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Induced triploidy in the common carp (*Cyprinus carpio* L.): a comparison of two methods

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Abstract

Cold shocks and hydrostatic pressure shocks, respectively, were applied for the induction of both triploidy and tetraploidy in the common carp. Cold shocks of 0-2°C, lasting 40 minutes and starting 2-5 minutes after the activation of gametes and hydrostatic pressure shocks with 1-3-5 minute exposures at 49.03-56.88 MPa (*i.e.* 7116-8225 psi – pounds per square inch), starting 5 minutes after the activation of gametes gave the best results. The tetraploidy induction by cold shocks or hydrostatic pressure shocks resulted either in negligible (1.54%), or in zero yields, respectively. The applicability of particular types of ploidy manipulation and the standardization of shock initiation were discussed.

Keywords : Carp, triploidy and tetraploidy induction, cold shocks, hydrostatic pressure shocks.

Triploïdie induite chez la carpe commune (Cyprinus carpio *L.*)*: comparaison de deux méthodes.*

Résumé

Des chocs thermiques froids et des chocs de pression hydrostatique sont appliqués respectivement afin d'obtenir des carpes triploïdes et tétraploïdes. Des chocs donnant les meilleurs résultats sont: les chocs thermiques de 0-2°C, d'une durée de 40 minutes et commençant 2 à 5 minutes après l'activation des gamètes; des chocs de pression hydrostatique, d'une durée de 1, 3 et 5 minutes de 49,03 à 56,88 MPa (équivalents à 7116 et 8225 psi) débutant 5 minutes après l'activation des gamètes. L'induction de la tétraploïdie au moyen de chocs thermique froids est négligeable (1,54 %) ou nulle au moyen de chocs de pression hydrostatique. Les conditions d'application de manipulations en vue d'obtenir des types particuliers de polyploïdie sont discutées ainsi que la standardisation de l'initiation des chocs.

Mots-clés : Carpe, triploïdie, tétraploïdie, induction génétique, chocs thermiques, pression hydrostatique.

INTRODUCTION

Two physical methods, viz. temperature shocks and hydrostatic pressure shocks, have been successfully applied by various authors for the retention of the 2nd polar body during meiosis and for the suppression of chromosome disjunction before the 1st and/or 2nd mitosis. Thorgaard (1983, 1986), Chourrout (1987), Donaldson and Benfey (1987), Benfey (1989) and others reviewed the respective experiments and discussed the possibilities of application of these manipulations in fish culture. It was previously believed, that

heat shocks are more efficient for induction of polyploidy in cold-water fishes than cold shocks and vice versa. However, the experiments of Streisinger *et al.* (1981), Chourrout and Itskovich (1983), Bidwell *et al.* (1985) and Hollebecq *et al.* (1988) have shown, that heat shocks can be efficient in warm-water fishes also.

The optimization of ploidy manipulation parameters (*i.e.* temperature or hydrostatic pressure used, duration of the shock and the timing of the initiation of the shock after gamete activation) in both types of physical shock is the key point of the whole procedure because the egg sensitivity changes rapidly during early embryonic development (Cherfas *et al.*, 1990). In various experiments with ploidy manipulations the fertilized eggs were kept at various temperatures prior to shock which itself has been applied in different time intervals after fertilization. In order to optimize the shock initiation, Gomelsky *et al.* (1989) and Cherfas *et al.* (1990) have tested a dimensionless unit of embryonic age τ_0 [the duration of one mitotic cycle during synchronous cell division (Detlaff and Detlaff, 1961; Ignatyeva, 1974)] related to the water temperature only. Artificial triploidy in common carp or koi carp has been successfully induced in experiments of Ojima and Makino (1978), Gervai *et al.* (1980), Ueno (1984), Taniguchi *et al.* (1986), Wu *et al.* (1986), Hollebecq *et al.* (1988), Linhart *et al.* (1989) and Cherfas *et al.* (1990). Most of these authors used cold shocks, except Hollebecq *et al.* (1988) and Rekoubratsky *et al.* (1989), who used the heat shock. Artificial tetraploidy was successfully induced by Rekoubratsky *et al.* (1989) by heat shock only. To the best of our knowledge, the application of hydrostatic pressure shock has not been used for ploidy manipulation of common carp or other closely related species except by Cassani and Caton (1986) for grass carp.

We report in this paper the results of our experiments with artificial induction of triploidy and tetraploidy in common carp by both cold shocks and hydrostatic pressure shocks.

MATERIAL AND METHODS

All experiments were carried out at the Experimental Station of Research Institute of Fish Culture and Hydrobiology, (RIFCH) at Vodňany in 1987 and 1989. Brood carps, originating from strains kept in RIFCH at Vodňany, were held in warmed water prior to artificial stripping. The duration of this period varied according to the degree of ripeness of the oocytes in females used in single experiments. Fish were stripped by the conventional dry method. Gametes were checked macro- and microscopically in order to exclude sperm with poor motility, sperm contaminated with urine or faeces or overriped oocytes. Heterosperm, after mixing from individual males, was maintained in glass tubes in refrigerator at 4-5°C prior to the experiments. Oocytes mixed from individual females were maintained in plastic bowls covered with a wet cloth in order to prevent drying. Gametes were also collected for estimation of the amount of spermatozoa in 1 ml of sperm and the number of oocytes in 1 g.

Generally, the schedule of all experiments was as follows: oocyte batches (5-10 g) were weighed in glass dishes, sperm was added, mixed and activated with 20 ml of degumming solution consisting of milk: water (1:40). After 2 minutes a more concentrated (1:30) milk solution was added. The batches of eggs were incubated in Kannengieter incubation jars (2 l volume) in non-recycled warmed water at an average temperature of 20°C. Each jar was placed in one aquarium, into which the larvae were transfered after hatching. Just before starting exogenous feeding, the total number of larvae was checked and a representative sample was taken to determine ploidy level by quantification of Ag-stained nucleoli in interphase cells and/or Ag-stained nucleolar organizer regions (NORs) in the metaphase chromosomes. For this determination, the method of Phillips *et al.* (1986) has been utilized but modified according to Flajšhans *et al.* (in press *a*). Briefly, the whole larvae were smeared on ethanol-cleaned microscopic slides in one drop of 60% acetic acid, fixed with methanol, dried and Ag-stained according to Howell and Black (1980), modified by Gold (1984) and Ráb and Roth (1988). Chromosome preparations were made as described by Baksi and Means (1988).

Cold shocks

The experimental batches were treated with crushed ice dispersed in milk solution (1:20) with the temperature 0-2°C. The shocks were initiated from 0 to 65 minutes, respectively, after gamete activation (see *tables* 1 and 2), the exposure lasted for 40 minutes, (Linhart *et al.*, 1987). The average temperature prior to the exposure was 24°C.

Hydrostatic pressure shocks

A hydrostatic pressure device developped for RIFCH at Vodňany by Moravian Chemical Enterprise at Ostrava (courtesy Dr. Theobald Olschak; device under patent control) has been used. In this device pressurized nitrogen is used to provide the hydrostatic pressure, so that the required pressure level can be reached in less than two seconds.

The temperature of water and the milk solution in the pressure chamber ranged from 19 to 21°C. Egg batches were treated in the pressure chamber in bags made from plastic mesh. The eggs were incubated after shocking at an average temperature of 20°C. Three experimental series were realized, in each series one shock parameter (starting time of shock after activation, pressure level, exposure time, respectively) was tested, while the remaining two were kept constant in order to optimize the parameters at the temperature used.

As inexact shocks can significantly affect the survival of incubated eggs, the results reported in this paper were calculated from the number of living eggs at morula stage in each experimental batch as follows.

Living eggs at morula stage:

number of eggs in experimental batch × average percentage of living eggs at morula stage from 5 analyzed random samples.

Percentage of hatched viable fry:

number of hatched viable fry/number of living eggs at morula stage.

Percentage of hatched viable polyploid fry:

percentage of hatched viable fry × percentage of polyploids from the analyzed samples.

RESULTS

Cold shocks

The results of experiments for the induction of triploidy are given in *table* I and in *figures* 1 and 2.

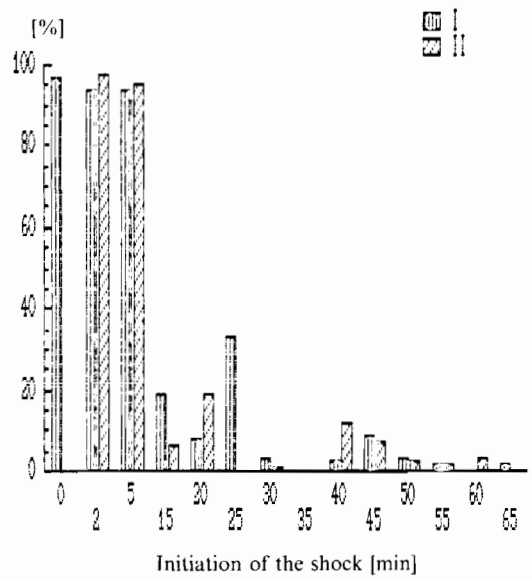

Figure 1. – Cold shocks: percentage of viable fry for two replicated groups (I and II series).

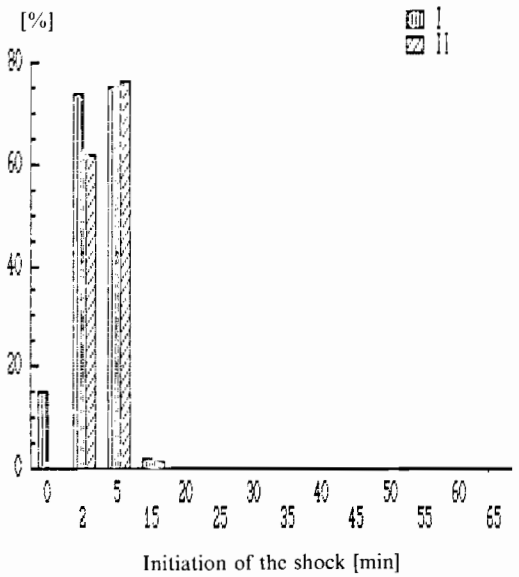

Figure 2. – Cold shocks: percentage of viable triploid fry ($3n$) for two replicated groups (I and II series).

Table I. – Cold shocks.

		Initiation of the shock after gamete activation (min)														
		0	2	5	15	20	25	30	35	40	45	50	55	60	65	C
Living eggs	I.	94.0	88.0	81.0	82.0	61.0	71.0	80.0	55.0	1.0	2.0	5.0	55.0	16.0	6.0	–
at morula stage (%)	II.	–	86.0	86.0	89.0	85.0	83.0	82.0	66.0	1.0	4.0	28.0	17.0	8.0	–	93.0
Viable fry (%) *	I.	93.56	93.56	93.42	18.98	7.68	33.32	3.44	0.32	2.42	8.64	3.00	1.79	0.38	1.85	–
	II.	–	97.68	94.96	6.45	18.67	0.34	1.15	0.29	12.12	7.18	2.22	1.45	3.43	–	95.5
Viable 3n fry (%) *	I.	15.26	73.87	74.74	2.11											
	II.	–	61.69	75.97	1.38		0.05									
Viable 3n fry (%) **	I.	15.79	78.95	80.00	11.11											
	II.	–	63.16	80.00	21.43		5.00									
Viable 4n fry (%) *	I.														0.13	
	II.												0.22	1.54		
Viable 4n fry (%) **	I.														7.14	
	II.												15.00	45.00		

Numbers I, II are numbers of experimental series.
C is the abbreviation of control group (untreated group).
* percentage was calculated from the percentage of living eggs at morula stage.
** percentage was calculated from the absolute number of viable fry.

The best results were obtained after shocks 2 and 5 minutes after activation of gametes *i.e.* 93.56 and 97.68% of hatched viable fry with 73.87 and 61.69% of hatched viable triploid fry, respectively for two replicated groups shocked after 2 minutes; and 93.42 and 94.96% of hatched viable fry with 74.74 and 75.97% of hatched viable triploid fry, respectively, for the shock after 5 minutes (*table* 1, *fig.* 2). If expressed in τ_0 unit, it equals 0.1 and 0.26 at the incubation water temperature of 24°C for 2 and 5 minutes shocks, respectively.

The percentage success for the induction of tetraploidy proved to be negligible (*e.g.* 1.54, 0.22 and 0.13% of hatched viable tetraploid fry from cold shocks after 55,60 and 65 minutes of incubation, respectively) and they were reached in one experiment only with no successfull replication (*table* 1, *fig.* 3). However, these tetraploids were safely produced by cold shock, according to the fact, that nothing but diploids were found in the respective control groups.

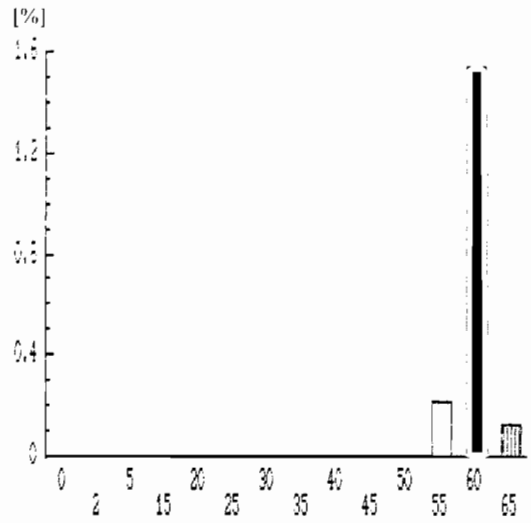

Figure 3. – Cold shocks: percentage of viable tetraploid fry (4*n*).

Hydrostatic pressure shocks

The results of experiments for the induction of triploidy are given in *table* 2, *in figures* 4 and 5. The best results (63.49% and 52.29% of hatched viable triploid fry) were obtained using the shortest exposure

Table 2. Hydrostatic pressure shocks, 5 min after gamete activation.

Experiment No.	Pressure (MPa)	Exposure (min)	Living eggs at morula stage (%)	Viable fry (%) *	Viable 3*n* fry (%) *	Viable 3*n* fry (%) **
1	50.99	0.03	22.0	82.35	20.59	25.0
2	10.79	1	10.5	19.52	0	0
3	20.10	1	7.5	21.22	0	0
4	30.89	1	8.0	18.75	0	0
5	39.23	1	13.5	56.60	11.79	20.83
6	49.03	1	5.5	1.09	0	0
7	51.98	1	6.5	18.46	7.69	41.67
8	52.96	1	24.0	76.74	19.19	25.0
9	56.88	1	29.0	61.01	52.29	85.7
10	49.03	2	19.5	76.28	30.51	40.0
11	49.03	3	13.5	80.19	63.49	79.17
12	48.25	4	13.5	49.38	39.50	80.0
13	47.37	5	15.5	50.65	42.21	83.33
14	45.60	6	9.5	28.16	23.26	82.0
15	44.13	7	5.0	17.17	13.44	78.26
16	43.15	8	6.0	54.03	36.02	66.67
17	55.90	10	0	0	0	0
18	9.81	20	4.5	0	0	0
19	20.59	20	8.0	82.18	60.07	73.10
20	30.40	20	3.5	14.97	0	0
21	39.72	20	2.5	0	0	0
22	50.99	20	0	0	0	0
23	51.98	20	0	0	0	0
24	53.94	20	0	0	0	0
25	53.94	30	0	0	0	0
26	55.90	40	0	0	0	0
C1		--	39.0	76.3	–	–
C2	–	–	24.6	94.08	–	–

C is the abbreviation of control group (untreated group, 2 replications).

* percentage was calculated from the percentage of living eggs at morula stage.

** percentage was calculated from the absolute number of viable fry.

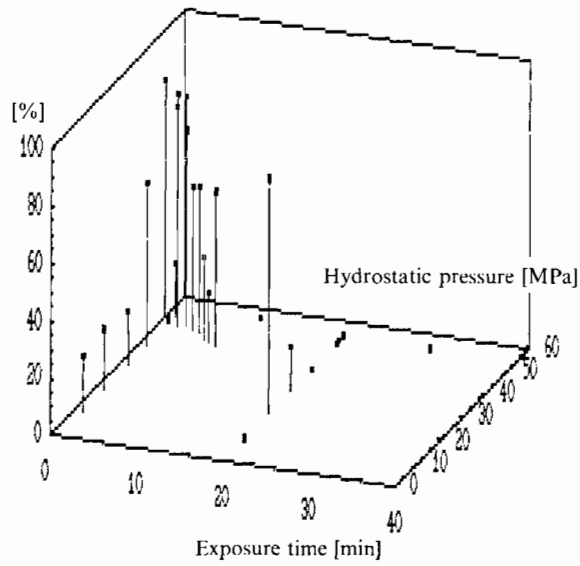

Figure 4. – Hydrostatic pressure shocks related to exposure time: percentage of viable fry.

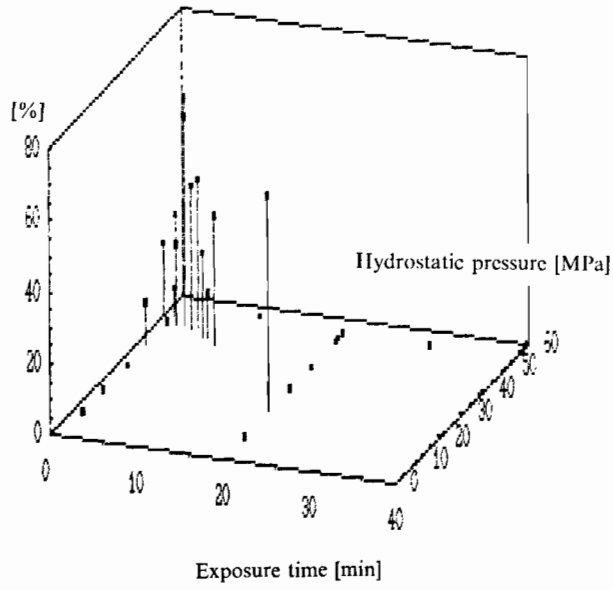

Figure 5. – Hydrostatic pressure shocks: percentage of viable triploid fry (3 *n*).

times (1-3-5 minutes) for the highest hydrostatic pressure levels (49.03-56.88 MPa, *i.e.* 7116-8255 psi-pounds per square inch) after 5 minutes incubation, *i.e.* in 0.17 τ_0 unit at an incubation water temperature of 20°C. Shocks starting after 2, 10, 15, 20, 25 and 30 minutes incubation did not result in any viable fry at all. Shocks with long exposure times at lower pressure levels did not result in significant numbers of hatched viable fry or in triploids at all, except for 60.07% triploids from the shock at 20.59 MPa for 20 minutes exposure and for 14.97% viable fry after 20 minutes exposure to a shock of 30.40 MPa. However, these data were not repeatable.

Hydrostatic pressure shocks of 49.03 MPa, starting at 40, 45 and 50 minutes after gamete activation, *i.e.* at 1.43; 1.61 and 1.79 τ_0 and lasting 1 minute were not successful for the induction of tetraploidy in any of our experiments.

DISCUSSION

The fertilization rates in each experiment were theoretically at the same level as in control groups and they were reduced to the observed level by the shock treatments (*tables* 1 and 2). We tried to transform the results of the experiments to a mode, which would reflect the correlation between the fertilization rate and the influence of the shock treatment itself *i.e.* the survival, expressed in the percentage of hatched viable fry and the "yield", expressed as the percentage of hatched viable polyploid fry. Therefore, the percentage of hatched viable polyploid fry was calculated from the number of living eggs at the morula stage and not directly from the number of hatched viable fry; the latter mode of calculation was taken only as a subsidiary item (*tables* 1 and 2).

Cold shocks

If the results of previously published cold shock experiments are transformed to τ_0 parameter, the data for the retention of the second polar body range from 0.03 (Cherfas *et al.*, 1990) to 0.45 (Taniguchi *et al.*, 1986), with the peak in 0.03-0.32 interval (Gervai *et al.*, 1980; Ueno, 1984; Taniguchi *et al.*, 1986; Linhart *et al.*, 1989; Cherfas *et al.*, 1990). The data of our experiments (0.1 and 0.26 τ_0 for shocks at 2 and 5 minutes, respectively) thus correspond with them.

The best data for suppression of the first mitosis were reported by Rekoubratsky *et al.* (1989) at 1.7 τ_0. In our experiments hatched viable tetraploid fry were produced at a very low percentage (up to 1.54%) and without successfull replication, if expressed in τ_0 unit, it ranged from 2.89 to 3.42. We suppose, that this could be a result of suppression of the second mitosis, while shocks applied before the first mitosis did not induce tetraploidy at all.

Hydrostatic pressure shocks

As no data on the application of hydrostatic pressure shocks for induction of polyploidy in carp are available, we have arranged our experiments according to Cassani and Caton (1986). It is evident from *figures* 4 and 5, that triploidy inducing shocks with the shortest exposure times for the highest pressure levels applied shortly after the gamete activation are

more efficient than longer shocks with lower pressure. The observation of 60.07% hatched viable triploids after 20 minutes shock of 20.59 MPa may be a result of a mistake in a process of fish handling (e.g. replacement of aquaria, etc.).

Hydrostatic pressure shocks were not successful for the induction of tetraploidy in carp in any of our experiments. In contrast, very similar experiments on tench (*Tinca tinca* L., Cyprinidae) resulted in significant numbers of tetraploids (up to 50%) by the supression of the first mitosis (Flajšhans *et al.*, in press *b*).

Hydrostatic pressure shocks do not seem to be the most efficient way for induction of triploidy in carp, not only due to lower yields reached, but also due to difficulties with handling during the treatment process. In previous experiments, where the pressure chamber was filled with water, all eggs of each treated batch were stuck together and it was not easy to release them, therefore we filled the chamber with degumming milk: water (1 : 20) solution to reduce the stickness of the eggs, which is the strongest in the first minutes after gamete activation, when contemporarily the triploidy-inducing treatments start. This arrangement proved to be partially successful.

We believe that temperature shocks are more effective than hydrostatic pressure shocks in carp, especially the heat shocks, with shock exposures causing lower mortality (Hollebecq *et al.*, 1988; Gomelsky, pers. comm.). For the standardization and the comparison of data on shock initiation, the application of the τ_0 unit (Detlaff and Detlaff, 1961; Ignatyeva, 1974; Gomelsky *et al.*, 1989 Cherfas *et al.*, 1990) seems to be essential.

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REFERENCES

Baksi S. M., J. C. Means, 1988. Preparations of chromosomes from early stages of fish for cytogenetic analysis. *J. Fish Biol.*, **32**, 321-325.

Benfey T. J., 1989. A. bibliography of triploid fish, 1943 to 1988. *Can. Tech. Rep. Fish. Aquat. Sci.*, **1682**, 33 p.

Bidwell C. A., C. L. Chrisman, G. S. Libey, 1985. Polyploidy induced by heat shock in channel catfish. *Aquaculture*, **51**, 25-32.

Cassani J. R., W. E. Caton, 1986. Efficient production of triploid grass carp (*Ctenopharyngodon idella*) utilizing hydrostatic pressure. *Aquaculture*, **55**, 43-50.

Cherfas N. B., O. Kozinsky, S. Rothbard, G. Hulata, 1990. Induced diploid gynogenesis and triploidy in ornamental (koi) carp, *Cyprinus carpio* L. I. Experiments on the timing of temperature shock. *Bamidgeh*, **42**, 3-9.

Chourrout D., 1987. Genetic manipulation in fish: review of methods. *In*: FAO/EIFAC Symp. Selection, Hybridization, and Genetic Engineering in Aquaculture, Bordeaux, 1986. Heenemans Verlags, Berlin, Vol. **II**, 111-126.

Chourrout D., J. Itskovich, 1983. Three manipulations permitted by artificial insemination in tilapia: induced diploid gynogenesis, production of all triploid populations and intergeneric hybridisation. *In*: Int. Symp. on Tilapia in Aquaculture. L. Fishelson, Z. Yaron Eds., Tel Aviv Univ., Tel Aviv, 246-255.

Dettlaff T. A., A. A. Dettlaff, 1961. On relative dimensionless characteristics of the development duration in embryology. *Arch. Biol.* (Liège), 72, 1-16.

Donaldson E. M., T. J. Benfey, 1987. Current status of induced sex manipulations. *In*: Proc. Third Int. Symp. on the Reproductive Physiology of Fish, D. R. Idler, L. W. Crim, J. M. Walsh Eds., Memorial Univ. of Nfld., St John's, 108-119.

Flajšhans M., P. Ráb, S. Dobosz (in press *a*). Frequency analyses of active NORs in nuclei of artificially induced triploid fish. *Theor. Appl. Genet.*

Flajšhans M., O. Linhart, P. Kvasnička, P. Ráb (in press *b*). Methods of artificial induction of triploidy and tetraploidy in tench (*Tinca tinca* L.). *In*: 4th Int. Symp. on Genetics in Aquaculture, Wuhan, China, April 29-May 3, 1991.

Gervai J., S. Péter, A. Nagy, L. Horvath, V. Csányi, 1980. Induced triploidy in carp, *Cyprinus carpio* L. *J. Fish Biol.*, **17**, 667-671.

Gold J. R., 1984. Silver staining and heteromorphism of chromosomal nucleolus organizer regions in North American cyprinid fishes. *Copeia*, **1**, 133-139.

Gomelsky B. I., A. B. Rekubratsky, O. V. Emelyanova, E. V. Pancratyeva, T. I. Lekontzeva, 1989. Obtaining of carp diploid gynogenetic offspring by heat shock on developed eggs. *Voprosy Ichthyology*, **28**, 168-170 (in Russian).

Hollebecq M. G., F. Chambeyron, D. Chourrout, 1988. Triploid common carp produced by heat shock. *In*: Reproduction in fish-Basic and applied aspects in endrocrinology and genetics, Tel Aviv, Israel, 1986. Les Colloques de l'INRA, Ed. INRA, Paris, **44**, 208-212.

Howell W. M., D. A. Black, 1980. Controlled silver staining of nucleolus organizer with a protective colloidal developer: a 1-step method. *Experientia*, **36**, 1014-1045.

Ignatyeva G. M., 1974. Dependence of cleavage rate on temperature in the carp, the pike and the peled. *Ontogenez* (USSR), **5**, 27-32 (in Russian with English summary).

Linhart O., V. Šlechtová, P. Kvasnička, P. Ráb, J. Kouřil, J. Hamáčková, 1987. Rates of recombination in LDH B^1 and MDH loci phenotypes after "pb" and "m" gynogenesis in carp, *Cyprinus carpio* L. *In*: FAO/EIFAC Symp. on Selection, Hybridization, and Genetic Engineering in Aquaculture, Bordeaux, 1986. Heenemans Verlag, Berlin, Vol. **II**, 335-345.

Linhart O., V. Šlechtová, P. Kvasnička, P. Ráb, I. Přikryl, 1989. Chromosome manipulations in tench (*Tinca tinca* L.) and carp (*Cyprinus carpio* L.) in Czechoslovakia. Papers of RIFCH Vodňany, **13**, 53-60.

Ojima Y., S. Makino, 1978. Triploidy induced by cold shock in fertilized eggs of the carp. A preliminary study. *Proc. Jpn. Acad.*, **54**, Ser. B., 359-362.

Phillips R. B., K. D. Zajicek, P. E. Ihssen, O. Johnson, 1986. Application of silver staining to the identification of triploid fish cells. *Aquaculture*, **54**, 313-319.

Ráb P., P. Roth, 1988. Methods of chromosome analyses. Cold-blooded vertebrates. *In*: Cytogenet. Sekce Čs. Biol. spol., ČSAV, Brno, 115-124 (in Czech).

Rekoubratsky A. V., B. I. Gomelsky, O. V. Emelyanova, E. V. Pankratyeva, 1989. Obtaining triploid and tetraploid carp progeny with heat shocks. *In*: Sbornik naucnych trudov voprosy selekcii, genetiki i plemennogo dela v rybovodstve. VNIIPRCH, Moscow, 91-95 (in Russian with English summary).

Streisinger G., C. Walker, N. Dower, D. Knauber, F. Singer, 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature*, **291**, 293-296.

Taniguchi N., A. Kijima, T. Tamura, K. Takegami, I. Yamasaki, 1986. Color, growth and maturation in ploidy manipulated fancy carp. *Aquaculture*, **57**, 321-328.

Thorgaard G. H., 1983. Chromosome set manipulation and sex control in fish. *In*: Fish Physiol., W. Hoar, D. J. Randall and E. M. Donaldson Eds. Academic Press, N. Y., **9**, 405-434.

Ueno K., 1984. Induction of triploid carp and their haematological characteristics. *J. Genet.*, **59**, 585-591.

Wu C., Y. Ye, R. Chen, 1986. Genome manipulation in carp (*Cyprinus carpio* L.). *Aquaculture*, **54**, 57-61.