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Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females – Potential of tetraploid fish

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Summary. First generation tetraploids were produced by hydrostatic pressure treatment before the first cleavage and raised until the adult stage. Their survival and growth were severely depressed when compared to the diploid control: after two years, no ovulated females were found although males produced sperm at 1 and 2 years of age and were mated individually with diploid females. The progenies were consistently normal with high survival rates. They were found to be almost all triploids by karyology, which failed to detect a significant rate of aneuploidies. However, the fertilizing ability of tetraploid males was always low (0 to 97% of the control; average 40%). Several arguments presented here support the hypothesis that diploid spermatozoas, which are wider than haploid ones, would be frequently blocked during their penetration through the micropyle canal. Second generation tetraploids were produced after such matings by heat shocks, causing the retention of the second polar body. Their survival and growth were much more satisfactory than in the first generation, although still lower than in diploid and triploid controls issuing from diploid parents. Performances of second generation triploids were comparable to those of diploids, and slightly better than those of conventional triploids issuing from diploid parents. 94.5% of the second generation tetraploids were male.

Key words: Artificial polyploidy – Salmonid fish – Fertility – Sex ratio

Introduction

With the discovery of the colchicine technique for inducing chromosome doubling, the era of polyploid breeding in plants started (Blakeslee and Avery 1937; Nebel 1937). In several cases breeders have actually got a breakthrough in plateaus of yield with autopolyploidy, while in other crops such a manipulation has simply been of no practical use.

The eventual high performances of autotetraploids (gigantism, higher tolerance to difficult environments . . .) are usually well correlated with their level of heterozygosity; therefore, the potential of a new tetraploid (mono-or diallelic for all loci) cannot be evaluated immediately, and several cycles of reproduction are required before such an estimation. More generally, maximizing heterozygosity is one of the major goals of polyploid breeders (Bingham 1980): a cross-pollinating situation is therefore an important condition favoring success in polyploid breeding. Another objective attained by polyploidy is the production of sterile seedless triploid fruits by mating tetraploids and diploids (Kihara 1951).

One of the major limitations of autopolyploidy is the reduced fertility of autopolyploids, which provide a significant proportion of unbalanced gametes. However, this partial sterility cannot be entirely due to meiotic irregularities associated with multivalents (Randolph 1942) but also to other factors curiously not well identified. It has become almost axiomatic that polyploid breeding has its greatest opportunities in crops that are grown primarily for their vegetative parts. In this group, the perennial crops and those possibly propagated vegetatively on a commercial scale have an additional advantage over those depending too much on seed production for their propagation.

Tetraploidy is not viable in mammals and birds, but is viable in amphibians. Obtaining tetraploids started by mating diploid males and triploid females which produce a significant proportion of triploid ova (Humphrey and Fankhauser 1949; Beetschen 1962; Nishioka and Ueda 1983; Kawamura et al. 1983), and then by heat shocks applied at first cleavage (Fischberg 1958; Jaylet 1972) and pressure shocks before first cleavage (Reindschmidt et al. 1979). The fertility in tetraploid amphibians is usually lower than in diploids: in anurans, both sexes can be fertile to some extent; in urodeles, no progeny has ever been obtained from tetraploid males. Fertile tetraploids produce, as in plants, a majority of diploid gametes and a minority of aneuploids. Tetraploidy, for obvious reasons, has

not been investigated in amphibians for the purpose of performance improvement.

Several preliminary observations suggest that the potential of tetraploids deserves an evaluation in commercial fishes: 1) all of them are cross-fertilizing species, 2) their high fecundity could afford an eventual reduction due to tetraploidy, 3) on the other hand, fish farmers are extremely interested in sterile triploid animals because the problems connected with sexual maturation (decreased growth, flesh quality, mortalities) limit appreciably the profit possibly expected from the production of large size animals.

The first massive production of tetraploid fingerlings was achieved in rainbow trout (Chourrout 1984) by using pressure shocks, a technique already successful in producing tetraploid anurans (Reindschmidt et al. 1979) and homozygous diploid zebrafish (Streisinger et al. 1981). This operation results in tetraploid progenies in trout, however, with a highly varying hatching rate.

We report here the results of survival and growth of the first generation of tetraploids; a serial of observations concern the reproductive capacity of tetraploid males, in terms of fertilizing ability and chromosome counts of the embryos in their offsprings. Second generation triploids and tetraploids obtained by mating tetraploid males and diploid females have also been grown and studied for their survival, growth and sex ratio.

Materials and methods

Diploid breeding animals

The diploid breeding males and females were 2, 3 and 4 years old and came from the fish farm of Gournay sur Aronde (temperature: 6 to 15 °C). They belonged to standard winter-spawning, or selected summer, fall and spring-spawning strains. Eggs (2,000 to 4,000 per female) were collected by abdominal pressure in the week after ovulation; the males produced several cc of milt every week collected also by abdominal squeezing into dry tubes. Many experiments involved pools of eggs issuing from several females and pools of sperm from several males, but sperm or eggs of individual animals have also been kept and used separately. In one experiment, the sperm was collected from homozygous golden males (autosomal mutation of depigmentation; dominant over the black wild type).

Fertilization, incubation, early survival counts

Dry fertilization was performed as follows, except when a modification is specified in the text: after elimination of the coelomic fluid the sperm was added to the eggs (usually 0.5 to 5 cc per 1,000 eggs, depending on the experiment); a saline buffered diluent (pH 9, 250 mosm) was immediately added and the batches were allowed to stand for 10 min before being poured in recirculated 10 °C water (Billard 1974); this instant was considered as the zero development time.

The early survival rates were scored either on live embryos at the eyed stage, i.e. 3 weeks after fertilization, or earlier (8 to 12 days) after fixation in the Stockard's solution; the hatching rates were counted approximatively 5 weeks after fertilization; groups to be raised were sent at the eyed stage to the fish farm.

Growth evaluation and sexing

The different batches of fish were grown separately in 2 m square Ewos tanks in the fish farm and fed ad libitum with commercial pellets. At one year of age, they were tagged and mixed into larger raceways. The determination of sex was performed on two batches of hundred tetraploids of second generation at the immature stage: both gonads were removed and examined in toto under a dissection microscope.

Triploid and tetraploid production

Pressure shocks

The initial production of triploids and tetraploids of the first generation was achieved using 7,000 psi pressure shocks and involved 8 males and 5 females. The treatment started 40 min after fertilization (9.6 °C) and lasted 4 min for the triploid production. In order to obtain tetraploids, eggs were shocked after 315 min of incubation for 3–5 min (Chourrout 1984).

Heat shocks

In later experiments, triploids of the first generation and tetraploids of the second generation (in this case, using tetraploid fathers) were produced by 26 °C – heat shock, starting after 25 min and lasting 20 min (Chourrout 1980; Chourrout and Quillet 1982).

Ploidy evaluation

Karyological examinations were performed at the tail bud stage (14 to 17 days). Eggs were kept overnight in 0.02% colchicine; the embryos were separated from their yolk sac by dissection in NaCl 0.8%, put for 15 min in trisodium citrate (0.8%) for hypotonic treatment and finally fixed for 2 to 10 h in cold fresh ethanol 3 : acetic acid 1 (periodically changed) at 4 °C; epithelial cells were dissociated from the embryos by a 5 min-immersion in 50 µl of 50% acetic acid and then dropped onto a prewarmed (50 °C) microscopic slide, which was stained in 4% buffered Giemsa for 30 min. Because of the robertsonian polymorphism in rainbow trout, the precise arm number was considered in evaluating the ploidy level. Due to its relatively high value (156 in triploids and 208 in tetraploids), the accuracy of the counts depended on the quality of the chromosome spreads and will be specified.

The efficiency of the initial pressure treatments in producing triploids and tetraploids was ascertained by measuring the size of erythrocyte nuclei on samples of 10 fishes (25 nuclei per individual). For this purpose, blood smears were photographed and slides were projected onto a screen.

Size of spermatozoas in diploid and tetraploid males

Both the width and length of the sperm head were estimated by similar procedures: after fixation of 0.1 cc milt in 1 cc of a 2% formalin solution prepared in the fertilization diluent containing, in addition, 2 g/l potassium chloride, the smears were stained 15 min in 4% Giemsa, photographed and measured by projection (magnification: × 12,000).

Size of the inner opening of the egg micropyle

Samples of unfertilized eggs issuing from 5 females were fixed overnight in 2% glutaraldehyde prepared in the fertilization diluent at 8 °C and then rinsed in the diluent. The eggs were cut with a sharp double-edged razor blade and the chorion was lifted off from the animal pole; the micropyle was located under the dissection microscope, and a small square containing the micropyle was cut and later on post-fixed with

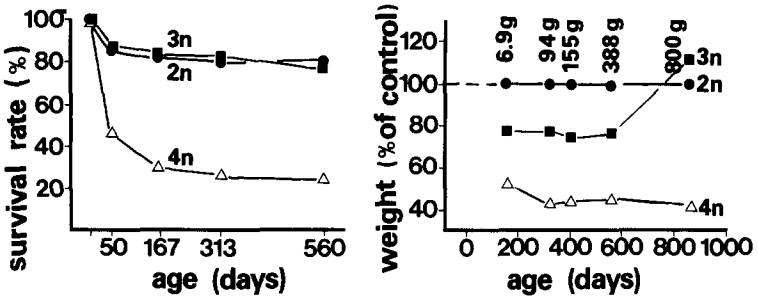

Fig. 1. Survival and growth of diploids, first generation triploids and tetraploids. Successive weights of diploids are indicated above the 100% line of the *right graph*

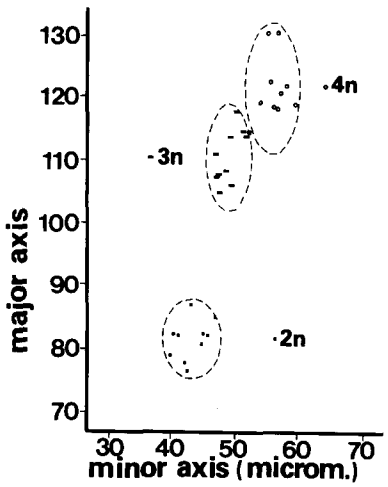

Fig. 2. Bidimensional measurements of erythrocyte nuclei for 10 diploids, 10 triploids and 10 tetraploids of first generation. *Dotted lines* delimit the 95% confidence areas for each ploidy level

osmium tetroxyde, dehydrated in successive baths of acetone and a graded series of ethanol; after the critical point, the specimens were metallized and examined in a Hitachi scanning electron microscope. Depending on the females, 12 to 34 specimens could be correctly measured. A similar procedure recently permitted the observation of trout micropyle (Szollosi and Billard 1974).

Results

Production and testing of first generation triploids and tetraploids

Preliminary experiment

In order to confirm the optimal time for pressure-shocking the eggs in order to produce tetraploids, an experiment was conducted using eggs from 34 females and sperm from 64 males. Treatments starting in the interval 270–420 min were tested on small batches of eggs (600 each; every 10 min between 300 and 360 min; 3 replications per treatment). A peak of survival was obtained, reaching its optimum (20 to 25% hatching rate) in the interval 310–340 min. Such a peak (Chourrout 1984) is supposed to represent only tetraploid embryos.

Mass production of triploids and tetraploids

A similar treatment was applied 315 min after fertilization on larger egg batches in order to test the performances of tetraploids in comparison with their diploid and triploid controls. The yields of embryos and hatching larvae in the tetraploids (respectively 10.5% and 10%) were lower here: in the diploid and the triploid groups, the corresponding yields were respectively 69.5% and 52% at the eyed stage, and 66.5% and 47% after hatching. These yields are the averages of five females, and it should be mentioned that two of them did not provide any tetraploid larvae. The number of larvae available in the fish farm, in the diploid, triploid and tetraploid batches, were respectively 1,343, 1,651 and 439.

Late survival

Figure 1 shows the evolution of survival calculated in percentage of original larvae number. Few mortalities occurred in the diploid and triploid batches: 80% of the larvae were survivors 18 months later. At that time, only 25% of the larvae had survived in the tetraploid batch. The corresponding mortalities were mainly observed during the yolk resorption, eliminating most of the abnormal larvae but they occurred later as well, affecting apparently normal individuals. The survival was stabilized after about six months.

Ploidy estimation

Preliminary examinations of 3 blood smears prepared from each of three diploid animals revealed non-significant differences between the average size of their erythrocytes, as well as between the 3 smears prepared from each individual. The same procedure applied on samples of 10 individuals allowed the discrimination between the diploid, triploid and tetraploid batches (Fig. 2).

Growth evaluation

The growth curves in Fig. 1 correspond to batches in which the density was approximately equalized at 200 individuals per tank, shortly after feeding start. Due to

the continuation of the mortalities in the tetraploid group, its density became quickly inferior to those of the diploid and triploid groups. However, the weight of tetraploids remained considerably lower than in the control (less than 50%), the triploid batch suffering a 20% depression. The latter difference was progressively reduced after sexual maturation (at two years of age), the triploids getting even bigger than the diploids after the spawning season.

Maturation of tetraploids and chromosome numbers in their progenies

Sexual maturation

The first maturations recorded curiously affected 6 males (5.5% of the group) of the tetraploid batch, which therefore were tested with diploid females (males A, 2, 3, B, C, D). Most of the diploid and the triploid males matured for the first time one year later (respectively 47% and 49.5% of these groups), but only the former provided normal milt. Half of the diploid females (26% of their group) matured and ovulated during the same season, while all the triploid females remained immature. In the tetraploid batch, 15 males (21% of the group) entered their first maturation process in their second year (males 1, 4, 5, 6 ... 17) and all produced sperm which was tested on diploid females as well. One female in this batch also entered maturation but died without having ovulated: its autopsy revealed a well developed ovary with full-growing oocytes.

Ploidy of progenies issuing from tetraploid males and diploid females (Table 1, Fig. 3 a, b)

The first examination concerned embryos obtained from the 6 males having matured in the first year (Table 1): most of them (97 out 104) were triploids, the rest being diploids (around 60 chromosomes; 104 arms). No aneuploids were detected. The accuracy was ± 3 arms in this experiment. The 7 diploids came from 4 of the 6 males and were therefore in each case only a minority. We did not examine here any diploid control which would have been provided by the same females fertilized by diploid males.

The accuracy of the counts performed one year later on progenies of 6 other males (1, 4, 5, 7, 8, 9) was ± 4 arms (Table 1): out of 134 embryos analysed, 131 were identified as triploids, 1 as diploid, and 2 abnormal ones as aneuploids (hypertriploid and hypotriploid). The diploid control was not checked.

The third examination was performed on a group issuing from a pool of milts provided by males of the first year, with an accuracy of ± 5 arms: the 34 embryos analysed were identified as triploids, while the 18 embryos of a control fertilized by diploid males were detected as diploids.

The last examination (accuracy ± 2 arms) concerned four samples obtained from the same 6 males; the groups, which they were belonging to, were grown up and their performances of survival, growth and sex ratio are presented in results, section 3 (first repetition): the 12 embryos analysed in the diploid control were actually diploids and the 15 embryos of the triploid control (same parents; eggs heat shocked) were triploids. From 15 embryos obtained after fertilization of the same females by the tetraploid males, 14 were triploids and 1 was mosaic, while in a group of 40 embryos issuing from the same parents, but after heat shock, 36 were tetraploids, 1 was triploid and 3 were aneuploids (1 hypertriploid, 1 hypotriploid, 1 hypotetraploid).

The progenies obtained from tetraploid males were composed of a large majority of normal embryos, and normal hatching larvae; their survival is presented in results, section 3.

Fertilizing capacity of sperm provided by tetraploid males

Fertilization rates and general features of sperm

The histogram of Fig. 4 groups the results of 99 attempts of fertilization by tetraploid males, performed at various dates and issuing from various combinations of parents. The fertilization rates were systematically lower than by using sperm of diploids on the same females: the batches contained 0 to 97% embryos, taking 100% as the reference for the diploid controls; the rest of the eggs in the batches were in a large majority eggs apparently unfertilized and in a small minority abortive eggs.

In contrast, checking the milt for spermatozoa motility prior to each fertilization was always positive, although the intensity of the motility was quite variable from one sample to the other. In addition, the spermatocrite (part of the milt occupied by the spermatozoas) was satisfactory, although lower than in sperm of diploids: 19% (10 to 35), an average of 14 independant measurements on tetraploids, versus 35% (17 to 50) an average of 8 measurements on diploids.

Formulation of a hypothesis explaining the low fertilization rates

The difference of spermatocrite values between the tetraploids and the diploids could possibly be attributed in part to the fact that the tetraploid males used were much smaller than the diploid males. As a matter of fact, the contamination of milt by urine during the collection in tubes is usually more difficult to avoid on small fishes.

Nevertheless, these observations of motilities and spermatocrites grouped together hardly explain the low fertilization rates recorded: the high amounts of sperm used in all these attempts should have guaranteed satisfactory yields in the case of diploid males having such sperm motilities and spermatocrites. Moreover, several of the lowest fertilization

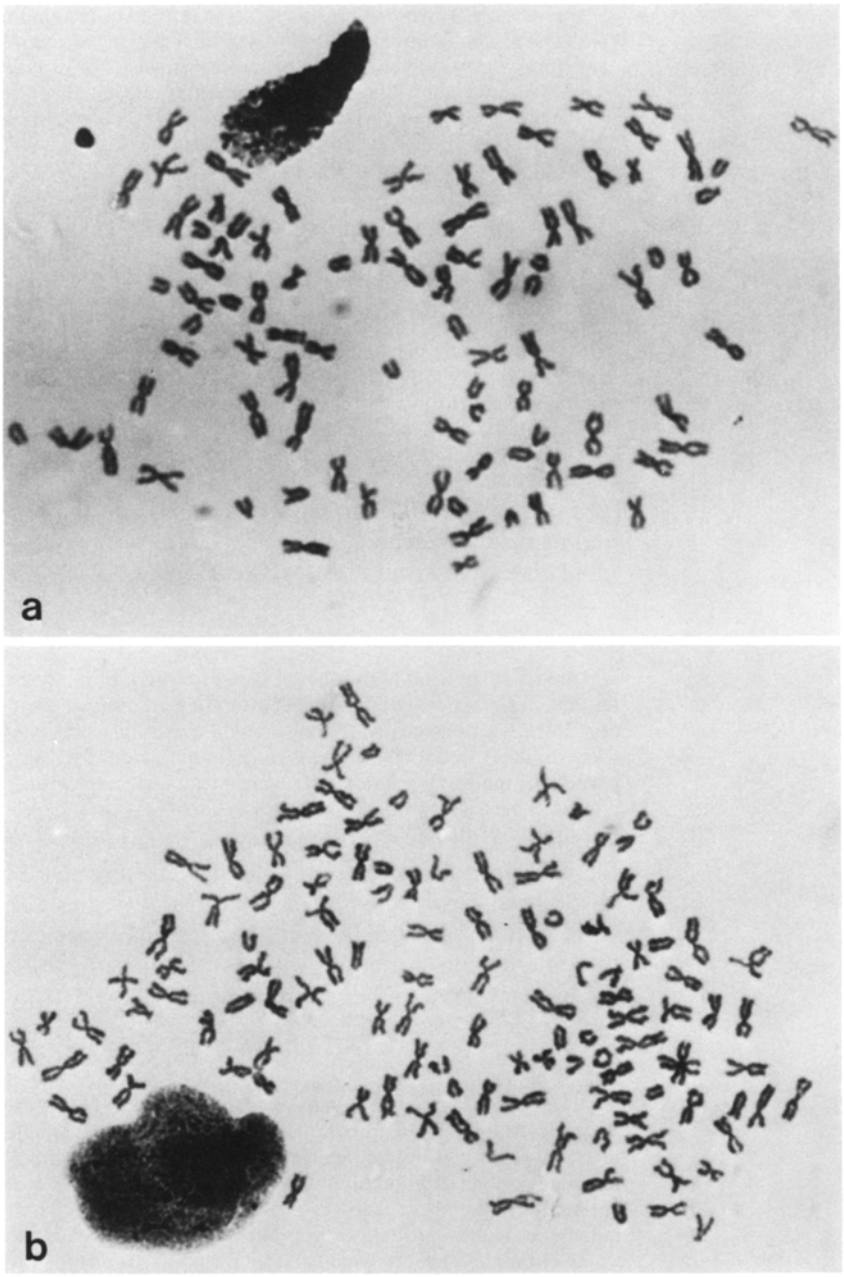

Fig. 3. Metaphases of second generation triploid (**a**) and tetraploid (**b**) embryos

Table 1. Karyological data obtained in progenic of 12 individual tetraploid males and pools of diploid females

Males	First year						Second year					
	A	2	3	B	C	D	1	4	5	7	8	9
Diploid	2	2	0	1	2	0	0	0	0	0	0	1
Triploid	18	18	17	15	16	16	26	20	20	22	27	16
Aneuploid	0	0	0	0	0	0	1	0	0	0	1	0
Total	20	20	17	16	18	16	27	20	20	22	28	17

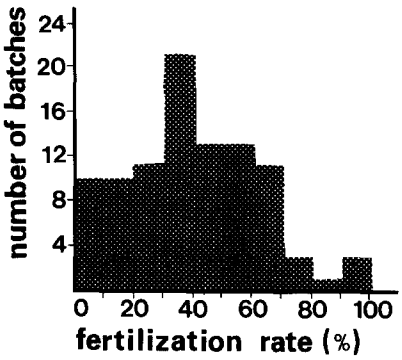

Fig. 4. Distribution of fertilization rates in 99 attempts using tetraploid males and diploid females (in% of the diploid controls)

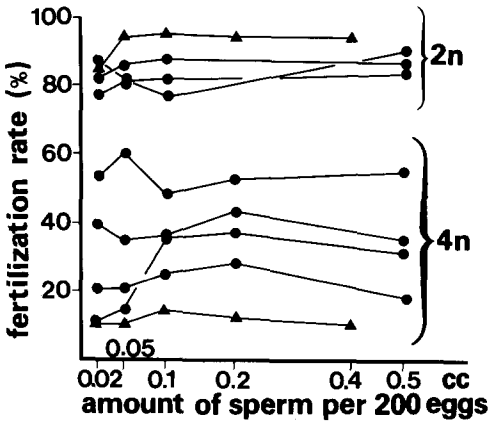

Fig. 5. Fertilization rates with various concentrations of sperm of tetraploid and diploid males (● individual males in exp. 1; ▲ pools of males in exp. 2)

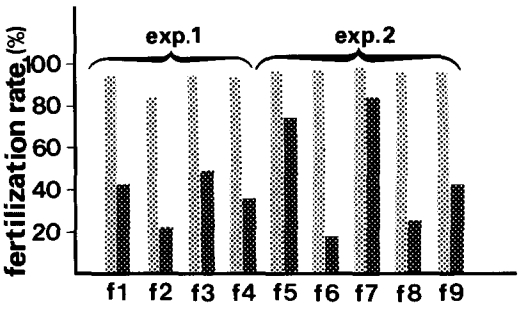

Fig. 6. Fertilizability of 9 individual females by pools of diploid and tetraploid males (pale and dark rectangles, respectively)

rates by tetraploid males have been recorded in cases of high motility and high spermatocrite.

These arguments led us to formulate and to test the following hypothesis: because of their supposed larger head size, the spermatozoas of tetraploids could be frequently blocked in the micropyle canal of the egg during their penetration down to the ooplasm.

Studies on brown trout (Ginsburg 1963) and chum salmon (Kobayashi and Yamamoto 1981) show that the inner part of this canal is just a bit wider than the sperm head of diploid males. Among the following experimental arguments, we analyze the sources of variation of fertilization rates, assuming that variations in micropyle canal and sperm head sizes could be the origins of eventual male and female effects.

Male effect; sperm amount effect

The low fertilization rates, if caused by sperm quality (spermatocrites, motility ...), could be improved by using larger amounts of sperm, and could possibly reach the yields of diploid males. On the other hand, in the case of satisfactory sperm quality (guarantee of a sufficient number of spermatozoas able to reach the micropyle canal), but of phenomena like a barrier created by the micropyle diameter, one should expect a plateau of fertilization rates for high sperm concentrations.

Figure 5 shows such plateaux occurring in two experiments and reaching levels dependant on the tetraploid males and lower than for diploid males.

Female effect

A female effect could also be a factor of variation of the fertilization rates, possibly connected with variations in average diameter of micropyles. Interfemale variations observed with diploid males could be greatly emphasized by using tetraploid males, the micropyle size being obviously more critical in the case of bigger spermatozoas.

Figure 6 clearly illustrates such a phenomenon: in the first experiment, a stronger female effect (range 21 to 49%) was obtained with tetraploid males (versus 83.5 to 95% with diploid males); in the second experiment, the interfemale variation was not significant using diploid males (96 to 99%), but considerable with tetraploid males (17.5 to 83%).

Male – female interactions

Again considering the previous hypothesis, male – female interactions should not be detected in diallel tables of fertilization rates obtained by mating individual tetraploid males and diploid females. As a matter of fact, a female with wider micropyles should be better than other females whichever the tetraploid male used, and vice-versa.

Table 2 summarizes the results obtained in 5 experiments performed over a six-month period; three diallel subtables can be isolated and statistically analyzed.

Because of the absence of intrabatch replication, the male-female interaction can only be overestimated by the variation after subtraction of the male and female main effects; the latter estimate is then compared to the minimized variation possibly recorded between intrabatch replicates, i.e. the variation of the binomial distribution generated by random effects (n = number of eggs; p = fertilization rate).

In the three diallels, no interaction between males and females could be detected in this way, which would have obviously concluded positively even in cases of non significant interactions. Significant male and

Table 2. Fertilizing capacity of 9 tetraploid males over a six month period, representing five experiments (data from experiments 2, 3, and 5 are used as diallel tables for measuring the male-female interactions)

No. of females	Exp. 1	Exp. 2					Exp. 3		Exp. 4	Exp. 5			
	10	1	1	1	1	1	1	1	8	1	1	1	1
Egg no. per batch	200	150	150	150	150	150	65	65	150	65	65	65	65
Male 1	64	97	35	97	49	66	48	36	59	–	–	–	–
Male 2	21	–	–	–	–	–	13	23	39	44	19	42	2
Male 3	42	64	19	84	19	37	29	27	46	49	31	36	11
Male 4	54	–	–	–	–	–	41	40	54	63	35	64	4
Male 5	68	87	25	94	41	55	31	38	61	–	–	–	–
Male 6	–	–	–	–	–	–	38	31	–	–	–	–	–
Male 7	39	–	–	–	–	–	2	0	32	–	–	–	–
Male 8	28	62	5	70	7	15	18	8	–	–	–	–	–
Male 9	31	50	2	68	6	30	6	13	–	–	–	–	–

female effects have been recorded in diallels 1 and 3, while only a male effect was detected in diallel 2 (for which only two females were tested).

Sperm head size; relative fertilizing capacity of various males

The length and width of 21 to 39 spermatozoa heads were measured in smears from 5 diploid and 9 tetraploid males, two or three smears being prepared for each of them (Table 3). The data on width only are detailed here since it is supposed to be the critical parameter for sperm penetration through the micropyle canal. The discrimination between tetraploid and diploid males was very clear, the ratio of their average widths being 1.30 (1.20 for the lengths).

The data were statistically analysed in a hierarchical model with three factors of classification (male, smear, spermatozoa); intramale intersmear variations were not significant in both types of males, while intermale variations were not significant in diploids but significant in tetraploids (97 to 104% of the average of all tetraploid males). The classification of males from the narrowest to the widest sperm heads was as follows: males 1, 4, 5 together, then male 3, then male 7, then males 8 and 9 together; the width in male 6 was significantly lower than in males 7, 8 and 9, while the width in male 2 was higher than in males 1, 4 and 5 but lower than in males 8 and 9. The intermale variation in width was negatively correlated with the intermale variation in length ($r = -0.7$), reflecting intermale differences in sperm head shape (the narrowest being the longest ones). It should be mentioned that these measurements were made in a blind manner (numbers of males and smears unknown by the operator), because of the frequent difficulty in determining the exact border of the spermatozoas on the screen.

These nine males had been tested over a period of six months (in five experiments) either on individual females or pools of several: the fertilization rates are indicated in Table 2. The males were compared two by two in two-way analysis of variance (male; attempts) using the data obtained on the same eggs. The nine males could not always be used on the same day. The extremely consistent ranking of the males over the period of testing permitted an easy classification of eight of them into three groups: the best composed by males 1, 4 and 5, the intermediate by male 3, and the worst by males 2, 7, 8 and 9. Male 6 was tested only twice in one experiment could not be classified but was in these two attempts equal to male 5, behind males 1 and 4 and before all the others.

To summarize, the analogy between the classification in sperm head size and the latter ranking is striking and acts in favor or our hypothesis (the narrowest spermatozoas fertilize better).

Micropyle diameter; relative fertilizability of females

Variations between females might be correlated with variations in micropyle canal size. The observation of the chorion specimens by scanning microscopy showed that the inner opening of the canal can have various shapes – circular in most cases, but sometimes more or less ellipsoidal. In the latter cases, the minimum diameter was considered. Significant differences were detected between the mean size of populations of micropyles from different females, the extremes being female 2 with a small average diameter and female 5 with larger openings (1.4 times on an average). Female 5 was also the better fertilized by tetraploid males, and female 2 was the worst in the same respect (Fig. 7); the three other females were intermediate for both parameters.

Table 3. Measurement of sperm head size performed on 5 diploid and 9 tetraploid males

Male	*n*	Wi[a]	(SD)	$\bar{W}$	$\bar{L}$
2n1	35	2.16	(0.08)	2.15	2.71
	35	2.13	(0.08)		
2n2	35	2.10	(0.07)	2.15	2.81
	35	2.19	(0.08)		
2n3	35	2.22	(0.08)	2.22	2.71
	35	2.21	(0.09)		
2n4	35	2.21	(0.08)	2.19	2.73
	35	2.18	(0.09)		
2n5	35	2.19	(0.09)	2.17	2.77
	35	2.15	(0.08)		
4n1	30	2.75	(0.13)	2.75	3.34
	30	2.70	(0.16)		
	27	2.79	(0.15)		
4n2	30	2.87	(0.16)	2.83	3.27
	30	2.82	(0.16)		
	21	2.80	(0.16)		
4n3	32	2.80	(0.16)	2.81	3.27
	32	2.77	(0.25)		
	37	2.87	(0.22)		
4n4	32	2.76	(0.19)	2.73	3.37
	32	2.71	(0.15)		
4n5	30	2.73	(0.15)	2.73	3.32
	28	2.71	(0.13)		
	28	2.76	(0.22)		
4n6	30	2.77	(0.20)	2.78	3.33
	32	2.79	(0.22)		
4n7	38	2.86	(0.20)	2.86	3.38
	39	2.86	(0.16)		
4n8	32	2.90	(0.16)	2.93	3.20
	26	2.95	(0.17)		
	30	2.95	(0.24)		
4n9	33	2.91	(0.14)	2.94	3.21
	33	2.99	(0.18)		
	32	2.91	(0.21)		

[a] Wi: average width in one smear; W, L: average width and length respectively in one animal (μm)

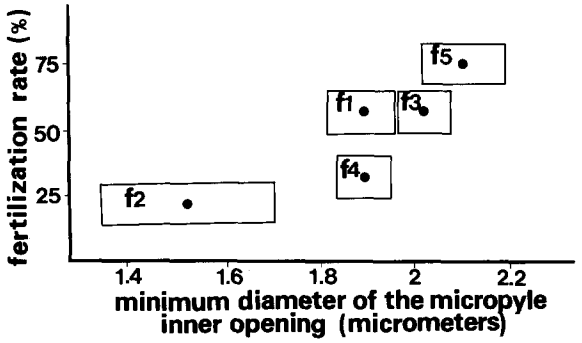

Fig. 7. Fertilizability of 5 females by a pool of tetraploid males (% of the diploid controls) in function of the average width of their micropyle inner opening. Rectangles delimit the 95% confidence areas

Successive fertilizations by sperm of tetraploids and diploids

After contact with the diluent of insemination, the spermatozoas quickly loose their motility but the eggs do not enter the cortical reaction and remain fertilizable. Let us consider the hypothesis that spermatozoas of tetraploids do not fertilize well because they are not able to reach the entrance of the egg micropyle: in this case, the unfertilized eggs left free remain fertilizable by an eventual addition of diploid sperm.

To test this assumption, we inseminated with diploid sperm of golden male eggs which had been mixed 5 min earlier with sperm of six tetraploid males and kept in the insemination diluent. Whether the eggs were washed or not between both inseminations, the yields of embryos were not significantly better (59 and 51%) than by the insemination with sperm of tetraploids alone (50%); moreover, none of the embryos issuing from successive fertilizations was yellow. In controls, diploid golden males alone provided 97% fertilization rates, all the embryos being yellow. This observation proves that even if the sperm of tetraploids does not fertilize all the eggs, it prevents systematically the later fertilization by diploid sperm.

Testing of second generation triploids and tetraploids

Evaluations of batches provided by tetraploid males and diploid females (in comparison with diploid and triploid controls provided by diploid males) were achieved twice in the fish farm. The first repetition involved 2 pools of sperm, one collected from 5 tetraploid males of the first year and one from 3 diploid males; the eggs came from 2 mixed female spawns. In the second repetition, we used a pool of sperms of 2 tetraploids of the first year and 6 of the second year, the eggs coming from 7 diploid females.

Yields of embryos at the eyed stage and karyology

In the first attempt, the rate of embryos were 95% of the initial egg number for the diploid control, 94% for the triploid control and 63% in the two batches inseminated by tetraploid males (second generation triploids and tetraploids). In the second experiment, the yields of embryos were respectively 93%, 91%, 24% and 23% for the same groups. A confirmation of the composition of the four groups in the first experiment was given by karyology (see "Results" section 2 b). Tetraploidy of the males used in the second repetition had been confirmed by examination of their progenies in other experiments (see same section; males 1, 2, 3, 4, 5, 7, 8, 9).

Survival

As illustrated in Fig. 8, the survival rate until one year of age was satisfactory in the four groups of the first experiment, although lower in the triploid control and the

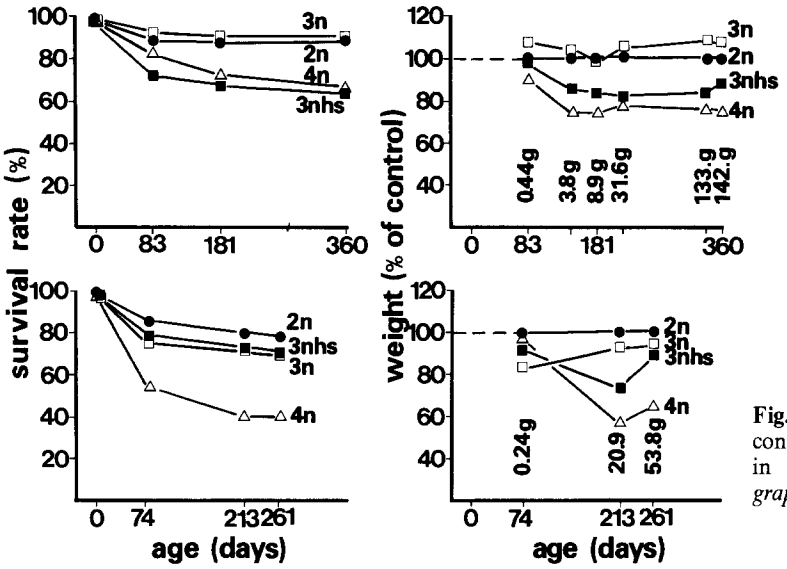

Fig. 8. Survival and growth of diploid and triploid controls, second generation triploids and tetraploids in two experiments (respectively, *upper and lower graphs*)

tetraploid batch. In the second attempt, the triploid groups has a similar survival rate until 8 months, slightly lower than the diploid control. The highest mortality occurred in the tetraploid batch, mainly before feeding but also later on; the survival in this group was stable after six months of feeding.

Growth evaluation (Fig. 8)

First experiment. After one month of feeding, the numbers of fish per tank were 177, 193 for the diploid and triploid controls, 527 and 726 for the triploids and tetraploids of the second generation. At that time, these numbers were reduced to 220 fish per tank in the two latter batches, but were left unchanged in the two controls. A final equalization to 156 fish in all tanks was accomplished three months later.

Therefore, the two controls were favourized by a lower density during their early growth. However, the best group over one year of testing was the second generation triploid followed by the diploid control and then by the triploid control; the tetraploid batch was the last group, but had a satisfactory growth rate.

Second experiment. The densities were equalized after 50 days of feeding, the numbers of fish in the groups being 436, 434 for the diploid and triploid controls, and 414 and 298 for the second generation triploids and tetraploids three weeks before this operation. The mortalities observed later occurred mostly in the tetraploid batch, which therefore was favourized in density during the rest of the testing period.

However, this group was again the last one (far behind the three others) although it showed a satisfactory weight gain in the last 45 days of testing; the control triploids grew initially slower than the second generation triploids, but this difference was reduced to 4% at the end of the testing period. The control diploids maintained a 6–7% advantage in weight over the second generation triploids during the last months of growth.

Sex ratio of second generation tetraploids

In the first experiment, a sexing was performed 11 months after hatching, revealing a large majority of the male sex: out of 94 individuals sexed, 90 were identified as males and 4 as females with ovaries full of oocytes.

In the second experiment, comparable results were recorded eight months after hatching; out of 86 individuals, 80 were identified as males, and 6 as females; only two of the latter presented ovaries full of oocytes.

Sexual maturation after one year

Several percents of maturing males were recorded in the four batches of the first experiment after one year; two second generation tetraploid males were mated with diploid females and both produced all triploid progenies (two samples of 20 embryos each were analyzed with an accuracy of ± 5 arms).

Discussion and conclusions

Production of first generation tetraploids

The use of pressure shocks had already permitted us to obtain the tetraploid embryos from which viable feeding larvae emerged (Chourrout 1984). The variable and usually low yields of embryos obtained might be partly attributed to the treatment itself: the optimal time of shock application has been determined on pools of several females but could vary between mothers. Analyzing the sources of such a variability in order to guarantee an optimal yield from a given female would certainly be a hard enterprise resulting in probably limited practical consequences. The shock intensity, i.e. pressure level and treatment duration,

should not yet be considered optimized; however, Thorgaard (personal communication), after having diploidized rainbow trout androgenetics with high pressure levels (9,000 psi; Parsons and Thorgaard 1985) obtained better rates of embryos using milder treatments (7,000 psi). The use of too high pressures might also explain the poor rates of tetraploid embryos in several species of *tilapia* (Myers 1985). The doubts emitted by the authors about the viability of tetraploidy in these species have to be taken very carefully until new pressure treatments are tested. Similar doubts have been expressed in the reports claiming the production of tetraploid rainbow trout embryos by heat shocks (Thorgaard et al. 1981; Chourrout 1982) which resulted in poor survivals and low frequencies of tetraploids, mixed with a majority of residual diploids and mosaics. In the same respect, the recent satisfactory rates of viable tetraploids obtained in channel catfish (Bidwell et al. 1985) with heat shocks underlines that treatments once proved unsatisfactory could be efficient in later attempts. The power of heat shocks in chromosome doubling has actually been well demonstrated in several amphibians (Fischberg 1958; Romanovsky and Spicarova 1961; Jaylet 1972). It is very likely that new claims of tetraploid viability in other fish species will appear within the next few years.

Up to now, growing up large numbers of first generation trout tetraploids is hard work because of their low early survival and poor growth. To explain this observation, one should not get rid of the eventuality of slight deviations from the tetraploid chromosome number in the fraction of the progenies issuing from pressure shocks; the high chromosome numbers involved here and the relatively low sophistication of karyological techniques used on fish embryos make the detection of minor aneuploidies more difficult than in amphibians and most crops. We showed recently (Chourrout 1984) that too short pressure shocks applied during second meiosis caused hypotriploidies; however, the stabilization of the survival and growth of the tetraploids after several months and the obtaining of fertile adults argue in favor of a limited occurrence or impact of such problems.

On the other hand, and as already mentioned, these low performances of first generation tetraploids would not surprise plant breeders reasoning in terms of correlations between yields and heterozygosity levels.

Some of them have interpreted the depression due to chromosome doubling as equivalent to several generations of selfing (Hougas and Peloquin 1958; Clement and Lehman 1962; Obajimi and Bingham 1973; Dunbier 1974; Mendoza and Haynes 1974). The situation is not so clear in amphibians, where the authors are not so much interested in a precise evaluation of growth with appropriate controls and often report observations collected on few individuals: Fischberg (1958) and Jaylet (1972) agree on a slower growth of tetraploids than that seen in diploids and triploids in urodeles; Reindschmidt et al. (1979) mention only their survival in *Xenopus* and the leopard frog, apparently comparable to diploids. Those tetraploids were produced by chromosome doubling, this event also being the probable origin of the few tetraploid *Xenopus* accidentally arising from nuclear transplantation (Gurdon 1958) which grew 20% slower than the diploids. The situation becomes evidently more complicated once other mechanisms are considered in the tetraploid production. As a matter of fact, the first generations of tetraploids issuing from matings between triploid females and diploid males may be tri- and tetraallelic in some loci: such individuals have a lower growth rate than diploids in axolotl (Humphrey and Fankhauser 1949; Fankhauser and Humphrey 1950) but equivalent to that found in treefrogs (Nishioka and Ueda 1983). This remark is a fortiori valid for the following generations of tetraploids issuing from first generation produced by either way.

Fertility of first generation tetraploids

The fertility of the first generation tetraploid males in trout is established in our study and preliminary data indicate that some males in their tetraploid progenies are fertile as well. The first problem is their low fertilizing ability, (lower than in diploid males), possibly due to the difficult penetration of diploid spermatozoas in the micropyle canal. The arguments presented here support this hypothesis rather than problems of insufficient sperm quality; among them we should emphasize the interfemale variation of fertilizability revealed by tetraploid males, the absence of tetraploid male-diploid female interaction in diallel matings, the plateau observed by increasing the sperm concentration, the negative correlation between sperm head width and fertilizing ability, and the fact that diploid spermatozoas prevent the later fertilization by haploid ones. On the other hand, the intermale variability per se does not favorize either hypothesis and the correlation between the micropyle canal size and the fertilizability of distinct females is still statistically weak. The absolute values of width of spermatozoas and micropyle openings should not be compared because they result from two very different techniques: the size of spermatozoas is probably overestimated when measured on a smear. In a practical respect, we should underline that the ranking of tetraploid males remained unchanged all over the testing period: therefore, the "worst" males can be eliminated after the first comparative assay in order to limit the egg loss in further attempts.

On the other hand, the fact that ovulation occurs only once a year did not permit us to observe whether the ranking of the females remains unchanged in successive assays: if the variability between diploid females is in part genertically determined, the fertilizability by tetraploid males might be improved in further generations by selective breeding of the "best" females (a progeny issuing from a pool of several

females will contain more individuals from the best ones). However, we should first consider that fertilizability by tetraploid males might not be restricted to intrinsic characteristics of the females but could possibly be connected to their physiological condition, itself partly resulting from the operator (egg aging depending on the time of stripping after ovulation).

The absence of progenies from tetraploid urodele males is still unexplained: the fact that they can produce spermatophores containing motile spermatozoas and that a significant minority of triploids can be provided by diplotetraploid males (mated with diploid females) indicates that the problem might be solved in the future (Jaylet 1972). A disadvantage here is that one of the ways to produce tetraploids, i.e. by mating triploid females with diploid males, results in all cases in tetraploid females, and therefore the chance of examining males is lower than in anurans, in which both sexes result from either method.

Fertile tetraploid males are usually found in anurans, with the exception of the *Xenopus* (Gurdon 1958). However, the tetraploid *Xenopus* results from nuclear graft, a technique known to induce chromosome aberrations (Di Bernardino 1979), themselves possibly affecting the fertility.

Fertile tetraploid females are encountered in urodeles, as well as in anurans; intermediates between sterile and fully fertile individuals are however mentioned in most reports but the eggs provided are fertilizable.

In trout, we have not got yet reliable information about the fertility of the tetraploid female. No ovulation occurred in the tetraploid group after two years, while many did in the diploids although the former fishes were much smaller than the latter. The discovery, although by autopsy, of one tetraploid female with developed ovaries makes us optimistic for using eggs of such individuals in the future.

Chromosome numbers of the progenies of tetraploids

The capacity of tetraploids to provide chromosomally balanced gametes will influence the survival of their progenies, either tetraploid or triploid.

It is a common (but not general) feature of tetraploid plants to produce, in addition to a majority of diploid gametes, a significant minority of hypo- and hypertriploid ones (Dawson 1962; Sakharov and Kuvarin 1970; Ellerstrom and Sjodin 1974), the latter resulting from part of the multivalents. Such a phenomenon will, of course, be overemphasized if the parent karyotype is slightly hypo- or hypertetraploid, and a precise identification of the parent is therefore of preliminary use to ascertain the origin of the unbalanced gametes. Viable hypotetraploids have been occasionally detected in progenies of triploid females and diploid males in anurans (Nishioka and Ueda 1983; Kawamura et al. 1983) however, even if the exact karyotyping of the parents has either not been performed or not been reported, it seems that tetraploid amphibians may provide a certain proportion of unbalanced gametes as well (Humphrey and Fankhauser 1949; Fankhauser and Humphrey 1950, 1959; Beetschen 1962). In other studies, only balanced karyotypes have been analyzed in the progenies of tetraploids (Nishioka and Ueda 1983; Kawamura and Nishioka 1967, 1983; Jaylet 1972) but eventual aneuploids may have disappeared before the stage of analysis. In the former study, the lower early survival in the group issuing from two tetraploid parents might reveal a depressive effect of the tetraploidy itself but the triploid groups resulting from one tetraploid parent, either male or female, also suffered a depression in survival, which possibly indicates the initial presence of aneuploid individuals.

In our study, the progenies of tetraploid males were composed of a large majority of normal embryos; the early survival of second generation tetraploids was sometimes lower than that of triploids from the same parents, but the survival of the latter was always comparable to the survival of triploid and diploid controls. The karyology performed very early, although with a variable accuracy, failed to detect a significant proportion of aneuploids. It is, however, still possible, as Beetschen (1967) suggested, that a triploid chromosome number may hide a severely unbalanced karyotype with, for instance, four exemplaries of one chromosome and two of another. Such abnormalities could only be detected by using very discriminant chromosome banding techniques which are not available in most fishes; they would certainly have a depressive effect on the survival of the second and following generations of polyploids. Our findings argue in favor of a limited occurrence of meiotic irregularities in the artificially produced tetraploid males.

In this respect, we should mention that salmonids are of tetraploid origin. The appearance, in the evolution of natural polyploids, of multivalent suppressors has been suggested to explain the nearly systematic bivalent pairing found in some plants of polyploid origin. One striking example is found in polyploid chrysanthemums (Watanabe 1983): the latter hypothesis is clearly supported by the fact that colchiploids issuing from a species of hexaploid origin exhibit much lower frequencies of multivalents than colchiploids derived from a diploid species. Additional investigations are needed before drawing any analogy with our present case. The description of meiotic multivalents in male chromosomes of the salmonids examined (at metaphase I) seems to argue against such a parallel (Ohno et al. 1965; Gold and Gall 1975; Lee and Wright 1981) but at least part of them might be explained by the robertsonian polymorphism frequently described (Thorgaard 1976). On the other hand, it is noteworthy that duplicate loci of salmonids are inherited disomically rather than tetrasomically (Allendorf and Utter 1975; May 1980) assuming that diploidization might be complete, and that no multivalents have been found in female meiosis observed at pachytene stage (Davisson 1969; Lee and Wright 1981).

The first experiment of karyology in embryos provided by tetraploid males revealed a minority (7%) of diploid embryos, the rest being exclusively triploid. In further attempts, only one diploid has been identified in 186 examinations of similar progenies (49 coming

from the same males). The most likely explanation for such diploids would be spontaneous androgenesis. If this accident was not specific of the fertilization by tetraploid males but rather due to the ova condition, we might have found a similar frequency of haploids in a control with the same females and diploid males, which was unfortunately not performed. Whatever the origin of these diploid embryos, we have to emphasize that matings between tetraploids and diploids, expected to produce all-triploid and sterile offsprings, may as well produce a minority of fertile diploids, possibly by accidents in the fertilization process; in the case of exotic fishes, which release in the natural waters depends upon a strict control of reproduction, a culling might remain necessary in progenies of tetraploid males and diploid females.

Second generation triploids and tetraploids

The present study poses the problem of how to propagate the initial tetraploids.

In urodeles, the only way was to alternate generations of triploids and tetraploids mated with diploid males (Fankhauser and Humphrey 1950, 1959; Beetschen 1962, 1967). Another method may be by successive gynogenesis induced in tetraploid females (Ferrier 1966; Jaylet and Ferrier 1978), a procedure, however, supposed to result in inbreeding, i.e. opposite to our general objectives. A tetraploid line was very recently obtained by successive mating of tetraploid males and females in anurans, the initial tetraploids having, however, resulted from triploid females (Nishioka and Ueda 1983). Another solution in anurans was to mate tetraploid males with diploid females and to retain the second polar body by thermal shock (Kawamura and Nishioka 1967).

The use of only one tetraploid parent is supposed to reduce the frequency of unbalanced karyotypes in the progenies, unless the thermal shock itself results occasionally in incomplete retentions of the polar body. In trout, we chose this method because of the present lack of mature tetraploid females. Our heat shock does not provide any additional aneuploidies (Chourrout 1985) nevertheless, this procedure is likely to be less efficient than mating between tetraploids for maximizing heterozygosity, due to the partial genetic similarity of the two female genetic sets fusing after thermal shock (Thorgaard et al. 1983; Guyomard 1984).

A remarkable observation is the improvement of survival and early growth in the second generation of tetraploids although it is still lower than that found in the diploid control. We may expect an increase of yields in further generations of tetraploidy, possibly connected with the increment of average heterozygosity.

Further generations of triploids issuing from the same matings will be compared to the "conventional" triploids resulting from diploid parents and thermal shock; the performances of the latter triploids have been extensively evaluated in rainbow trout (Chevassus et al. 1985) and can be summarized as follows: early survival and growth systematically slightly lower than in diploids, but better yields after sexual maturation connected with the complete sterility of the triploid female (the triploid male does not produce viable gametes but exhibits signs of maturation). The conventional triploids have not an optimal heterozygosity – for the reasons mentioned above – this goal being possibly attained in triploids provided by tetraploid males. It is noteworthy and encouraging in this respect that the second generation triploids were better than the diploid and triploid controls in one of the two testing performed.

Such an advantage of these new triploids will have to compensate the waste of ova caused by the low fertilization rates recorded by using tetraploid males. This problem might not occur in the matings between tetraploid females and diploid males, but a given production of triploid fingerlings will in this case involve a much larger tetraploid broodstock, difficult to create in the first generation.

Another problem is the sex ratio of the successive generations of tetraploids produced by the present method. The initial tetraploids issuing from chromosome doubling are certainly $XXYY$ and likely to provide more XY and YY than XX spermatozoas. The male prevalence observed in the second generation of tetraploids of this study is therefore not surprising: simple meiotic models of strict disomy and tetrasomy would respectively lead to all male progeny and 5 males : 1 female ratios. Quite similar ratios would result from more complicated situations of tetrasomy involving exchanges of sex determining factors by crossing-over (Demarly 1963); the 5.5% of females obtained would argue in favor of a preferential pairing of X,X and Y,Y chromosomes respectively, not necessarily with formation of sex tetravalents. The same sex ratios are expected in triploids having the same parents; therefore, most of the tetraploid males in the second generation are supposed to be $XXXY$ and likely to produce more equilibrated sex ratios in the next generations, if they are fertile.

A predominance of males has also been recorded in second generation tetraploids and triploids produced by the same method in two anurans (Kawamura and Nishioka 1967). However, males and females have been obtained in the following generations in *Rana nigromaculata*, while quite all-male progenies were consistently observed in *Rana japonica*. The authors interpret this latter observation by sex reversals occurring in polyploids of this species (as a matter of fact, conventional triploids provided by diploid parents were also all-males). In any case, the female monosexing of trout triploids will be more than ever required if they result from tetraploid males; in this purpose, we intend to initiate the first generations of tetraploids by using diploid XX males issuing from androgen treatment of gynogenetic progenies (Chourrout and Quillet 1982; Chevassus et al. 1984): this would result in a tetraploid $XXXX$ line maintained through sex reversal.

Finally, matings between tetraploid and diploid individuals could be very promising for fishes in which conventional triploidy is not practicable on a large scale: Among them, the

tilapias of commercial interest require a strict control of reproduction (which results otherwise in overpopulation of ponds). In these species, conventional triploids can be induced by a thermal shock applied shortly after fertilization (Valenti 1975; Chourrout and Itskovich 1983). This operation needs a control of the fertilization time and therefore the practice of in vitro insemination, which is still possible only on several individual females. The mass spawning of tetraploid males and diploid females would be the solution in this case. The fact that the resulting fingerlings would be predominantly male in the female homogametic tilapias is of a great interest because the males here grow faster than the females.

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