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Production of all-triploids, all-tetraploids, and
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PRESSURE-INDUCED RETENTION OF SECOND POLAR BODY AND SUPPRESSION OF FIRST CLEAVAGE IN RAINBOW TROUT: PRODUCTION OF ALL-TRIPLOIDS, ALL-TETRAPLOIDS, AND HETEROZYGOUS AND HOMOZYGOUS DIPLOID GYNOGENETICS

DANIEL CHOURROUT

Laboratoire de Physiologie des Poissons, CNRZ-INRA, 78350 Jouy-en-Josas (France)

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ABSTRACT

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Retention of the second polar body in rainbow trout eggs was induced by 7000 psi early pressure shocks (applied 40 min after insemination) lasting 4 min. This resulted in all-triploid progenies after fertilization with functional sperm, and in high yields of heterozygous diploid gynogenetic fry after insemination with gamma-irradiated sperm.

All-tetraploid progenies were produced by 7000 psi late pressure shocks (applied 5 h 50 min after insemination) lasting 4 min, if normal sperm had been used. With eggs activated by gamma-irradiated sperm, these shocks resulted in diploid gynogenetic embryos that were supposed to be homozygous at all their loci because obviously they result from the fusion of two mitotic products. This fact may explain their very low post-hatching survival, but the occurrence of residual chromosome fragments of paternal origin in their cells, demonstrated by an extensive karyological investigation, could also be responsible of the poor yields; such an observation suggests that we abandon the use of gamma irradiation for sperm inactivation.

Finally, tetraploid hybrid embryos were produced with similar late pressure shocks applied after heterospecific inseminations.

INTRODUCTION

In the reproduction of most domestic birds and mammals, two diploid parents of the same species give rise to a diploid offspring after meiosis and fertilization; in such conditions the breeder has to determine which parents will be involved in the reproduction of his population.

In commercial fish, deviations from the normal reproduction pattern can be induced easily: parents of two different species (hybridization), only one parent (gynogenesis and androgenesis) or increase of chromosome number (polyploidy). These phenomena can be exploited in planning new schemes of genetic improvement for animal breeding (but already classical

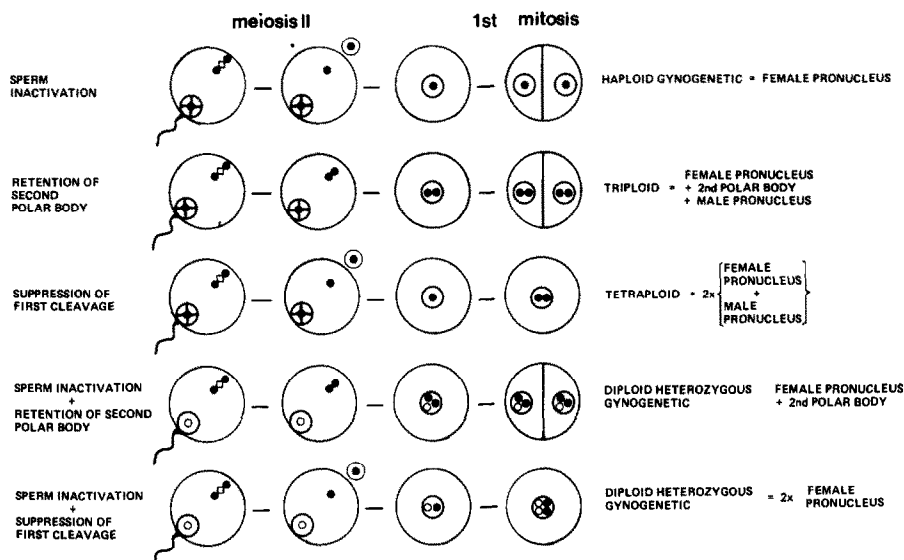


Fig. 1. Three operations are required for producing gynogenetic individuals, triploids and tetraploids in lower vertebrates: sperm inactivation, retention of second polar body and suppression of first cleavage.

in plants), such as the crossing of inbred lines (produced by gynogenesis) or the use of polyploids.

In this respect, diploid gynogenetic individuals and polyploids could be produced if three techniques were available, viz. genetic inactivation of sperm, induced retention of the second polar body, and induced suppression of first cleavage (see Fig. 1). Diploid gynogenesis requires the combination of sperm inactivation and diploidization of the maternal chromosome set. If the latter is achieved by retention of the second polar body, the resulting embryo starts from two differing terminal products of the same meiosis and so is not homozygous at all the loci (such embryos are called heterozygous diploid gynogenetics in the text). If suppression of first cleavage is used to diploidize the maternal set, the collected embryos are considered to be homozygous at all the loci because they result from the fusion of two mitotic products.

Such techniques have been applied recently in many fish species (Table I) with the object of producing inbred lines or monosex populations by gynogenesis and sterile animals by polyploidy. These investigations are based on methods first developed with amphibians (review Chourrout, 1982a).

In the case of salmonid fishes, sperm inactivation is usually performed with gamma rays, a choice discussed in this paper. Retention of the second polar body is systematically induced by treating eggs with short heat shocks soon after insemination, resulting in all-triploid progenies if functional sperm are used, and in high yields of heterozygous gynogenetics after in-

TABLE I

Authors having induced sperm inactivation, retention of the second polar body and the suppression of first cleavage in fish. Parentheses Indicate studies resulting in low yields

Sperm inactivation

Agents used	
	Ionizing rays Ultraviolet rays
Fish other than salmonids	Lasher and Rugh, 1962 Stanley, 1976
	Romashov et al., 1963 Streisinger et al., 1981
	Golovinskaja et al., 1963 Chourrout and Itskovich, 1983
	Cherfas, 1965
Salmonids	Purdum, 1969
	Purdum, 1969 Chourrout, 1982c
	Chourrout et al., 1980
	Onozato, 1982

Retention of second polar body

Agents used			
	Cold shocks	Heat shocks	Pressure shocks Colchicine
Fish other than salmonids	Swarup, 1958	Swarup, 1958	Streisinger et al., 1981
	(Cherfas, 1965)	Romashov and Belyaeva, 1965	
	Purdum, 1969	Valenti, 1975	
	Nagy et al., 1978	Chourrout and Itskovich, 1983	
	Ojima and Makino, 1978		
Salmonids	Wolters et al., 1981		
	Chourrout, 1980	Chourrout, 1980	Smith and Lemoine 1979
	Lemoine and Smith, 1980	Thorgaard et al., 1981	
	Refstie et al., 1982		

Suppression of first cleavage

Agents used		
	Cytochalasin B	Heat shocks Pressure shocks
Fish other than salmonids		Streisinger et al., 1981
Salmonids	Refstie et al., 1977	(Thorgaard et al., 1981) (Chourrout, 1982b)
	Allen and Stanley, 1979	
	Refstie, 1981	

semination with irradiated sperm. However, chromosome doubling by suppression of first cleavage has not yet been satisfactorily achieved in spite of several attempts with heat shocks and cytochalasin B. This paper summarizes our successful attempts at inducing suppression of first cleavage by means of hydrostatic pressure shocks, which have been shown to be effective for doubling haploid eggs in the fish *Brachydanio rerio* (Streisinger et al., 1981) and of both haploid and diploid eggs in the leopard frog *Rana pipiens* (Reindschmidt et al., 1979). Because of the difficulty in optimizing three parameters (pressure level, shock duration and time of application, the latter being difficult to estimate a priori), we calibrated the two parameters of shock intensity in attempts to retain the second polar body. In fact, we could test a limited number of times of application in this case, according to previous results obtained with heat shocks (Chourrout, 1980).

MATERIALS AND METHODS

Fertilization

In order to get enough eggs for large experiments, we used in most cases mixtures of ova collected from several females (at least four). In one case, yields of gynogenetic individuals provided by six females were compared, fertilization was carried out by mixing 500 eggs with 0.05 to 0.50 ml of pure milt and 25 ml of a saline-buffered diluent (Billard, 1974); 10 min later, the eggs were transferred into a thermoregulated (9.4°C) recirculating system for incubation (zero development time).

Sperm irradiation

In order to induce gynogenesis, pure sperm was collected in tubes and submitted to Cobalt-60 irradiation (135 krad except in one case where effects of 100 and 135 krad were compared, see Results, section 3).

Pressure treatments

In order to induce retention of the second polar body and suppression of first cleavage, we tested early pressure shocks (EP shocks) applied in the first hour of development, and late pressure shocks (LP shocks) applied between 4 and 8 h after insemination. Each treatment consisted in placing 250 to 500 eggs in a stainless steel cylinder full of water at 9.4 °C and closed by a piston; the pressure level was raised with an external hydraulic press and reached 6000 or 7000 psi after 3 s; decompression was instantaneous at the end of the treatment which lasted 2 to 12 min; the machine used here belongs to the Laboratory of Animal Biology of Toulouse University and had already been used in the induction of second

polar body retention in the newt *Pleurodeles waltlii* (Jaylet and Ferrier, 1978; Ferrier and Jaylet, 1978).

Survival rates

Counts of survivors were made in the different batches at tail bud stage (eyed stage; day 18 to day 26), after hatching (day 35 to day 40) and, in many cases, at the start of feeding (day 55 to day 60).

In the experiments on gynogenesis, the hatching rate was the first criterion for estimating the success of induced diploidization, since all the haploid embryos die before this stage. It must, in any case, be confirmed by karyological examinations.

Karyology

In attempts at polyploidization and at sperm inactivation, the effectiveness of treatments was ascertained by chromosome counts on embryos or on hatched fry.

Embryos. On day 17, the eggs were placed for 6 h in a 0.02% colchicine solution, and then dissected in 0.7% NaCl for removal of the embryo. The tail was cut and subjected to a hypotonic treatment (15 min in distilled water), fixed in ethanol 3/acetic acid 1 for 3 min and rinsed in distilled water; the tail epithelium was gently dissociated in a drop of 50% acetic acid.

Hatched fry. On day 45, the fry were kept swimming for 15 h in a 0.01% colchicine solution. After killing, the gills were removed, put in distilled water for 45 min, fixed for 3 min and finally dissociated.

In both cases, the cellular suspension was dropped onto a warmed (50 °C) microscopic slide, air-dried, and stained in 4% Giemsa for 10 min. Each investigation concerned at least 15 individuals in a batch.

RESULTS

(1) Retention of second polar body induced by early pressure shocks

A. Fertilization with irradiated sperm

Initial experiments showed that 6000 psi EP shocks lasting 8 min resulted in high hatching rates, whereas all the embryos died in the control (irradiated sperm; no pressure shock). Fig. 2a shows a maximum survival with shocks applied at 20 and 40 min after insemination for hatched fry and at 40 min for fry at the start of feeding.

At 7000 psi, EP shocks applied 40 min after insemination gave the best results when shock duration was 3.5–5 min (Fig. 2b).

Karyological investigations of progenies revealed only diploid complements, whereas all the analysed embryos of the control proved to be haploid.

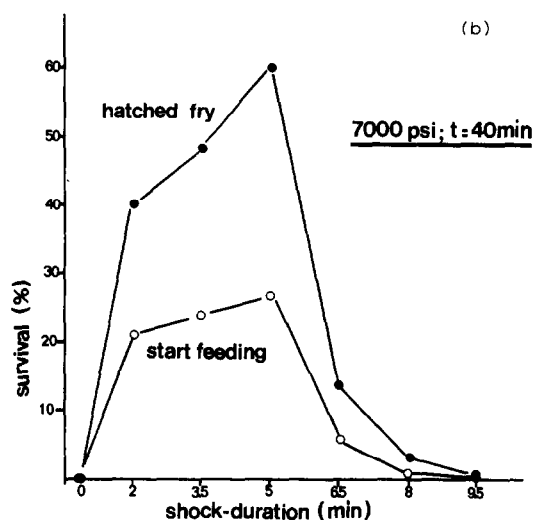
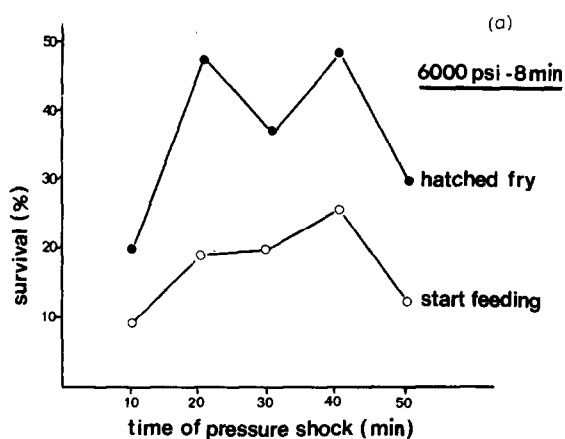


Fig. 2. Percentages of viable gynogenetic individuals obtained from eggs treated with various EP shocks (applied after fertilization with irradiated sperm: (a) optimization of preshock duration with 6000 psi shocks; (b) optimization of shock duration at 7000 psi for shocks applied 40 min after insemination.

Table II shows that yields of viable gynogenetic individuals at hatching time and at the feeding stage varied appreciably according to the female used.

B. Fertilization with functional sperm

Treatments with 6000 psi applied 10 min after fertilization with untreated sperm, and lasting 2 to 12 min, provided the following results:

(a) The embryonic survival rate was not appreciably decreased by shocks lasting up to 8 min. Nevertheless, most of the embryos from the 4-min treatment were abnormal and died without hatching, whereas most of

TABLE II

Percentages of treated eggs giving viable hatched fry (H) and feeding fry (F) for six females subjected to gynogenesis with retention of the second polar body (7000 psi shocks lasting 4 min applied 40 min after insemination with irradiated sperm)

		Females					
		1	2	3	4	5	6
Diploid control	H:	100	98	92	86	99	58
	F:	96	88	92	76	95	55
Gynogenetic individuals	H:	43	82	79	36	73	18
	F:	37	61	78	24	73	17

TABLE III

Karyological results provided by the examination of embryos after 6000 psi shocks lasting 2 to 8 min applied 10 min after fertilization with normal sperm

Shock duration (min):		0	2	4	6	8
Abnormal embryos	analysed	0	8	8	8	0
	diploids	—	3	2	2	—
	triploids	—	1	0	0	—
	aneuploids	—	4	6	6	—
Normal embryos	analysed	10	8	6	8	15
	diploids	10	8	1	3	0
	triploids	0	0	5	5	15
	aneuploids	0	0	0	0	0

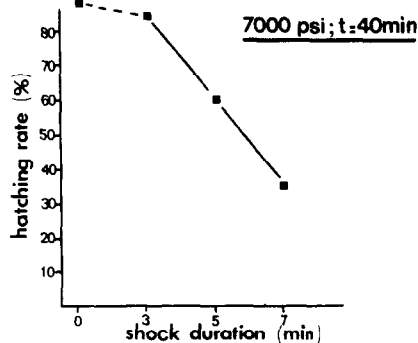
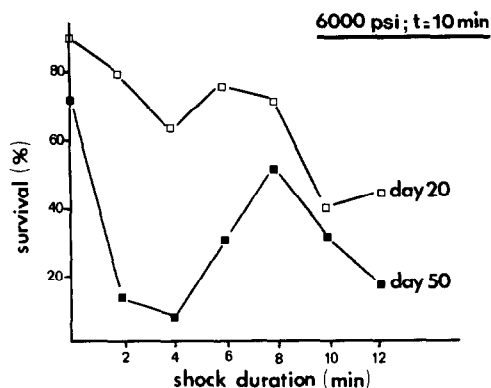


Fig. 3. Survival at tail bud stage (day 20) and after hatching (day 50) following 6000 EP shocks applied 10 min after fertilization with normal sperm (percentage of treated eggs).

Fig. 4. Hatching rates following 7000 psi shocks applied 40 min after fertilization with normal sperm. All the fry analysed were triploid.

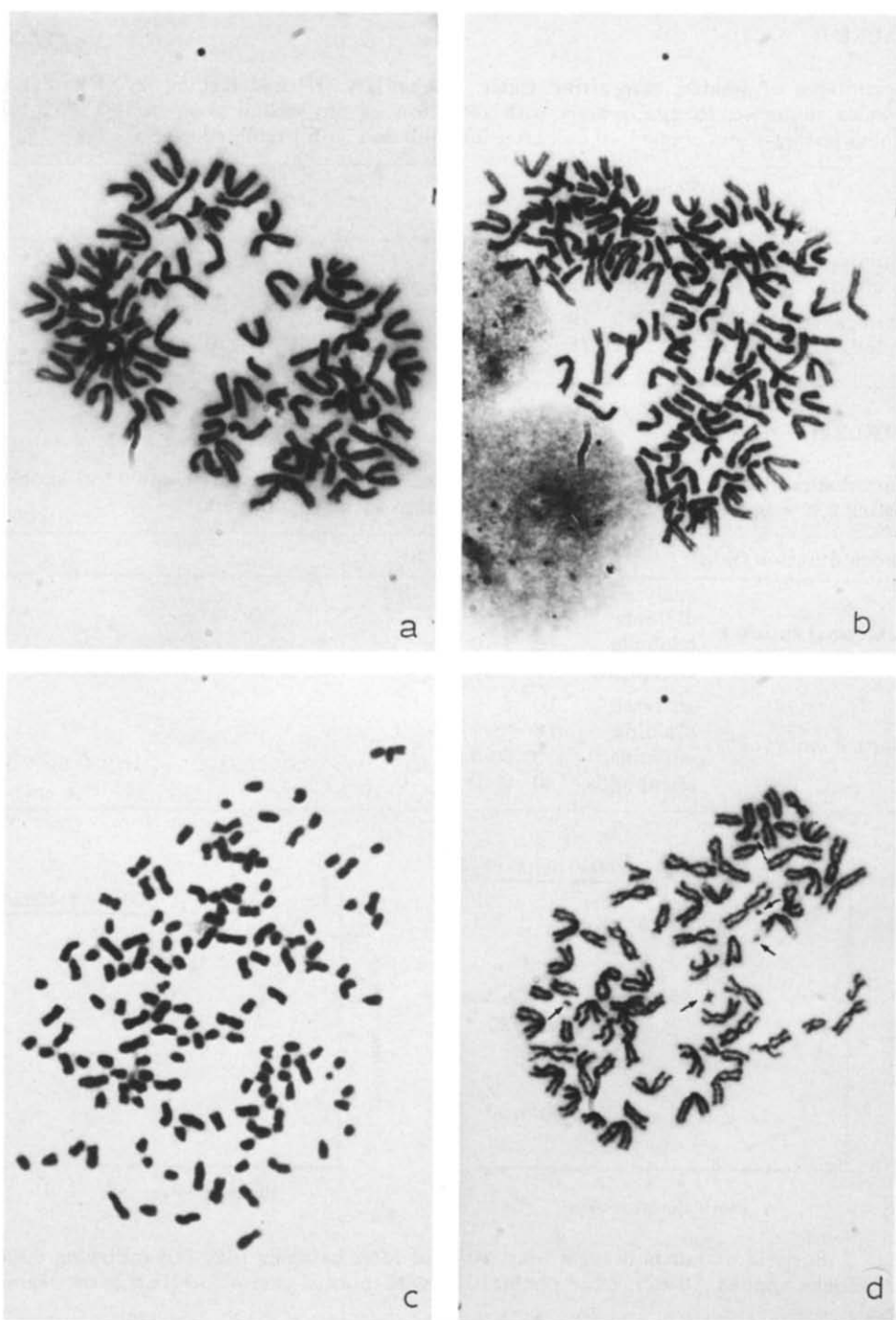


Fig. 5. Metaphases obtained on embryos produced by EP shocks (a: triploid) and by LP shocks (b: tetraploid; c: allotetraploid "female rainbow trout \times male brown trout"; d: homozygous diploid gynogenetic individuals with residues of paternal chromatin — arrows).

the embryos from the 8-min shock were normal and survived after hatching (Fig. 3).

(b) These observations were supported by the karyological data (Table III): in the 4- and 6-min batches, most of the abnormal embryos analysed were aneuploids (hyperdiploids and hypotriploids), and most of the normal ones were triploid; in the 8-min batch, all the embryos analysed were triploid (Fig. 5a).

Treatments with 7000 psi EP shocks (the optimum found in the experiments on gynogenesis) resulted here in all-triploid offspring, with satisfactory hatching rates (Fig. 4).

(2) Suppression of first cleavage induced by late pressure shocks

A. Fertilization with functional rainbow trout sperm

Treatments with various LP shocks provided the following results, illustrated by Figs. 6 and 7:

(a) Treatments applied earlier than 5 h and later than 7 h after fertilization systematically resulted in rapid abortions.

(b) High survival rates were counted at tail bud stage for shocks applied 5 h 20 min and 5 h 50 min after insemination and lasting 4 and 5.5 min. This procedure, when repeated, gave lower but satisfactory survival rates at the same stage, but the same peak of survival between 5 and 7 h was always registered.

(c) All the embryos analysed were tetraploid (Fig. 5b).

(d) Fig. 7 shows that significant mortalities occurred until the feeding stage; they consisted mostly in an elimination of abnormal fry, the survivors at this time being almost all normal in appearance and proving to be all-tetraploid by a new karyological investigation.

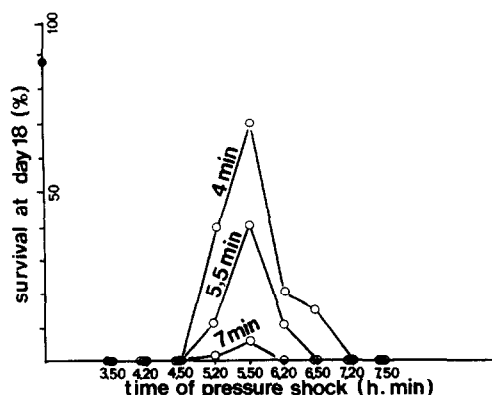


Fig. 6. Survival at tail bud stage (percentage of treated eggs) after 7000 psi LP shocks lasting 4, 5.5 and 7 min, and applied between 3 h 50 min and 7 h 20 min after fertilization with normal sperm.

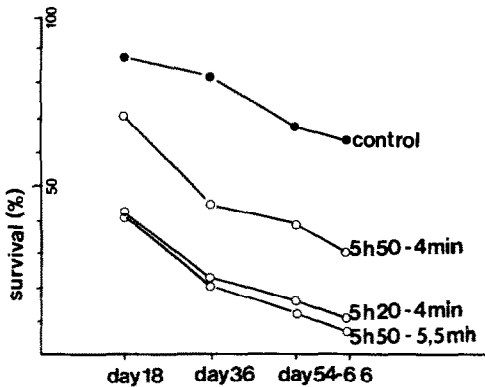


Fig. 7. Survival until the start of feeding (percentage of treated eggs) of four batches subjected to LP shocks after insemination with normal sperm.

B. Fertilization with functional heterologous sperm

The optimal LP shock, applied 5 h 50 min after fertilization with brown trout and coho salmon sperm, respectively, provided 12% and 19% survivors at the tail bud stage. This preliminary experiment was stopped before hatching but all the embryos analysed proved to be allotetraploid (Fig. 5c).

C. Fertilization with irradiated rainbow trout sperm

Various LP shocks were tested after insemination with inactivated sperm and some of them, lasting 3 min, appreciably increased the hatching rate,

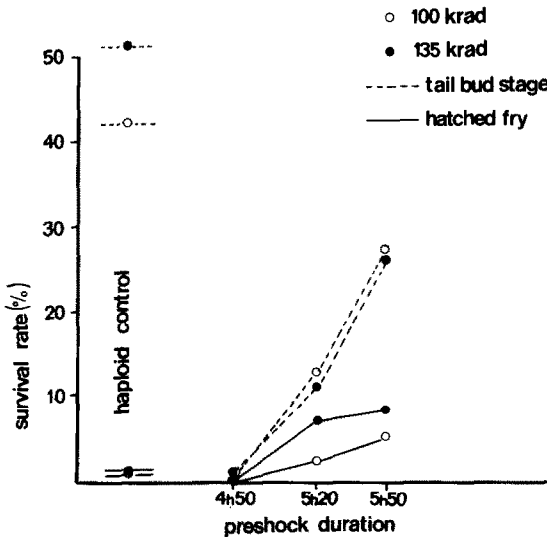


fig. 8. Survival at the tail bud stage and after hatching (percentage of treated eggs) after 7000 psi LP shocks lasting 3 min and applied 4 h 50 min, 5 h 20 min and 5 h 50 min after insemination with irradiated sperm.

if compared with the haploid control (Fig. 8). At the tail bud stage, karyological examinations revealed only diploid embryos (Fig. 5d) versus only haploid ones in the control. However, the yield of these supposed homozygous diploid individuals was considered very low (32 fry collected from 389 treated eggs in the best case: 7000 psi lasting 3 min and applied 5 h 50 min after insemination).

(3) Karyological investigations of various types of gynogenetic individuals and their corresponding survival rates

Supernumerary chromosome fragments were detected in the cells of all the gynogenetic embryos analysed; their number varied considerably according to the observed metaphase in a given embryo. For a given batch, the distribution of their frequency per metaphase was determined from observation of 15 embryos and grouping of these results. This investigation was performed for six batches of gynogenetic individuals: three types (haploid, heterozygous diploid obtained after retention of the second polar body, homozygous diploid obtained after suppression of first cleavage) and two doses of sperm irradiation for each type (100 and 135 krad). The results are presented in Fig. 9:

(a) For a given type of embryo, the mean number of fragments per

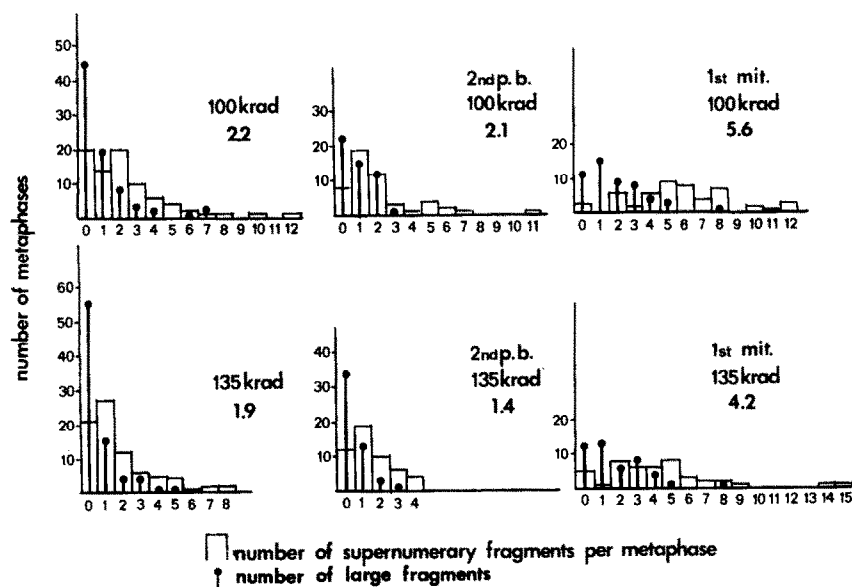


Fig. 9. Large numbers of supernumerary fragments were detected in the metaphases of gynogenetic embryos by karyological examination. Their distribution is presented here for six batches (two doses of sperm irradiation \times three types of gynogenetics: haploids, heterozygous diploids and homozygous diploids). For each graph, the mean number of fragments per cell is given.

TABLE IV

Survival at tail bud stage, hatching time and start of feeding of gynogenetic individuals produced by EP and LP shocks

Time after fertilization of shock	Shock duration (min)	Sperm irradiation dose (krad)	Treated eggs (no)	Tail bud stage	Hatching stage	Feeding stage	Tail bud stage to feeding stage
40 min	3	100	235	70	66	54	84
		135	271	61	59	56	91
40 min	4	100	261	67	59	52	77
		135	270	60	54	47	78
40 min	5	100	300	63	58	48	77
		135	248	68	50	45	67
Total EP shocks		100	796	66	61	51	77
		135	789	63	54	49	79
5 h 20 min	3	100	386	13	2	0	2
		135	403	11	7	4	42
5 h 20 min	4	100	447	8	2	0.5	6
		135	436	6	2	1	15
5 h 50 min	3	100	367	27	5	1	2
		135	389	26	8	3	10
5 h 50 min	4	100	374	16	4	1	5
		135	397	14	5	3	19
Total LP shocks		100	1574	16	4	1	5
		135	1615	20	5	3	19
Control, no shock		100	313	42	1	0	0
		135	334	51	1	0	0

cell was not appreciably decreased by the use of 135 krad instead of 100 krad: 1.9 versus 2.2 for haploids, 1.4 versus 2.1 for heterozygous diploids, and 4.2 versus 5.6 for homozygous diploids.

(b) However, we could ascertain, with the help of a rather arbitrary distinction between small fragments (point size) and larger ones (but always smaller than trout chromosomes), that the mean size of the supernumerary fragments was reduced by the use of 135 krad.

(c) For a given irradiation dose, it is evident that cells of homozygous embryos contained on average many more fragments (about twice as many) than did cells of heterozygous and haploid embryos.

These results have to be related to the survival rates recorded in the batches of the same experiment. In fact, many batches were studied, corresponding to one control, three EP shocks, and four LP shocks, with a comparison of the effects of 100 and 135 krad in each case; the results from tail bud stage to the start of feeding are presented in Table IV:

(a) The yields of heterozygous gynogenetics are much higher than the yields of homozygous ones (particularly if the numbers of tail bud embryos providing feeding fry are compared).

(b) It is important to note that the yields of homozygous individuals were improved by the use of 135 krad, whereas yields after EP shocks did not differ according to the irradiation dose.

DISCUSSION AND CONCLUSION

Technical aspects

The results demonstrate that hydrostatic pressure is very effective in inducing the retention of the second polar body. The yields of heterozygous gynogenetic individuals at the start of feeding can be compared with those following heat shocks, and all-triploid offspring are easily obtained with the same optimized EP treatments. Treatments of lower intensities induce incomplete retention of the second polar body, leading to inviable aneuploids. In this respect, a high frequency of abnormal embryos resulting from a pressure treatment can indicate that the tested shock is not strong enough: in such a case, an increase of intensity should improve the rate of normal individuals.

Most of the rainbow trout eggs treated with LP shocks at 5 h 50 min after fertilization with normal sperm gave viable embryos which were all tetraploid. The low rates of tetraploidy previously obtained with late heat shocks (Chourrout, 1982b) had been interpreted by supposing that the eggs are sensitive to such heat shocks only during a very short period of their development. The induction of tetraploidy by heat shocks in amphibians requires a preliminary selection of sensitive eggs, based on observation of the cleavage furrow with the naked eye: this is impossible in salmonids because the egg shell is not transparent at this stage. Pressure treatments are far more effective in our case, and probably act on less critical stages of first mitosis, but it could be possible to obtain good results with heat shocks in species having transparent eggs. Late pressure shocks are also effective for the production of allotetraploid embryos, without requiring any additional optimization, at least in the case of the two hybridizations tested here which use the rainbow trout as the maternal species.

With similar LP shocks following an insemination with gamma-irradiated sperm, we have obtained high frequencies of diploid gynogenetic embryos. They were supposed to be completely homozygous, a condition that could explain their high mortality before hatching and during yolk resorption. However, the karyological investigations performed here demonstrate the occurrence of supernumerary chromosome fragments in the cells of these homozygous embryos, probably due to their replication before the inhibition of first cleavage. The fact that the use of 135 instead of 100 krad for sperm inactivation, that decreases the mean size of these fragments, was associated with an improvement in survival suggests a depressive effect caused by a contamination of the maternal genome by residual chromatin. Therefore, the poor survival of homozygous individuals could in part be attributed to the use of gamma rays for sperm inactivation. Our primary objective was to determine a way of suppressing first cleavage, but the work also demonstrates the inefficiency of gamma rays for

inducing a "clean" sperm inactivation and suggests abandoning the use of this agent, although a recent study on chum salmon does not mention any supernumerary fragments in embryos following a 100-krad sperm irradiation (Onozato, 1982).

Similar karyological investigations have to be repeated in the case of ultraviolet irradiation, but the results obtained up to now in trout are very encouraging because no fragments have been detected with the optimal UV treatments (Chourrout, 1982c). In addition, the induction of gynogenesis in tilapia, recently performed with ultraviolet irradiation of sperm (Chourrout and Itskovich, 1983), has not resulted in the detection of any residual fragments.

Future prospects of chromosome doubling in fish

Tetraploidy. Tetraploidy is not considered to be an effective method for improving growth in animals. This idea is based in part on the observation of very low numbers of tetraploid amphibians produced directly from diploid eggs, but strictly these should be considered as double-diploids. Careful comparison with appropriate diploid controls is required, and several generations of mating between tetraploids (a necessary procedure for optimizing the level of heterozygosity) should be achieved before drawing any conclusion about this important question.

If tetraploids actually produce high rates of diploid gametes, they could be used in matings to produce triploid sterile animals. The resulting heterozygosity would be higher than by direct retention of the second polar body. In addition, the possibility of tetraploid females producing larger oocytes could justify this method.

Homozygous diploidy. Homozygous diploid gynogenetic individuals can be mated in order to produce clones for various experiments (Streisinger et al., 1981), even if they are produced in low numbers.

Our opinion is that production of inbred strains for genetic improvement of commercial performances will be difficult if started with gynogenesis by suppression of the first cleavage. The low survival rates resulting from this operation probably do not permit the maintenance of lines until reproduction and an eventual selection at this stage.

We would prefer to start such a program by subjecting several females to gynogenesis by retention of the second polar body, although this method is not effective for fixing genes distant from the centromere and although a process of selection for the most "heterozygous" genotypes may occur. The fixation of the lines could be performed in the next generation of gynogenesis, by using the suppression of first cleavage. In this case, better yields could be recorded if in the first generation of inbreeding, natural selection in the lines had eliminated part of the lethal genetic factors.

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