H	10.2	97.5	2.5	46.8	4.2
	D	9.2	-	97.3	68.2	35

\*Number of examined embryos.

<sup>#</sup>The remaining 10% were triploid.

Fertilization rates at five days of development were similar in control and haploid groups, reaching about 80%. The diploid gynogenetic eggs presented 35% and 65% mean fertilization rates in 1989 and 1990 experiments, respectively.

A control embryo karyotype with  $2n=59$  is shown in Figure 2a. The sex chromosome pair in this embryo is heteromorphic, the X subtelocentric (st) and Y

telocentric (t), which corresponds to a male karyotype. Three haploid metaphase plates with 29, 30 and 31 chromosomes are shown in Figures 2b, 2c, and 2d, respectively. All showed NF52.

Table II - Results of experimental gynogenesis in rainbow trout. 1990.

Female	Group	Ploidy (%)			Survival (%)	
		N*	n	2n	eyed-stage	hatching
988	C	10	10	90	87.6	74.3
	H	8	100	-	31.6	0
	D	10	-	100	100	52.2
989	C	10	-	100	94	87
	H	11	100	-	66.1	0
	D	9	-	100	100	70
1011	C	10	-	100	93	81
	H	10	100	-	23.1	0
	D	12	17	83	38	11
1012	C	10	-	100	84.5	73.4
	H	9	100	-	18.8	0
	D	10	-	100	63.9	15.9
Mean	C	10	2.5	97.5	89.7	78.9
	H	9.5	100	-	34.9	0
	D	10.2	4.2	95.7	75.4	37.2

\*Number of examined embryos.

Two X chromosomes were identified in the metaphase plates of embryos treated with heat shock. Figure 3a,b shows metaphase plates of two diploidized embryos stained with Hoechst 33258/AMD and Giemsa, respectively. A subtelocentric chromosome pair (Figure 3b) shows a characteristic bright fluorescent band near the centromere and in the short arms (Figure 3a).

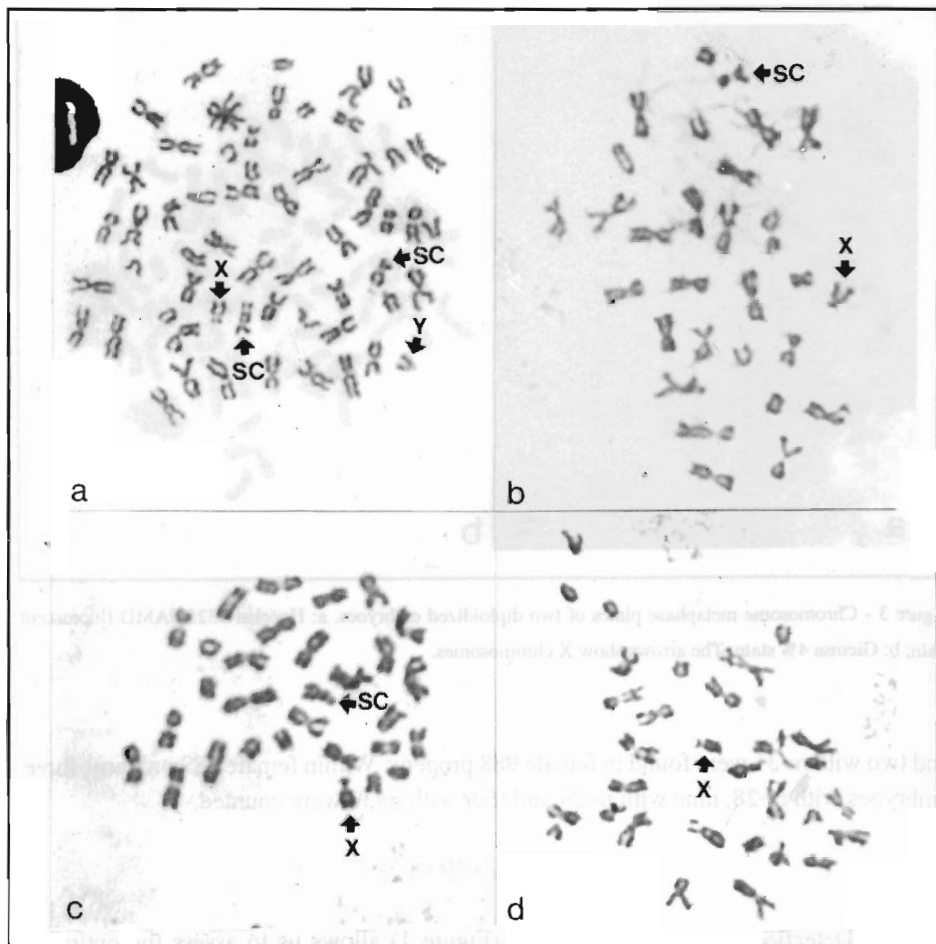


Figure 2 - Chromosome metaphase plates. a: control embryo with  $2n=59$ ; the heteromorphic chromosome pair, the subtolocentric (X) and telocentric (Y), which correspond to the male sex chromosome pair; b, c and d: metaphase plates of haploid embryos with  $n=29$ ,  $n=30$  and  $n=31$ , arrows show the X chromosome. SC: secondary constriction carrier chromosomes.

Nine haploid embryos of female 988 progeny and sixteen embryos of female 989 were analyzed to determine modal chromosome number. In both progenies individuals with different chromosome numbers but the same NF52 are represented. Chromosome number ranged between 28 and 31. One embryo with  $n=29$ , six with  $n=30$

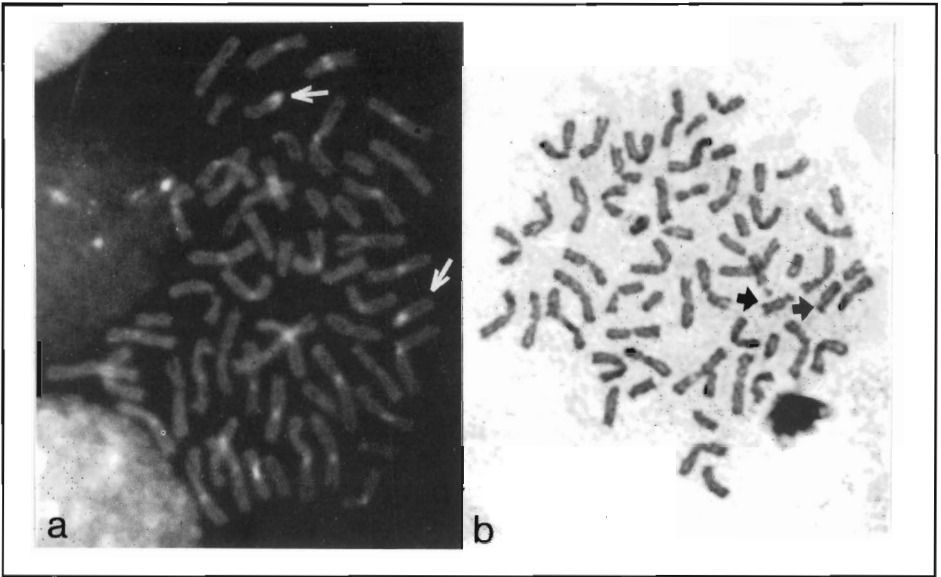


Figure 3 - Chromosome metaphase plates of two diploidized embryos. a: Hoechst 33258/AMD fluorescent stain; b: Giemsa 4% stain. The arrows show X chromosomes.

and two with  $n=31$  were found in female 988 progeny. Within female 989 progeny, three embryos with  $n=28$ , nine with  $n=29$  and four with  $n=30$  were counted.

## DISCUSSION

Detection of the Hertwig effect (Figure 1) allows us to assess the optimum irradiation dosage for gynogenesis induction. Increased embryo survival at low UV irradiation dosage may be explained by whole DNA sperm inactivation (Ijiri and Egami, 1980). Sperm could have been also affected in fertilizing capacity after 12 min of UV treatment due to excessive irradiation and/or by the long period of mechanical stirring of the semen sample.

Eggs fertilized with irradiated sperm showed a high rate of haploidy (over 91%). Interestingly, we did not observe chromosome fragments in haploid metaphases of embryos, as Chourrout (1984) reported. Our results are in agreement with a whole DNA sperm inactivation hypothesis, which means no male genetic contribution to embryo development. Few hatched embryos were seen in haploid groups from 1989 experiments (4.2%). We assume that their survival was related to a diploid condition. There is some evidence that such embryos arise by spontaneous retention of the second polocyte

(Stanley, 1976; Nagy *et al.*, 1978; Chourrout *et al.*, 1980; Streisinger *et al.*, 1981; Purdom *et al.*, 1985; Kaastrup and Horlyck, 1987). Our results suggest that hatched embryos obtained in the haploid groups (Table I) could arise by a similar process. This is supported by the high rate of haploidy and lower frequency of surviving embryos in our experiment. Thorgaard and Gall (1979) found non-induced triploids in a rainbow trout family. It is possible that their occurrence could be related to the spontaneous retention of the second polocyte.

The mortality of haploid embryos could be explained by their genetic constitution and the expression of lethal recessive genes. The presence of only one nucleolar organizer region in these embryos does not involve less RNAr availability (Strelkov *et al.*, 1976). The maternal lethal genes are in the haploid genome of the embryos. Differential lethal gene expression throughout rainbow trout ontogeny can explain the mortality in advanced developmental stages.

The brood stock is from the Aguas Claras fishfarm. This population shows an XX/XY sex chromosome determination system (Veloso *et al.*, 1990). Thus, the identification of two X chromosomes in diploidized embryos suggests their gynogenetic origin. The X chromosomes were distinguished unequivocally from the rest of the chromosome complement by a pericentromeric brilliant fluorescent band with Hoechst 33258/AMD stain. Hoechst 33258 stain demonstrates AT base composition in DNA (Schweizer, 1981). Our results, therefore indicate AT richness of the constitutive heterochromatin of these chromosomes (Veloso *et al.*, 1990). Fluorescent stain is more accurate than morphological identification of the sex pair because of the larger chromatin fold in some plates and the morphological similarity with the autosomes. Nevertheless, it is important to consider that males can contribute an X chromosome carrying gametes with 50% probability.

The hatching survival rate of gynogenetic diploids (37%) (Tables I and II) is within the range reported for heat shock treatments. (Chourrout, 1982; Refstie, 1983; Kaastrup and Horlyck, 1987; Lou and Purdom, 1984). Since the use of hydrostatic pressure for egg diploidization gives better survival rates at hatching stage than heat shock (Chourrout, 1984; Lou and Purdom, 1984), the lower survival of the diploidized embryos can be partially explained as the effect of the heat shock. Also, the genetic constitution of the diploidized embryos must be considered. Theoretically, the retention of the second polocyte should lead to a homozygosity gain and to the expression of lethal recessive genes hidden in heterozygous condition. Some diploidized progenies showed a high survival rate at hatching stage (females A and 989), suggesting the participation of individual factors, like egg quality and age of spawning females.

The higher mean fertilization rates in the diploidized groups of the 1990 experiments over 1989 experiments may be explained by the use of a lower number of eggs to be inseminated. This result suggests that the sperm volume vs. egg number

relationship is important to obtain high fertilization rates. Our results show that when 700 eggs (7.4  $\mu\text{l}/\text{egg}$ ) are used at least 40% fertilization may be obtained. Even with a 100% fertilization rate, a survival rate of diploidized embryos similar to controls will be not achieved because of the use of temperature in egg manipulation and because of the genetic constitution of the eggs.

Chromosome counting of gynogenetic progenies is an important part of our analysis. In this paper, we stress the use of haploid gynogenetic individuals in rainbow trout chromosome studies. The lower chromosome number (around 30) and distinct chromosome morphology makes it easy to establish the NF, in comparison with diploid individuals.

Haploid chromosome sets in embryos represent the gamete segregation of the female parent. Our results indicate that haploid progenies of  $2n=60$  females range between 28 and 31 chromosomes, but with a conservative NF52. This chromosome number variability can be explained as a result of arm rearrangements in the female germ line. Chromosome number polymorphism reported in the Aguas Claras population ranged between  $2n=58$  and  $2n=61$  (Veloso *et al.*, 1990). It is likely that during female gamete segregation, cells with different chromosome numbers were produced, which possibly can explain the detected polymorphism.

Experimental gynogenesis has a potential use in fish farms, as a method to control sex ratios. Salmonid culture can also be improved by gynogenesis, since female sexual maturity occurs later than male. With the production of "all female" progenies, it is possible to avoid changes in meat quality which parallel sexual maturity. It is also possible to homogenize and improve the size of commercial fishes.

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## RESUMO

Proles experimentais de *Oncorhynchus mykiss* foram identificadas através de análises cariotípicas. Embriões haplóides foram obtidos fertilizando ovos com esperma irradiado (UV) por 10 min. Embriões diplóides ginogenéticos obtidos por choque de temperatura, mostraram dois cromossomos X, identificados com corante fluorescente Hoechst 33258/AMD. A sobrevivência média dos embriões diploidizados foi 37% ao eclodir. A variação no número de cromossomos nos embriões haplóides sugere rearranjos de cromossomos "Robertsonianos" na linhagem de células maternas germinativas, o que pode explicar polimorfismos no número de cromossomos, previamente relatados para trutas arco-íris.

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