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Techniques of chromosome manipulation in rainbow trout: a new evaluation with karyology

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Summary. Experiments, supported by extensive karyology, were carried out to evaluate the different techniques used for chromosome manipulation in rainbow trout. Eggs, when subjected to early heat shocks, changed from haploidy to diploidy and from diploidy to triploidy. In this respect heat shocks differ from pressure shocks which induce gradual transitions between successive ploidy levels. Sperm treatment with dimethylsulphate yields haploid embryos containing residual sperm chromatin fragments, in contrast to treatment with ultraviolet rays.

Key words: Gynogenesis – Ploidy – Salmonids – Heat shocks

Introduction

Most of the research in fish chromosome manipulation has concentrated on methods of efficiently inducing triploidy and gynogenesis. Triploidy has been of interest because triploid fish were supposed to be sterile. Gynogenesis results in the production of diploid individuals with both chromosome sets from the female parent. It may be valuable as a method for rapid inbreeding and, also, in the production of all-female populations.

Triploid females are entirely sterile so the production of large trout combines both approaches and involves the triploidization of monosex female offspring (Lincoln and Scott 1983; Chevassus et al. 1984), themselves derived possibly from sex-inverted gynogenetic males.

Gynogenetic developments are triggered by sperm inactivated either by gamma-rays (Purdom 1969; Chourrout et al. 1980) or ultraviolet rays (Chourrout 1982; Onozato and Yamaha 1983; Thorgaard et al. 1983). The choice between these agents is governed by two factors: gamma-rays are very penetrating and permit the treatment of large milt amounts while UV-rays are effective only on thin layers of diluted milt. On the other hand, UV rays are readily available in contrast to gamma-rays which are often unavailable near a laboratory.

The availability of viable fertile gynogenetic fish depends on their diploidization, performed on a large scale either by heat shocks (Chourrout 1980; Thorgaard et al. 1981; Chourrout and Quillet 1982) or by pressure shocks (Benfey and Sutterlin 1984; Chourrout 1984; Lou and Purdom 1984). These two agents suppress the second meiotic disjunction in the egg, and are equally effective in trout. In practice heat shocks are preferred because they permit the treatment of very large egg numbers while pressure chambers can accomodate only several hundreds or a thousand eggs. The numerous triploids are obtained by the same shocks applied shortly after fertilization with normal sperm.

It becomes evident, therefore, that the choice of technique is related largely to practical considerations. Consequently, it is worthwhile comparing in more detail the action of the two agents of sperm inactivation and, also, the action of the two agents inhibiting the second division of meiosis in the egg.

Pressure shocks of a suboptimal intensity result in high numbers of aneuploids, indicating that transition from diploidy to triploidy by pressure is a gradual process (Chourrout 1984). This had been suspected from observations of reduced survival following shocks of medium durations. Such a study has not yet been performed with heat shocks.

We also showed that gamma-irradiation of sperm, although giving all haploid progenies over a wide range of doses, results in residual supernumerary fragments of paternal origin in most cells. In a previous study (Chourrout 1982), no fragments were detected in haploids produced by UV-rays, but the karyology described at the blastula stage without prior colchicine treatment was not entirely reliable.

An attractive alternative might be the use of chemical mutagens to inactivate the sperm since, like UV-rays, they can be used everywhere and, as with gamma-rays, they permit the treatment of large sperm volumes. Dimethylsulphate has already been tested on salmonids and provided haploids (Tsoi 1969) but it is not clear whether supernumerary fragments were present in their cells.

In the present report, the process of haplodiploidization and diplotriploidization by heat shocks was ex-

amined. Sperm inactivation by dimethylsulphate was optimized and compared to ultraviolet ray treatment.

Materials and methods

Breeding animals, fertilization and incubation

Sperm and ova were collected from animals grown in the fish farm of Gournay sur Aronde. Dry fertilization involved batches of 150 to 500 ova mixed with 0.1 to 1 cc of milt and 20 cc of dilutent 532 (Billard 1974) and was followed 10 min later by incubation in freshwater at 10 °C. Early examination of eggs (day 10) using Stockard's fixative permitted counts of gastrulated embryos, abortions and unfertilized eggs; later counts at the eyed stage (day 20) did not require the sacrifice of embryos which were studied for their ability to hatch (day 40).

Chromosome preparations

The karyology concerned mostly 14 to 22 day-old embryos, and also one batch of hatched larvae. It involved colchicine treatment (0.02% overnight), hypotonic treatment (0.8% trisodium citrate for 20 min) after dissection of the embryo in 0.8% NaCl, and successive fixations in ethanol 3 : acetic acid 1. Preparations of slides were performed according to Chourrout (1984). Because of the Robertsonian polymorphism found in domestic strains of rainbow trout, the arm number (104 in diploids) was considered for ploidy evaluation.

Heat shocks and UV-sperm-irradiation

In order to suppress the second division of meiosis, we used 26 °C shocks starting after 25 min of incubation and lasting 2 to 20 min. UV-sperm-irradiation lasted from 30 s to 10 min and was performed according to Chourrout (1982).

Dimethylsulphate sperm treatment

After preliminary experiments, we concentrated on the use of a 18.75 mM DMS solution prepared by the direct dilution of a 5 M solution in ethanol into the fertilization diluent with 0.2% KCl. 0.5 cc of sperm was added to 2 cc of this solution and treated 10 to 150 min at 10 °C. In one experiment, the treated sperm was centrifuged 10 min at 1,500 g (4 °C), 10 min more being spent in resuspending all the samples in the diluent without DMS.

Results

1 Ultraviolet irradiation

Survival (Fig. 1). The eggs were divided into 10 batches, 9 being fertilized with sperm irradiated for different durations and 1 by non-irradiated sperm. The rate of embryos was stable over most of the range tested but slightly reduced for durations 30 s, 1 min and 1 min, 30 s (Hertwig effect) which provided a minority of retarded gastrulae. High survivals for longer treatments were maintained until the hatching stage, beyond which half of the embryos could not develop. The

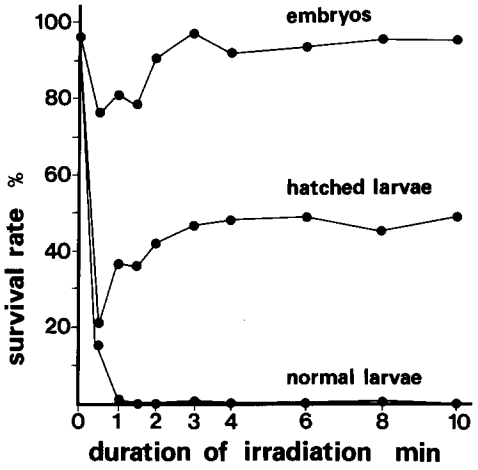

Fig. 1. Yield of eyed embryos, hatched larvae and normal larvae for increasing durations of UV irradiation. In this particular experiment, about half of the haploids hatched

hatched larvae were, however, abnormal and less than 1% of them proved viable in each batch.

In a repetition of this experiment, the reduced survival at the eyed stage was recorded at the same durations, but was slightly more pronounced (60%). For durations longer than 2 min, many embryos died before the hatching stage.

Karyology (Table 1). Extensive examinations at the eyed stage indicated that sperm inactivation was accomplished at durations of 2 min and more when only haploids resulted. In addition, 18 larvae resulting after 4 min of irradiation were haploids. Diploids and hypodiploids were recorded in the batches fertilized with sperm given the shortest irradiation.

Twenty-five somatic metaphases were carefully examined in each of the 63 haploid embryos found in the different samples analyzed in order to detect and to count chromosome fragments. The data grouped for each batch indicate that such fragments were abundant at the shortest durations but neglectable from 3 min onwards. The analysis of 101 haploids (500 cells) produced by irradiation lasting 3 min or more in other experiments also did not reveal any fragments in addition to the haploid chromosome set.

Irradiated sperm amount. In the experiments described above, all the batches were inseminated by one irradiated sample (2.5 cc of diluted milt). Here, batches of 200 eggs received decreasing amounts of diluted milt, either irradiated for 4 min or not irradiated (2.5 to 0.0025 cc). The fertilization rate with non-irradiated milt did not vary for the complete range of sperm volume tested; with irradiated sperm, it decreased significantly when the volume used was under 0.25 cc.

Table 1. Karyological examinations of embryos resulting from UV irradiated sperm

UV duration	Total embryos	Haploids	Haploids with fragments	Total cells	No. of cells with fragments							Average fragment no. per cell
					0 fgt	1 fgt	2 fgts	3 fgts	4 fgts	5 fgts	6 fgts	
30 sec	15	4	2	100	80	9	7	3	0	0	1	0.38
1 min	15	14	10	351	282	46	12	4	4	1	2	0.33
2 min	15	15	3	375	368	7	0	0	0	0	0	0.02
3 min	15	15	0	375	375	0	0	0	0	0	0	0.00
4 min	15	15	1	375	374	1	0	0	0	0	0	0.00

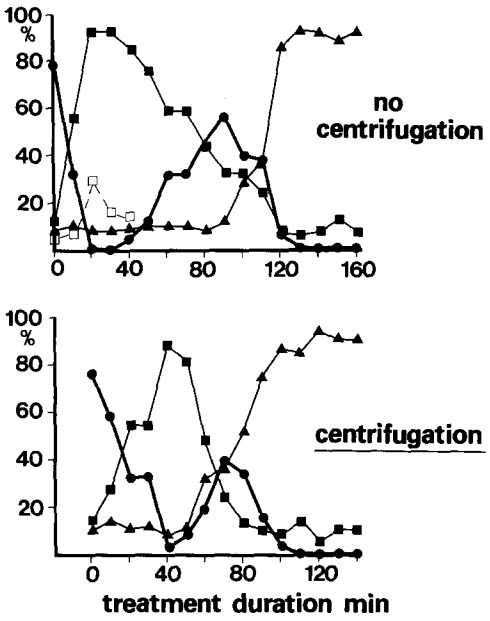

Fig. 2. Hertwig effect obtained by increasing durations of 15 mM DMS treatment in experiment 2: (● embryos continuing their development at Day 17; ■ abortions before Day 17; □ abortions before Day 8; ▲ unfertilized eggs); the position of the abcissa in the lower graph is due to the 20 min spent in centrifugation and resuspension

2 Sperm inactivation by dimethylsulphate

Survival. Two similar experiments (1 and 2) permitted the test of increasing durations of DMS treatment, and the induction of a very pronounced Hertwig effect (Fig. 2 corresponds to experiment 2): at day 17, the rate of developed embryos dropped down to zero for the 20–30 min treatments and gradually increased to an optimum of 35% in experiment 1 (90–110 min) and of 55% in experiment 2 (80–100 min). Longer durations (120 min and more) again reduced drastically the rate of embryo development.

The loss of embryos at short durations was caused by a peak of abortions detectable from the gastrula stage which dramatically increased one week later, affecting very short and deeply abnormal gastrulae.

Longer durations were associated with an improvement of embryo morphology, most but not all the gastrulae apparently being normal above 80 min. The relatively low value of the optima at day 17 was caused by abortions continuing even later on. At that stage, all the survivors were abnormal, resembling haploids produced by gamma-rays, and they all died before hatching.

The progressive loss of embryos consecutive to treatments longer than the optimal ones was caused mainly by non-fertilization but also by abortions occurring after the eyed stage. In all the batches, the hatching rate was close to zero.

Half of the batches of experiment 2 were inseminated by sperm treated in the same way but centrifuged prior to insemination in order to remove the mutagen. In this case, an Hertwig effect was again recorded, but the result differed from experiment 1: survival at day 17 was slightly better for the shortest treatments but decreases in the fertilization rate appeared for durations shorter than when the sperm had not been centrifuged; the peak of survivors at day 17 was consequently much narrower and lower.

Karyology. Batches of embryos from experiments 1 and 2, inseminated by sperm treated for the longest durations (around the optima at day 17) were examined for chromosome counts and presence of supernumerary fragments. The results in Table 2 can be summarized as follows: 92 embryos were all haploids, and a total of 485 fragments were detected in 2,481 metaphases scored (Fig. 3). The average number of fragments was significantly higher at treatments shorter than the optima of survival, and much lower only at durations leading to low rates of fertilization.

3 Test of suboptimal heat shocks

In a preliminary experiment, eggs of one female fertilized by normal sperm were subjected to heat shocks lasting 2, 4, 6, 8, 10, 15 and 20 min. The survival rate at the eyed and hatching stages were over 90% in all cases. The karyology of 10 embryos in each batch revealed only diploids at durations 2 to 10 min, and only

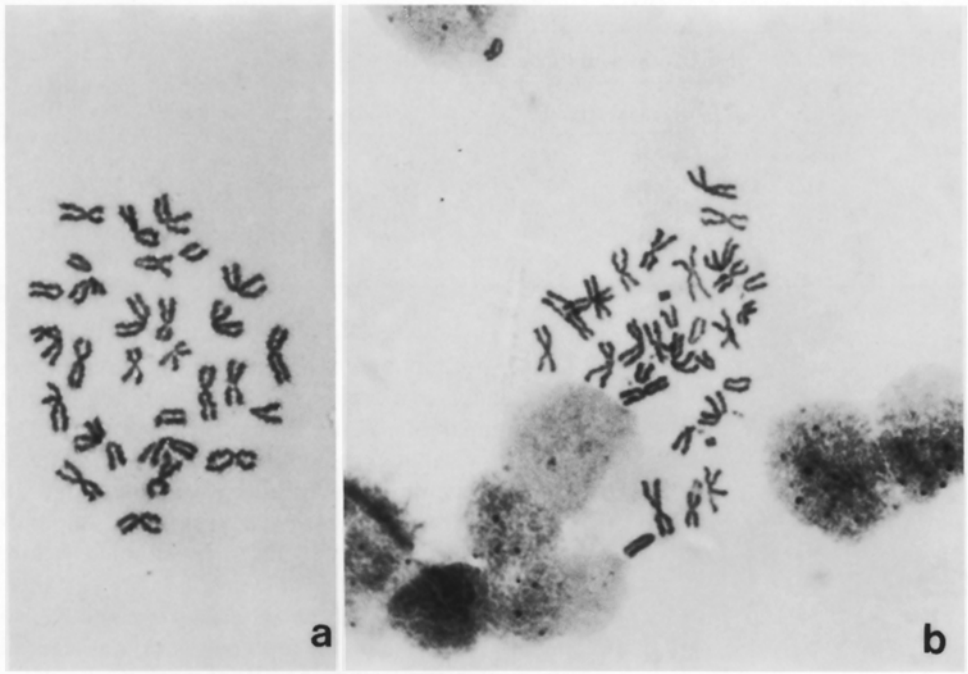

Fig. 3. Haploid metaphase without supernumerary fragments (**a**); Haploid metaphase with supernumerary framgents (**b**)

Table 2. Karyological examinations of embryos resulting from two experiments of DMS sperm treatment

DMS duration	Total embryos	Haploids	Haploids with fragments	Total cells	No. of cells with fragments							Average fragment no. per cell
					0 fgt	1 fgt	2 fgts	3 fgts	4 fgts	5 fgts	More	
90 min	16	16	15	483	384	36	31	12	13	6	1	0.45
120 min	16	16	12	488	445	35	11	6	0	0	0	0.16
80 min	15	15	8	375	337	10	13	10	3	0	2	0.24
90 min	15	15	8	375	347	10	9	4	3	1	1	0.19
100 min	15	15	7	375	364	9	1	1	0	0	0	0.04
110 min	15	15	3	375	371	4	0	0	0	0	0	0.01

triploids at 15 and 20 min, indicating that transition from diploidy to triploidy happened in the interval 10–15 min.

A similar experiment examined in detail the process of diplotriploidization. The haplodiploidization was also investigated by adding a set of batches inseminated by UV-irradiated sperm:

In both series, the survival at the eyed stage in all the batches was between 90 and 100% of the survivals in the non-heat shocked controls (Fig. 4); most of the embryos following fertilization with normal sperm became viable larvae; in contrast, the hatching rate following irradiation was not significant at durations shorter than 8 min, 30 s, but gradually increased with longer treatments.

The karyology of embryos revealed that the increase in shock durations induced a progressive replacement of haploids by diploids, and of diploids by triploids

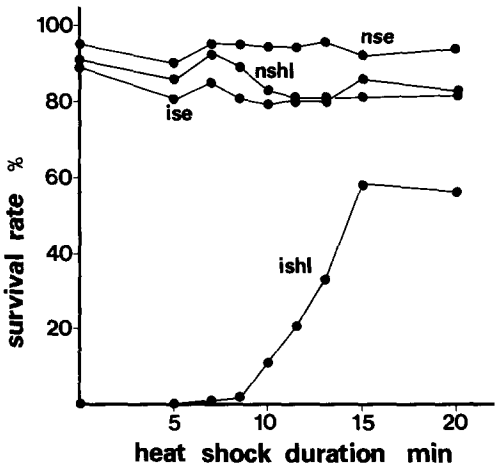

Fig. 4. Yield in embryos (e) and hatched larvae (hl) for increasing durations of heat shocks after fertilization by normal sperm (ns) and irradiated sperm (is)

Table 3. Karyological examinations of embryos resulting from increasing durations of heat shocks, after fertilization by normal or irradiated sperm

Heat shock duration		Normal sperm					Irradiated sperm				
		Collected and examined	2n	3n	*n*	Mosaic or aneuploid	Collected	Examined	*n*	2n	Aneuploid
0 min	Normal	14	13	–	–	1	0	0	–	–	–
	Abnormal	1	–	–	1	–	50	15	15	–	–
8 min 30	Normal	14	14	–	–	–	0	0	–	–	–
	Abnormal	1	–	–	1	–	50	14	14	–	–
10 min	Normal	14	10	4	–	–	8	5	–	5	–
	Abnormal	0	–	–	–	–	42	15	15	–	–
11 min 30	Normal	13	9	4	–	–	13	8	–	8	–
	Abnormal	0	–	–	–	–	37	11	11	–	–
13 min	Normal	13	3	10	–	–	28	12	–	12	–
	Abnormal	1	–	–	1	–	22	7	5	–	2
15 min	Normal	14	–	14	–	–	47	13	–	13	–
	Abnormal	1	–	–	–	1	3	2	–	–	2
20 min	Normal	13	–	13	–	–	50	15	–	15	–
	Abnormal	2	–	1	–	1	0	0	–	–	–
Total	Normal	95	49	45	–	1	146	53	–	53	–
	Abnormal	6	–	1	3	2	204	64	60	–	4

when normal sperm had been used. Occasional mosaics and aneuploids were found, but in non significant proportions (Table 3).

Discussion and conclusions

1 Ultraviolet irradiation

Our data favour the choice of ultraviolet rays for sperm inactivation. Haploids are consistently obtained from irradiations lasting 2 min. The survival of haploids is stable up to hatching and their morphology is better than those produced by gamma-rays. Moreover, a large proportion of them occasionally survive after hatching, in contrast to our observations with gamma-rays and dimethylsulphate. This may be connected with the absence of supernumerary fragments in their cells.

The fertilization rate is not impaired by durations of treatment much longer than those necessary for haploid production; however, this depends on the use of sperm volumes much larger than those required for lower durations. The consequence in practice is that the utilizable range of irradiation durations is in fact narrower than what appears in our experiments. Calculations based on the testing of various sperm concentrations after a 4 min irradiation show that one sample (0.5 cc of pure sperm) permits the fertilization of one female (3,000 eggs) and probably more if the treatment lasts 3 min.

2 Dimethylsulphate treatment

The dimethylsulphate led to a typical Hertwig effect with haploid production at the longest durations tested. The survival reduction observed for the lowest treatments is analogous with that resulting from gamma irradiation, but mortality attains 100% more than one week later. However, the extreme morphological abnormalities of the resulting embryos indicate a development arrested before the completion of gastrulation and that diploids and haploids cannot be present in the same batches; good homogeneity of the treatment for the different spermatozoas is therefore probably achieved with dimethylsulphate, as with gamma-rays.

Optima of haploid production results from 90–100 min of treatment at 15 mM. This contrasts with the data of Tsoi (1969; 120 min at 3 mM) which were obtained at 4 °C; however, we also tested various concentrations at this temperature in preliminary attempts, and got the optima in the range 10–15 mM.

There are, altogether, several reasons against the use of this mutagen for performing the sperm inactivation:

a) The yield in surviving haploids after two weeks is lower than in the case of ultraviolet irradiation, the difference being mainly caused by abortions of highly abnormal embryos. Even later on, the survival is not stable and the morphology of the survivors is more aberrant than in the case of UV-induced haploids.

b) Supernumerary chromosome fragments are detected in the haploid embryos obtained after optimal treat-

ments. Their frequency is, however, lower than with gamma irradiation (Chourrout 1984) but is appreciably decreased only by durations associated with a loss of fertilization capacity, although high sperm concentrations are used. In addition, embryos having aborted before the analysis might have contained more chromosome fragments.

c) Some of the mortalities occurring for all the durations tested might have been caused by the brief contact of the mutagen with the ova at the time of insemination. This would explain why the mortalities for low durations were delayed when the treated sperm had been washed by centrifugation before fertilization. Unfortunately, no improvement in the yield of haploids was observed for the high durations by sperm washing because this procedure impairs the sperm fertilization capacity.

3 Heat shocks and pressure shocks

The direct replacement of diploids by triploids and of haploids by diploids observed here by heat shocks differs markedly from the gradual transition from diploidy to triploidy through aneuploidy previously reported with pressure shocks. It is difficult to generalize this distinction to all pressure and all heat treatments but our results may indicate that these two agents alter different cell mechanisms, even though both suppress the second division of meiosis. Heat shocks either permit or inhibit the entire disjunction and therefore may interfere with factors involved in the migration of all chromosomes, for example the centrioles. Suboptimal pressure treatments prevent only part of the meiotic disjunction, possibly by impairing processes involved in the migration of individual chromosomes, for example the spindle fiber formation.

In any case, two practical conclusions can be drawn: if the shocks used for triploidy are suboptimal, the contaminants will be mainly diploids in the case of heat shocks, and aneuploids in the case of pressure. Diploids which are fertile, will be undesirable in the case of exotic fishes whose release demands a strict control of reproduction. In aquaculture for consumption purpose, where eventual viable aneuploids may have low performances and inviable ones will cause a waste of eggs and larvae, diploid contaminants due to heat shocks might be preferred.

Another important consequence of using heat shocks as compared with pressure is related to the use of very inaccurate methods of ploidy estimation, like the measurement of nuclei size, by some workers. Such techniques certainly permit the distinction between diploids and triploids, but will not detect the presence of hypotriploids. Our results suggest that it is less dangerous to use them for setting heat shocks than pressure shocks.

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