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Optimization of Chromosome Set and Sex Manipulations in Common Carp, *Cyprinus carpio* L.

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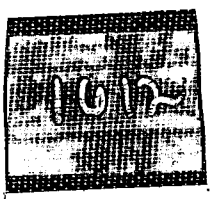

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Title:

"OPTIMIZATION OF CHROMOSOME-SET AND SEX MANIPULATIONS IN THE COMMON CARP *Cyprinus carpio* L."

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ABSTRACT

The optimum temperature shock timings for the suppression of meiosis II and the 1st cleavage were determined and standardized, using the relative unit of embryological age (τ_0). The highest production of 2n meiotic gynogenetic (or 3n) and of 2n mitotic gynogenetic (or 4n) larvae can be expected when heat shock is applied, respectively, at 0.10-0.25τ_0 and at 1.5τ_0 , after insemination. The optimum timings for cold shock initiation (investigated for meiosis II only) corresponds to 0.05-0.10τ_0 and to 0.30-0.40τ_0, respectively. The dynamics of survival and of 2n gynogens production have opposing tendency in cold and heat shocked progenies, suggesting that different intracellular mechanisms are responsible for the effects of high and low temperatures. Hydrostatic pressure shock for induction of 2nd polar body gynogens was most successful when applied at 7500-8000 PSI for 1 min between 0.25-0.30τ_0 (preliminary results). Meiotic and mitotic gynogens and 3n of Israeli common carp were mass produced (in heat shock experiments) and reared to adult stages. No advantage of culturing triploid common carp over diploid common carp was observed. All tetraploid fish obtained died, and the question whether or not it is possible to obtain adult tetraploid common carp remains unsolved.

The phenomenon of spontaneous diploidization in koi was shown to have a hereditary nature. Some transformation in early meiotic stages may, apparently, be responsible for unreduced eggs production.

Density-dependent growth affected gonadal differentiation. In general, stages are reached and passed at a younger age but at a larger size among more rapidly growing fish and vice versa. The methyltestosterone immersion treatments were ineffective in altering the sex ratio of any treatment group. Higher effectiveness of oral androgen treatment in recirculation water systems (than in the tanks with running water) was confirmed: the frequency of inverted males was 47-96% and 20-44%, respectively, in gynogenetic treatment groups under the two conditions.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The general objective of the research proposal was the further development of the methods of induced gynogenesis, polyploidy and normonal sex inversion in common carp *Cyprinus carpio* L., the important object of aquaculture. Each component has previously been accomplished for the common carp but the various phases lack optimization which has been an impediment to programmatic development and pragmatic application of these methods in the fish culture industry.

The main topics focused on in this research were:

1. Optimization of gynogenesis and polyploidy treatments for common carp, and, primariliy, further development of the "temperature shock" treatments which are used for inducing chromosome-set diploidization in various stages of early embryogenesis. These investigations included:

   a) Determination of the optimum temperature shock timing during the 2nd meiotic division and the 1st cleavage; evaluation of effectiveness of hydrostatic pressure for inducing 2nd polar-body retention; examination of the possibility to standardize shock timing by using the relative measure of embryological age τ_0.

   b) Specifying the protocols for production of gynogenetic and polyploid common carp progenies on a large scale;

2. Investigation of the phenomenon of spontaneous diploidization in ornamental (koi) common carp, including:

   a) Study of previously obtained spontaneous gynogenetic offspring;

   b) Testing of hereditary control of this phenomenon and its cytogenetic features.

3. Optimization of methods of hormone-induced sex reversal in common carp females, including:

   a) Evaluation of the effectiveness of hormone-induced sex reversal by immersion treatment, as an alternative to oral administration of androgen;

   b) Further development of a protocol for oral androgen treatment;

4. Production and culture evaluation of diploid and triploid unisexual (all-female) common carp progenies.

The investigations were carried out according to the project's program, and most of the planned tasks have been fulfilled. The following deviations from the research plan should be noted:

Induced gynogenesis and polyploidy

Evaluation of pressure shock effectiveness (U.S.A.) was delayed until year 3 because of technical complications in developing the equipment. Only preliminary trials were accomplished during the study. Karyological investigations (Israel) were substituted for less laborious flow cytometry analyses.

Investigations of spontaneous diploidization phenomenon (Israel)

Only part of the planned crossings were carried out due to death of some breeders.

Investigations on hormone-induced sex inversion (U.S.A.) were carried on bisexual common carp progenies, due to failure of obtaining unisexual gynogenetic progenies on a large scale.

Investigations on culture assessment of sterile triploid fish (Israel) were carried out on bisexual (but not unisexual) triploid progenies, since the latter have not been obtained; obtaining of unisexual diploid progenies (Israel) was delayed until year 3 due to the unfavourable weather conditions in 1992, thus the data obtained are restricted to the observations on fingerlings only (final results will be obtained in 1994, i.e., beyond project's termination).

BODY OF REPORT

Introduction

The BARD project "Optimization of chromosome set and sex manipulations in the common carp, *Cyprinus carpio* L." was aimed at further development of the methods for induced diploid gynogenesis, induced polyploidy and hormonal sex reversal techniques in the common carp. Theoretical and practical importance of these techniques were discussed in many reviews (e.g., Kirpichnikov, 1981; Thorgaard; 1983; Shelton, 1986, 1990; Nagy, 1987). The most important applied problems which could be solved by these methods are: obtaining unisexual diploid and/or triploid (sterile) progenies for the management of unwanted reproduction in grow-out ponds; increasing growth potential of carp in Israel by using all-female progenies; fast creation of homozygous progenies (with inbreeding coefficient =1); obtaining isogenic progenies, i.e., lines and clones whith fixed valuable traits. Some important theoretical problems in the field of fish cytogenetics and selection (e.g., gene mapping, estimation of inbreeding depression, analysis of chromosome constitution of sex) can also be investigated by these methods. Although there are a large number of investigations on induced of gynogenesis, polyploidy and sex reversal in the common carp, some important questions concerning these methods were not clear, and the data obtained appeared contradictory in some cases. The present project was aimed at closing some of these gaps.

According to the project program, the investigations were carried out in two principal directions. One was devoted to further elaboration of the methods of induced gynogenesis and polyploidy, and also to culture evaluation of progenies obtained by these methods. These investigations were carried out primarily in Israel. The second dealt with optimization of sex reversal techniques for the common carp, needed for propagation of all-female gynogenetic lines. These investigations were carried out primarily in the U.S.A.

Results

1. Induced diploid gynogenesis and polyploidy in ornamental (koi) carp *Cyprinus carpio* L.

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Induced diploid gynogenesis and polyploidy in ornamental (koi) carp, *Cyprinus carpio* L. 2. Timing of heat shock during the first cleavage

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ABSTRACT

Cherfas, N.B., Hulata, G. and Kozinsky, O., 1993. Induced diploid gynogenesis and polyploidy in ornamental (koi) carp, *Cyprinus carpio* L. 2. Timing of heat shock during the first cleavage. *Aquaculture*, 111: 281–290.

Experiments on timing of heat shock during the first cleavage in order to optimize induced diploidization of the chromosome set were conducted on ornamental (koi) carp, *Cyprinus carpio* L. The effect of heat shock on embryo survival and ploidy level was monitored in gynogenetic offspring and regular ones. Dimensionless measure of embryological age τ_0 was used to express the time of heat shock application. The heat shock was initiated within the limits of 0.6–3.0 τ_0 (0.9–2.0 τ_0 in most experiments) after insemination. Incubation temperature prior to treatment was either 25°C or 20°C. Water temperature of heat shock was 39.3–40.5°C, and duration 2–3 min, in different experiments. Embryo survival is characterized by alternating "sensitive" and "non-sensitive" periods with lowest survival when heat shock initiation corresponds to 1.6–1.8 τ_0. The highest frequency of 2n mitotic gynogenetic embryos (up to 15% relative to total number of inseminated eggs) was observed when heat shock was initiated at 1.5–1.6 τ_0.

INTRODUCTION

The elaboration of the method for the suppression of the first cleavage is a major component of studying chromosome set manipulations in fishes. This method allows completely homozygous specimens (in the case of induced diploid gynogenesis) and tetraploid specimens (in the case of normal fertilization) to be obtained. Both propagation systems are of great interest for practical and theoretical studies on fishes (Chourrout, 1987; Nagy, 1987). Mitotic gynogens and tetraploids have already been obtained in different fish species including aquacultured and non-commercial ones.

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Studies dealing with suppression of the first cleavage in the common carp have been conducted during recent years (Linhart et al., 1987; Nagy, 1987; Gomelsky et al., 1989; Recubratsky et al., 1989; Sumantadinata et al., 1990; Komen et al., 1991). Heat shock was used to suppress the first mitotic division in most experiments (except the investigation of Linhart et al. in which cold shock was applied). Diploid gynogenetic larvae or tetraploids were obtained in shock-treated offspring, and thus the efficiency of heat shock was confirmed.

Although some positive results from applying heat shock were obtained, the information concerning the time of heat shock initiation is still insufficient. The optimal timing of heat shock during the first cleavage was the main objective of this work.

MATERIALS AND METHODS

The experiments were conducted at the Fish and Aquaculture Research Station, Dor, from March to July 1990. Females of orange colored ornamental (koi) carp and males of wild type colored local edible carp were used as breeders. Standard methods of artificial propagation of common carp were applied.

Eggs were incubated in Petri dishes placed on trays within a circulating water system. Eggs of one female and sperm of several (usually 3–4) males were used in each experiment. The timing of heat shock was investigated in regular (intact eggs×intact sperm) and gynogenetic (intact eggs×genetically inactivated sperm) offspring. The method for obtaining gynogenetic offspring was described by Cherfas et al. (1990). Gynogenetic origin of the offspring was verified by the absence of wild type dominant color in the larvae.

The effect of heat shock applied at different phases of the first cleavage was monitored. Time of shock initiation (i.e. embryological age) was expressed in the relative dimensionless unit τ_0 which is equivalent to the duration of one mitotic cycle during synchronous cell divisions in the initial stages of embryogenesis (Dettlaff and Dettlaff, 1961). According to the theory, a certain embryological age expressed in τ_0 is a constant value (within the limits of the temperature optimum for a given species). The second meiotic division in the common carp is completed at 0.6 τ_0 and the first cleavage at 2.1–2.3 τ_0 after insemination (Ignatieva, 1979; Altasei, 1985, cited by Gomelsky et al., 1989).

Heat shock was applied from 0.6 to 3.0 τ_0 after insemination in experiments on regular offspring and from 0.9 to 2.0 τ_0 after insemination in experiments on gynogenetic offspring (at 0.1 τ_0 intervals in all experiments). The absolute time of shock initiation was calculated from the duration of one τ_0 at the water temperature prior to shock of 20°C or 25°C, which is equal to 29 min and 18 min, respectively (Ignatieva, 1974).

Small portions of inseminated eggs were placed every 0.1 τ_0 on separate Petri dishes and incubated at 25°C (or 20°C) prior to heat shock. After that, all Petri dishes were subjected simultaneously to heat shock by moving the trays carrying the Petri dishes to a hot bath. Thus, the time of heat shock initiation for the first and last Petri dishes corresponded to the end and the beginning of the investigated period of embryogenesis, respectively (Fig. 1). Water temperature during heat shock was 39.3–40.5°C, and shock duration 2–3 min, in different experiments. These values were selected on the basis of previous investigations (Nagy, 1987; Gomelsky et al., 1989; Komen et al., 1991).

The effect of heat shock on embryo survival was monitored in experiments on regular offspring (exp. 1 and 2; 20°C prior to heat shock), gynogenetic offspring (exp. 3 and 6; 25°C prior to heat shock) and both regular and gynogenetic offspring (exp. 4 and 5; 25°C prior to heat shock).

The influence of heat shock on ploidy level was estimated by the output of diploid larvae in the gynogenetic shock-treated offspring. In addition, the number of nucleoli per nucleus, as ploidy level index (Cherfas, 1975; Cherfas et al., 1990), was examined in 2-day-old post-hatch gynogenetic embryos (exp. 3) and shock-treated regular embryos (exp. 4).

The correspondence between τ_0 values and successive phases of the first mitotic division was investigated by histological examination (standard method).

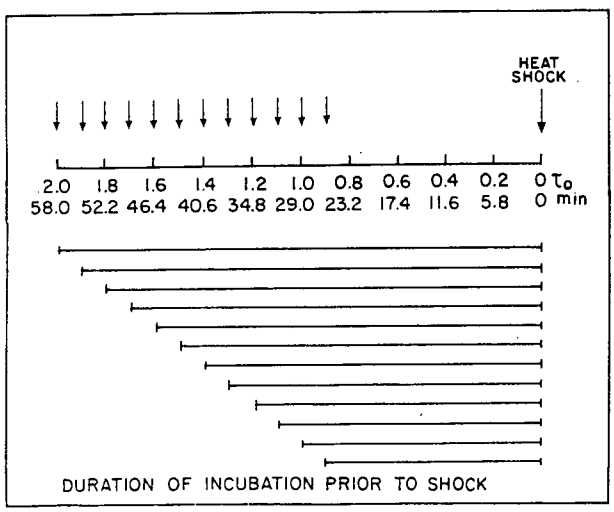

Fig. 1. An example of schedule for heat shock treatments. Here heat shock was initiated between 0.9 and 2.0 τ_0 after insemination at 0.1 τ_0 intervals. Time of insemination and the duration of incubation prior to heat shock are indicated, for each egg sample, by an arrow and a horizontal line below, respectively. The corresponding time for heat shock initiation in min is given for the pretreatment water temperature of 20°C and the duration of one τ_0 29 min.

RESULTS

Survival and normal embryo production

The survival of embryos in regular offspring prior to hatching was characterized by alternating "sensitive" and "non-sensitive" periods (Fig. 2). A sharp decrease in survival was observed when heat shock was initiated at 1.6–1.8 τ_0 after insemination in all shock-treated offspring, independently of the total sensitivity to heat shock in the eggs (embryos) of different females. Embryo survival was high (sometimes at the level of the control) when heat shock initiation corresponded to 1.0–1.4 τ_0 and 2.0–2.4 τ_0 after insemination. The output of morphologically normal post-hatch embryos was very low, even in the periods characterized by high embryo survival prior to hatching (the highest value was 10% at 2.1 τ_0 in exp. 4).

The survival curves obtained at the two different pretreatment water temperatures were almost identical for shock initiation up to 1.7–1.8 τ_0 after insemination, and the beginning of the most sensitive period coincided in these

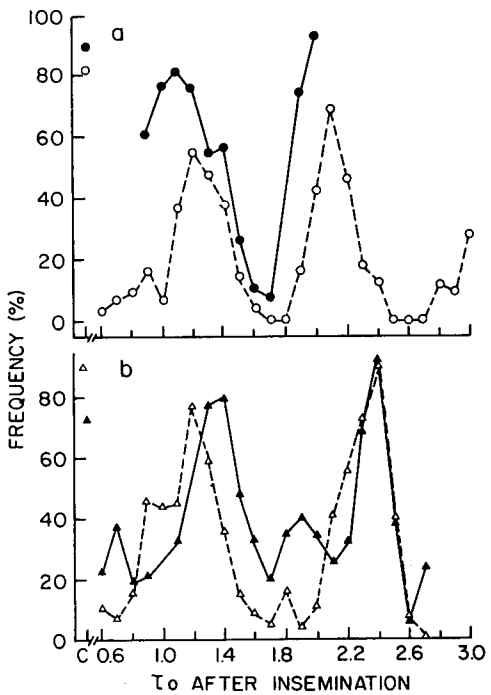

Fig. 2. Survival of shock-treated embryos prior to hatching in experiments on regular offspring: (a) exp. 4 (○) and 5 (●); (b) exp. 1 (△) and 2 (▲). Water temperature prior to heat shock was 25°C (a) and 20° (b). C on the abscissa indicates the control, without heat shock.

cases. Some discrepancy was observed when shock was initiated at the later stages (Fig. 2).

The dynamics of survival of the gynogenetic shock-treated embryos was, as a whole, similar to that of the regular shock-treated offspring (Table 1; Fig. 3). Heat shock initiated at 1.7–1.8 τ_0 resulted in death of almost all embryos. In exp. 3 the drop in survival was observed at 1.8–1.9 τ_0.

Apparently normal (i.e. diploid) larvae were regularly detected in gynogenetic shock-treated offspring, in contrast to gynogenetic offspring obtained without thermal shock (Table 1; Fig. 3). They were found in all experiments when heat shock was initiated at 1.3–1.6 τ_0. Heat shock applied later or earlier was not effective in most cases. The highest output of normal mitotic gyno-

TABLE 1

Experimental data of incubation of the gynogenetic shock-treated offspring (25°C prior to heat shock)

	Initiation of shock (τ_0 units) after insemination													
Estimate	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	G*	C**
Experiment 3. 39.8°C; 3 min***														
Total no. of inseminated eggs	–	–	–	–	–	–	252	153	251	128	150	167	236	207
Survival (%) prior to hatching	–	–	–	–	–	–	43	24	18	4	2	8	85	95
Total number of normal embryos	–	–	–	–	–	–	37	12	15	0	0	0	0	182
Experiment 4. 39.8°C; 3 min														
Total no. of inseminated eggs	161	224	213	197	217	145	266	234	211	221	211	218	211	218
Survival (%) prior to hatching	3	4	4	10	22	32	19	5	0	0	0	8	75	94
Total number of normal embryos	0	0	0	0	3	4	4	2	0	0	0	0	1	200
Experiment 5. 39.5°C; 3 min														
Total no. of inseminated eggs	236	227	190	214	276	218	191	216	201	231	197	145	220	305
Survival (%) prior to hatching	12	34	38	46	43	28	16	6	1	1	6	12	73	91
Total number of normal embryos	0	0	0	0	2	2	6	1	0	0	0	0	0	190
Experiment 6. 39.3°C; 3 min														
Total no. of inseminated eggs	380	366	341	227	298	341	242	252	287	230	122	134	296	340
Survival (%) prior to hatching	66	58	50	51	66	49	43	11	2	21	46	56	72	79
Total number of normal embryos	2	2	1	3	7	14	15	7	0	0	0	0	0	206

*G — Gynogenesis without heat shock. **C — Control (intact offspring). ***Period only from 1.5 τ_0 to 2.0 τ_0 was investigated in exp. 3.

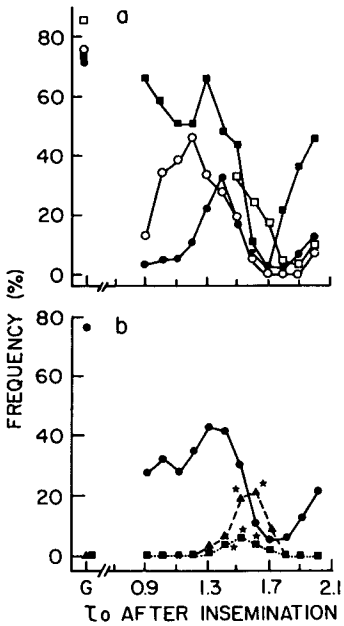

Fig. 3. Experiments on gynogenetic offspring. (a) Survival in shock-treated embryos prior to hatching, in four experiments: (□) exp.3, (○) exp.4, (●) exp.5, (■) exp. 6. The incubation water temperature was 25°C prior to heat shock in all experiments. (b) Mean values of survival prior to hatching (●) and output of apparently normal embryos relative to the number of live embryos prior to hatching (▲) or to the number of inseminated eggs (□). G — control, gynogenesis without heat shock. Data points with asterisk are significantly different from the no-heat-shock control (G) values.

gens was observed after heat shock initiation at 1.5 τ_0 and 1.6 τ_0. It varied in different experiments, with mean values of about 6% (1.5 τ_0) and 3% (1.6 τ_0) relative to total number of inseminated eggs or 19% (1.5 τ_0) and 21% (1.6 τ_0) relative to number of live embryos prior to hatching. The best result was equal to 14.6% and 34.2%, respectively (exp.3, 1.5 τ_0).

Histological data indicated that the period of 1.5–1.6 τ_0 corresponds to metaphase of the first mitotic division.

Nucleoli per nucleus

Results of cytological analysis are summarized in Table 2 and Fig. 4. As was determined earlier, the mean number of nucleoli per nucleus in diploid embryos in common carp is equal to 1.8 (i.e. 0.9 per genome), and haploid embryos have one nucleolus per nucleus (Cherfas, 1975; Cherfas et al., 1990). The control individuals in exp. 3 and 4 had cells with two (rarely one) nucleoli with a mean number of 1.78–1.79 nucleoli per nucleus (i.e. "diploid number"); Fig. 4c,e.

All the gynogenetic embryos obtained without thermal shock in exp. 3 had

TABLE 2

Number of nucleoli per nucleus in some shock-treated and control offspring (100 cells scored for each individual)

Type of offspring	Mean ± s.e.	Range	CV	No. of embryos	Interpreted as
Exp. 3					
Control	1.79 ± 0.01	1.64–1.91	3.93	27	2n
Gynogenesis:					
abnormal[1]	1.01 ± 0.00	1.00–1.05	1.60	30	n
abnormal[2]	1.75 ± 0.02	1.49–2.01	5.08	28	2n[3]
Exp. 4					
Control	1.78 ± 0.01	1.67–1.91	3.65	31	2n
Shock-treated:					
normal[4]	3.33 ± 0.05	2.36–3.59	7.41	29	3n, 4n, mosaics
abnormal[4]	3.28 ± 0.05	2.75–3.83	7.90	21	3n, 4n, mosaics
abnormal[5]	3.20 ± 0.05	2.64–3.46	7.15	21	3n, 4n, mosaics

[1]Without shock.
[2]Heat shock at 1.5–2.0 τ_0.
[3]One haploid embryo was also found (see text).
[4]Heat shock at 1.5 τ_0; one diploid embryo was also found in this offspring.
[5]Heat shock at 1.6 τ_0.

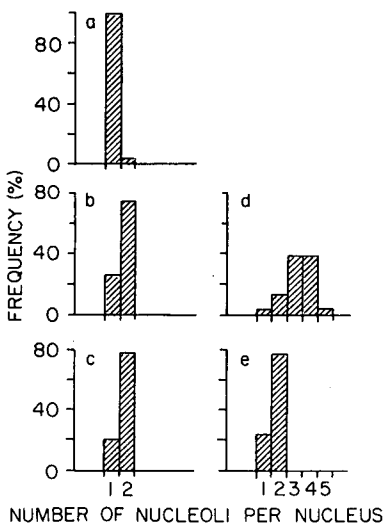

Fig. 4. Mean proportions of cells having various numbers of nucleoli per nucleus in embryos of the shock-treated and control offspring. a, b, c — exp. 3: (a) gynogenesis without heat shock; (b) shock-treated gynogenetic offspring (abnormal embryos); (c) control regular embryos. d, e — exp.4: (d) shock-treated regular offspring, (e) control embryos.

one nucleolus per nucleus and thus were haploids (Fig. 4a). Among the 29 investigated shock-treated gynogenetic embryos, suspected to be haploids due to abnormal morphological appearance, only one was haploid as judged by nucleoli number. The rest were diploids, and did not differ from the control (Fig. 4b).

Only one of 71 investigated embryos was diploid in the regular shock-treated offspring (exp. 4). All others differed from their diploid control. About 80% of cells in these embryos had 3 or 4 nucleoli per nucleus with a mean of 3.2–3.3 (Fig. 4d) (expected mean tetraploid number 3.6). Thus, these offspring could consist of polyploid embryos including mainly tetraploids, triploids, and mosaics. No significant difference was observed between normal embryos and those with morphological deviations (Table 2).

DISCUSSION

The embryo survival in shock-treated offspring (both regular and gynogenetic) was dependent on the time of heat shock initiation. The cyclic nature of this dependence is apparently associated with phases of the mitotic cycle, as the time interval between two adjacent "sensitive" (or "non-sensitive") periods is equal to approximately 1 τ_0. The period of greatest sensitivity to heat shock is at 1.6–1.8 τ_0 after insemination under the conditions of two pretreatment water temperatures.

The number of spontaneous diploid gynogens was negligible in shock-treated gynogenetic offspring as indicated by the results of incubation of the no-heat-shock gynogenetic controls. Thus, diploid gynogenetic larvae obtained in the experimental offspring can be attributed to the effect of heat shock at the first cleavage (however, we can not exclude the presence of some spontaneous diploids among gynogenetic larvae). The output of diploid gynogens was comparable with that obtained in other investigations on the common carp, in which heat shock was applied at the first cleavage (e.g. Komen et al., 1991). The actual frequency of chromosome set diploidization was undoubtedly higher than estimated by the number of morphologically normal larvae in gynogenetic offspring. According to the number of nucleoli, the abnormal gynogenetic larvae obtained in shock-treated offspring were also diploids, and almost all investigated larvae in regular shock-treated offspring were polyploids.

The output of diploid gynogenetic embryos was clearly dependent on heat shock timing. The best results were obtained when heat shock initiation corresponded to 1.5–1.6 τ_0 (coinciding with the beginning of the period of highest sensitivity to heat shock). The periods of 0.9–1.3 τ_0 and 1.7–2.0 τ_0 could be considered as not effective.

Using τ_0 it is possible to compare the results of different experiments on the first cleavage suppression in common carp. In theory we can expect the

same (close) optimum τ_0 values for heat shock initiation under the conditions of different pretreatment water temperatures, i.e. standardize this parameter. The coincidence of embryo survival dynamics at 20°C and 25°C prior to heat shock in our experiments supports this assumption. In the experiment of Gomelsky et al. (1989) the highest output of 2n gynogenetic larvae was observed after heat shock initiation at 1.7–1.9 τ_0. The same timing was used for mass production of tetraploids of common carp, and the best results were obtained at 1.7 τ_0 (Recubratsky et al., 1989). Water temperature prior to heat shock of 20°C was used in both cases. The most effective timing of Nagy (1987) corresponds to 1.8 τ_0 and that of Komen et al. (1991) for gynogenetic offspring to 1.5–1.6 τ_0 (22°C and 24°C prior to heat shock, respectively) [our calculation of τ_0 for the results of Nagy (1987), Komen et al. (1991) and Sumantadinata et al. (1990) is based on one τ_0 duration at the corresponding pretreatment water temperatures (Ignatieva, 1974)]. Thus, our results coincide with those of Komen et al. (1991) whereas the optimum timing was observed at the later stages in other experiments. The data of Sumantadinata et al. (1990) obtained in experiments on Indonesian common carp are markedly distinguished from those obtained by the other authors. The highest output of diploid gynogenetic larvae was observed at 2.2–2.5 τ_0 and 3.0 τ_0 after insemination in this case (water temperature prior to heat shock was 25°C). The results are not understood and have to be verified as the authors also point out.

As a whole, the optimum τ_0 values for heat shock initiation are concentrated within the limits of 1.5–1.9 τ_0. The data indicate some correlation between optimum value of τ_0 and water temperature prior to heat shock, since the highest τ_0 values (1.8 and 1.9) are obtained at 20–22°C prior to heat shock and the lowest values (1.5 and 1.6) at pretreatment water temperatures of 24°C and 25°C. This observation has to be examined in a special investigation. The difference in the τ_0 optimum may also be due to genetic and physiological peculiarities in common carp of different stocks (and variation in the rate of development resulting from this), egg quality and some technical reasons.

The available experimental data are contradictory, and further empirical supports are required to obtain accurate data concerning heat shock timing at the first cleavage in common carp. According to our preliminary results, the period of 1.5–1.6 τ_0 could be considered as the most likely "optimum" timing for heat shock initiation during the first cleavage. This embryological age corresponds to metaphase of the first cleavage as has been shown by Ignatieva (1979) and confirmed in the present investigation. The data are in accordance with the results of Komen et al. (1991) in experiments on common carp, and investigations on zebra fish *Brachydanio rerio* and medaka *Oryzias latipes* in which the optimum timing was also observed after heat shock applied at metaphase of the first cleavage (Streisinger et al., 1981; Naruse et al., 1985).

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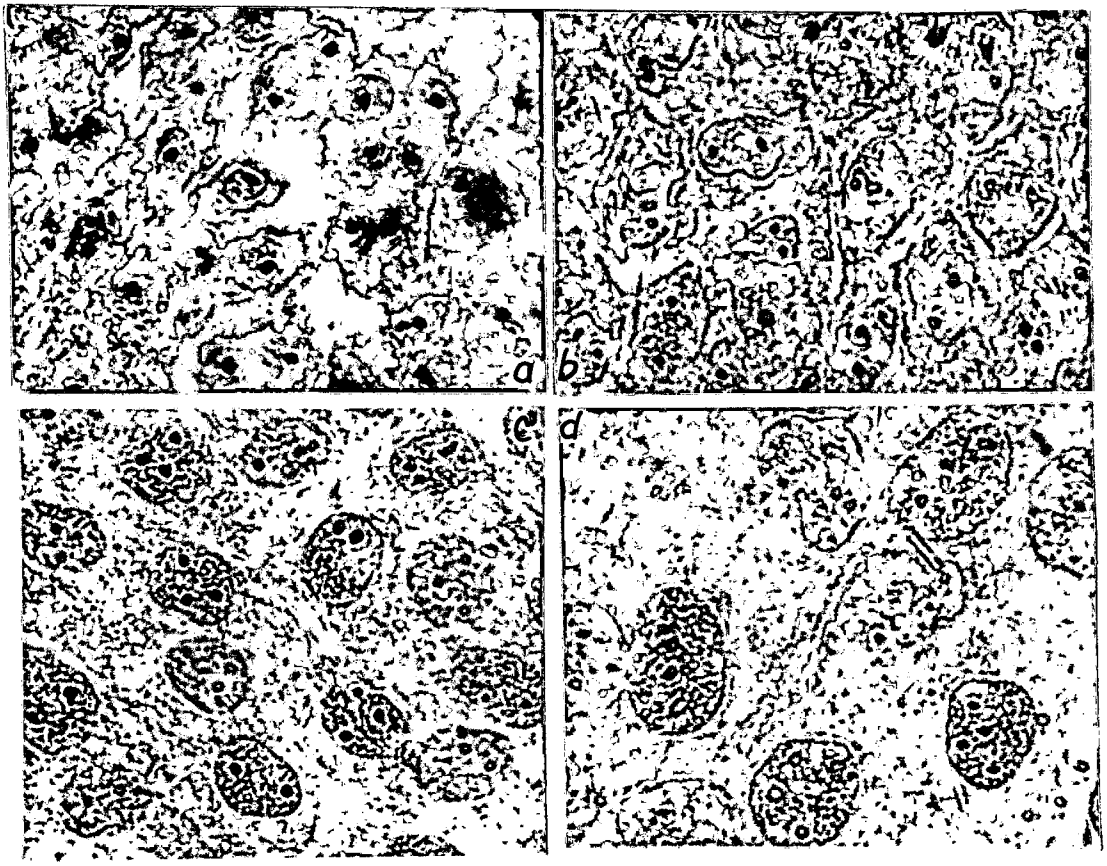

Suppl. 1. Ploidy level estimation by the nucleoli number in the nuclei of epithelial cells. The mean individual nucleoli number per nuclei is: 1 in haploid (a), 1.8 in diploid (b), 2.7 in triploid (c) and 3.6 in tetraploid (d) embryos. Aceto-carmine method.

Induced diploid gynogenesis and polyploidy in ornamental (koi) common carp *Cyprinus carpio* L. 3. Optimization of heat shock timing during the 2nd meiotic division and the 1st cleavage.

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Summary. In spite of a number of successful experiments on induced gynogenesis and polyploidy in common carp (*Cyprinus carpio* L.), the methods for obtaining gynogenetic and polyploid progenies were in need of optimization. Series of experiments were conducted in our laboratory on ornamental common carp (koi) dealing with heat shock during the second meiotic division (for obtaining meiotic gynogenetic and triploid progenies) and during the first mitotic division (for obtaining mitotic gynogenetic and tetraploid progenies). Determination of the adequate heat shock timing for supression of the 2nd meiotic division and the 1st cleavage was the main objective of these investigations. Time of heat shock initiation was expressed in the relative unit of embryological age (τ_0) in order to standardize this parameter. Heat shock was applied within the period of 0.05-2.20 τ_0 which covers the successive phases of the 2nd meiotic division and the 1st cleavage. The absolute time for heat shock initiation was calculated from the duration of one τ_0 at any given pre-treatment water temperature. The effectiveness of heat shock was estimated according to frequency of diploid gynogenetic larvae in shock treated offspring. The highest production of diploid gynogens was observed when heat shock was initiated at 0.15-0.25 τ_0 and at 1.5 τ_0, after insemination. The first age

corresponds to anaphase of meiosis II, and the second one to metaphase of the 1st cleavage. These results were obtained in the condition of different pre-treatment water temperatures, thus confirming the possibility of standardization heat shock timing by τ_0. Analysis of published data has shown that optimum heat shock timing in common carp from other origins was identical, or very close to those obtained for koi (when expressed in τ_0). The results can be used for obtaining two types of gynogens, tri- and tetraploid progenies in common carp.

Key words: Common carp - chromosome-set manipulations - heat shock - induced gynogenesis and polyploidy

Introduction

Heat shock as an agent for inducing chromosome set diploidization was applied in several experiments on diploid gynogenesis in the common carp, *Cyprinus carpio* L. (Hollebecq et al. 1986; Nagy 1987; Gomelsky et al. 1989; Sumantadinata et al. 1990; Komen et al. 1991; Rothbard 1991). Its effectiveness was shown to depend on initiation time after insemination, as also known for other treatments. In spite of some important results obtained in these experiments, data concerning heat shock timing were not conclusive (especially in relation to heat shock timing at the 1st cleavage) due to varying water temperature prior to heat shock. We have, therefore, concluded that additional investigations to obtain accurate data on this subject were needed for further development of the methods of chromosome-set manipulations in the common carp.

Several series of experiments on heat shock timing during the 2nd meiotic division and the 1st cleavage have been conducted during 1990-1992. The main objective of these investigations was to standardize heat shock timing and

to obtain accurate data concerning optimum values of this parameter within the 2nd meiotic division and the 1st cleavage.

The possibility of resolving the first problem is based on the use of relative unit embryological age "τ_0", which is equivalent to the duration of one mitotic cycle during synchronous cleavage. According to the theory (Dettlaff and Dettlaff 1961), the duration in absolute time of one τ_0 is temperature dependent, but a certain embryological stage (within the optimal temperature range for any given species) is a constant value if expressed in τ_0 units. Consequently, it was supposed that embryological stages when temperature shock initiation will be effective may also be defined in terms of τ_0, irrespective of pre-treatment water temperature. This unit (as a measure for timing heat shock initiation) was used by Gomelsky et al. (1989), Rothbard (1991) and Cherfas et al. (1993) in experiments on induced gynogenesis in common carp. Our first experiments of 1990 dealt with heat shock at the first cleavage (Cherfas et al. 1993). The dynamic of embryo survival at different pre-treatment water temperatures in regular progenies was examined. Absolute time for heat shock initiation was calculated from duration of one τ_0 at the pre-treatment temperatures of 20°C and 25°C used by Ignatieva (1974). The similarity of the two survival curves obtained supported the above supposition concerning standardizing heat shock timing. Gynogenetic progenies were incubated only at 25°C prior to heat shock, and the optimum heat shock timing was determined to be 1.4-1.6τ_0 (after insemination).

The present paper describes the main results of the 1991-1992 optimization experiments. The dynamic of diploid gynogenetic larvae output has served as a main index for evaluating the result obtained. The experimental procedure was improved since the two pre-treatment water temperature variants were carried out simultaneously, using the eggs of the same female. Thus, the two series of each experiments differed in pre-

treatment water temperature only, while all other experimental conditions were identical. Besides, a new (more precise) data on the duration of τ_0 were used (Ignatieva and Saat, pers. comm.). Heat shock was initiated at the same embryological age after insemination, expressed in τ_0, but at different absolute times. Successive phases of the 2nd meiotic division and of the 1st cleavage were investigated, that allowed to determine optimum heat shock timing.

Materials and methods

The experiments were conducted at the fish and Aquaculture Research Station, Dor, during 1991-1992. Females of ornamental common carp (koi) and males of the Israeli Dor-70 carp line were used as breeders. Recessive orange body color in koi females and dominant wild-type body color in Dor-70 males were served as genetic markers to confirm gynogenetic origin in the experimental progenies, as in our previous investigations (Cherfas et al. 1990, 1991, 1993). All investigations were carried out on gynogenetic progenies of ornamental (koi) common carp obtained by insemination of koi eggs with genetically inactivated sperm of edible (wild-type colored) carp. A UV dose of 800j/m^2 was used for sperm genetic inactivation (Cherfas et al. 1990). The absence of wild-type colored embryos served as a confirmation for the gynogenetic origin of the progenies.

The experimental procedures were identical to those described by Cherfas et al. (1993). Standard technique of artificial propagation of common carp was used (Rothbard 1981). Eggs of a single female were used in each experiment. Inseminated eggs were incubated in Petri dishes (250-350 eggs on each one), placed on trays within a circulating water system. Three experiments (exp. 13/91, 14/91, 15/91) dealt with heat shock at the 2nd meiotic division and four (exp. 2/91, 5/91, 3/92, 4/92) with heat shock at

the 1st cleavage.

The timing of shock initiation was expressed in τ_0 (Detlaff and Detlaff 1961; Cherfas et al. 1993). According to recent data (Ignatieva 1974; Saat 1991), the 2nd meiotic division in the common carp is completed at $0.48\tau_0$ after insemination and the 1st mitotic division - at $2.1\text{-}2.3\tau_0$ after insemination. Heat shock was initiated (at $0.05\tau_0$ intervals) within $0.05\text{-}0.60\tau_0$, and from 1.2 to $2.2\tau_0$ (at $0.1\tau_0$ intervals), after insemination. The period from 0.6 to $1.2\tau_0$ was considered as non-effective (Gomelsky et al. 1989; Cherfas et al. 1993). Two synchronous series, differing only in pre-treatment water temperature (20°C in one series and 24°C in the other) were included in each experiment. The absolute time for heat shock initiation was calculated from the duration of one τ_0 at these temperatures, according to Ignatieva and Saat (pers. comm.) (Table 1).

Temperature of heat shock and shock duration were chosen according to the results of previous investigations (Gomelsky et al. 1989; Komen et al. 1991; Cherfas et al. 1993), and were not re-examined. They were as follows: 38.9-39.5°C for 2.0-2.5 min in experiments on shock timing at the 2nd meiotic division, and 39.5-39.8°C for 2.5-2.8 min in experiments on shock timing at the 1st mitotic division.

Non shocked samples of the same gynogenetic progenies were used as controls in each experiment to estimate quality of eggs and irradiated sperm, fertilization rate (i.e. the number of embryos starting gynogenetic development) and the frequency of spontaneous diploid gynogenetic larvae.

The results were analyzed according to embryo survival prior to hatching and the output of diploid gynogenetic larvae 2-3 days after hatching. Active, normally appearing gynogenetic larvae were considered as diploids. The effectiveness of heat shock applied at different τ_0 after insemination was estimated from the frequency of diploid gynogenetic larvae. The two series obtained at pre-treatment water temperatures of 20°C and 24°C were

compared by t-test.

Results

Heat shock at the 2nd meiotic division

The results of incubation are presented in Table 2 and Fig. 1. Heat shock resulted in decreased embryo survival, compared to the control, with lowest embryo survival observed when heat shock was applied prior to $0.10\tau_0$ or later than $0.30\tau_0$ after insemination (Fig. 1). Highest embryo survival (although lower than in the control) was observed when heat shock was initiated at $0.15\text{-}0.25\tau_0$ after insemination.

The effective period for heat shock initiation coincide with the period of high survival in shock treated offspring ($0.10\text{-}0.25\tau_0$). The highest output of diploid gynogenetic larvae was obtained when heat shock was initiated within $0.15\text{-}0.25\tau_0$ after insemination (Fig. 1). Only few, or no diploid larvae were obtained when heat shock was applied earlier or later than this period, even if embryo survival was relatively high (e.g. at $0.45\text{-}0.60\tau_0$ in exp. 14/91; Table 2). The highest production of diploid gynogenetic larvae was 36.5% of total number of inseminated eggs, or 52.0% of the number of live embryos prior to hatching, when heat shock was initiated at $0.15\tau_0$ (Exp. 15/91, Table 2). The dynamics of incubation results was almost identical in the two experimental series, and the discrepancies in both embryo survival and production of 2n-larvae were random. The two curves in Fig. 1 were not significantly different ($t_{(df=11)}=1.07$ for survival and 0.15 for 2n-larvae production).

Heat shock at the 1st mitotic division

The results of incubation are presented in Table 3 and Fig. 2. A decrease in embryo survival (compared to the controls) was observed in all shock-treated offspring. It was extremely low (even zero) when heat shock was initiated at 1.6-1.9τ_0 after insemination.

A peak of diploid larvae output was obtained when heat shock was initiated in the period 1.4-1.6τ_0 after insemination. A few diploids were also obtained at some other periods. Based on the output of gynogenetic diploids, the most effective heat shock timing corresponded to 1.5τ_0 after insemination (Fig. 2), which coincided with the beginning of the sharp decrease in embryo survival. The highest output of mitotic diploid larvae was 8.4% from total number of inseminated eggs or 15.3% from the number of live embryo prior to hatching when heat shock was initiated at 1.5τ_0 (Table 3). The output of mitotic diploid larvae was somewhat higher in the 24°C pre-shock water temperature series (Fig. 2, Table 3). Very similar survival and 2n-larvae production curves for the two experimental series of 20°C and 24°C pre-shock water temperatures were obtained (Fig. 2), with some irregular discrepancies in different experiments (Table 3). No significant difference was revealed between the curves of the two experimental series ($t_{(df=10)}$ =1.82 for survival and 0.28 for 2n-larvae production).

Discussion

A strong resemblance between the two curves of diploid larvae output, obtained at two pre-shock water temperatures, was revealed for both investigated periods, i.e. the 2nd meiotic division and the 1st cleavage. Thus, we can conclude that the time of heat shock initiation corresponded to the same phase in the cell cycle, at each investigated τ_0, irrespective

of pre-shock water temparture. The difference between two series of experiment in absolute time for the same τ_0 values during the 1st cleavage was considerable (e.g. 13.4 minutes for $1.5\tau_0$ after insemination; see Table 1). Therefore, the results obtained have shown that heat shock timing in experiments on chromosome-set manipulations in common carp can be effectively standardized using the parameter τ_0.

Two optimum periods for heat shock initiation were identified according to the results of these experiments: $0.15\text{-}0.25\tau_0$ and $1.5\tau_0$, after insemination (Table 4). The first period corresponds to mid-anaphase II, and the second one to metaphase of the 1st cleavage (Ignatieva 1979; Saat 1991). The output of diploid gynogenetic larvae was not high, especially in the experiments on mitotic gynogenesis when optimum heat shock timing preceded (or sometimes coincided with) the period of drastic decrease in embryo survival.

The results of other investigations on induced gynogenesis in common carp, in which heat shock was applied, were transformed to enable comparison with those of the present study (Table 5). This was done by deviding the absolute reported time of heat shock initiation divided with the duration of one τ_0 at any given pre-shock water temperature. Although results were obtained for common carp of different origins and age, optimum timing in most experiment lies within the same range of embryological age, concentrated within $0.10\text{-}0.25\tau_0$ (in experiments on meiotic gynogenesis) and $1.4\text{-}1.6\tau_0$ (in experiments on mitotic gynogenesis). No correlation between water temperature prior to heat shock (ranging from 20 to 25°C) and optimum timing was observed. The only obvious exception is the result obtained in expertiment on mitotic gynogenesis in Indonesian common carp (Sumantadinata et al. 1990). It seems that diploid gynogenetic larvae have resulted from supression of the 2nd cleavage while the 1st cleavage was not investigated in this experiment, or (though less likely) that the duration of one τ_0 in

the Indonesian common carp is different.

Based on the results of the present investigation, embryological age of 0.20 (0.15-0.25)τ_0 and 1.5 (1.4-1.6)τ_0 can be recommended for induction of chromosome-set diploidization at the 2nd meiosis and at the 1st cleavage, respectively. This timing can be used within the limits of optimum temperature for common carp reproduction (20-25°C) to obtain diploid gynogenetic and polyploid progenies. Saat (1991) has recently shown that the time-course of the fertilization process (expressed in τ_0) is similar in different teleosts. Thus, the data of heat shock timing obtained for common carp may be applicable also for other fish species.

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Table 1. Time for heat shock initiation after insemination

Pre-treatment water temperature (°C)	Duration of one τ_0, (minutes)*	Investigated period (min)	
		2nd meiotic division ($0.05-0.6\tau_0$, $0.05\tau_0$ intervals)	1st mitotic division ($1.2-2.2\tau_0$, $0.1\tau_0$ intervals)
20	29.4	1.5 - 17.6 (1.45 min interval)	35.3 - 64.7 (2.94 min interval)
24	20.4	1.0 - 12.2 (1.02 min interval)	24.5 - 44.9 (2.04 min interval)

*) According to Ignatieva and Saat (pers. comm.).

Table 2. Results of the experiments with heat shock applied at the 2nd meiotic division at 20°C and 24°C incubation temperature prior to heat shock: embryo survival and production of 2n-larvae

	20°C prior to heat shock				24°C prior to heat shock			
Shock initiation (τ_0)	No. of eggs	Survival prior to hatching (%)*	2n-larvae (No.)	2n-larvae (%)*	No. of eggs	Survival prior to hatching (%)*	2n-larvae (No.)	2n-larvae (%)*
Experiment 13/91								
0.05	306	19	3	0.9	251	3	0	0.0
0.10	281	39	0	0.0	290	21	2	0.6
0.15	317	49	3	0.9	181	48	2	1.1
0.20	269	59	16	5.9	278	60	6	2.1
0.25	233	60	13	5.6	210	66	22	10.4
0.30	258	45	5	1.9	215	40	5	2.3
0.35	356	35	0	0.0	238	44	1	0.4
0.40	484	18	0	0.0	218	27	3	1.3
0.45	347	20	1	0.3	203	20	0	0.0
0.50	234	20	0	0.0	204	14	0	0.0
0.55	220	24	0	0.0	239	23	0	0.0
0.60	290	33	0	0.0	229	28	0	0.0
C**	226	77	0	0.0				
Experiment 14/91								
0.05	315	18	1	0.3	261	21	1	0.3
0.10	295	38	7	2.3	212	44	9	4.2
0.15	470	32	1	0.2	231	52	18	7.7
0.20	273	57	18	6.5	242	46	12	4.9
0.25	253	53	11	4.3	245	49	5	2.0
0.30	241	52	10	4.1	321	42	8	2.4
0.35	172	46	3	1.7	216	45	2	0.9
0.40	153	39	2	1.3	188	42	0	0.0
0.45	370	37	1	0.2	226	38	0	0.0
0.50	322	36	0	0.0	231	42	0	0.0
0.55	226	45	0	0.0	205	40	0	0.0
0.60	239	62	0	0.0	201	61	0	0.0
C	287	71	0	0.0				
Experiment 15/91								
0.05	205	26	6	2.9	390	25	18	4.6
0.10	258	30	13	5.0	238	40	34	14.2
0.15	178	70	65	36.5	278	63	65	23.3
0.20	304	54	18	5.9	280	25	22	7.8
0.25	200	18	9	4.5	269	16	8	2.9
0.30	195	13	1	0.5	195	10	2	1.0
0.35	285	10	0	0.0	466	6	0	0.0
0.40	332	13	0	0.0	274	5	0	0.0
0.45	389	8	0	0.0	233	12	0	0.0
0.50	321	6	0	0.0	222	7	0	0.0
0.55	258	4	0	0.0	267	8	0	0.0
0.60	326	6	0	0.0	348	11	0	0.0
C	284	93	1	0.4				

*) From total number of inseminated eggs

**) No-shock gynogenetic control

Table 3. Results of the experiments with heat shock applied at the 1st mitotic division at 20°C and 24°C incubation temperature prior to heat shock: embryo survival and production of 2n-larvae

Shock initiation (τ_0)	20°C prior to heat shock: No. of eggs	Survival prior to hatching (%)*	2n-larvae (No.)	2n-larvae (%)*	24°C prior to heat shock: No. of eggs	Survival prior to hatching (%)*	2n-larvae (No.)	2n-larvae (%)*
Experiment 2/91								
1.2	264	23	3	1.1	275	39	3	1.0
1.3	424	39	6	1.4	259	53	3	1.1
1.4	443	47	23	5.1	309	54	7	2.2
1.5	500	55	42	8.4	208	42	16	7.6
1.6	375	50	27	7.2	297	19	12	4.0
1.7	366	22	11	3.0	276	5	2	0.7
1.8	337	11	4	1.1	235	3	0	0.0
1.9	526	7	1	0.1	360	6	0	0.0
2.0	383	9	0	0.0	322	19	0	0.0
2.1	131	2	0	0.0	282	25	0	0.0
2.2	206	3	0	0.0	372	48	3	0.0
C**	289	75	1	0.3				
Experiment 5/91								
1.2	223	43	1	0.4	284	51	0	0.0
1.3	168	45	1	0.6	246	44	0	0.0
1.4	320	26	4	1.2	251	29	1	0.4
1.5	275	16	2	0.7	183	12	4	2.2
1.6	336	13	2	0.6	277	7	1	0.4
1.7	380	2	1	0.2	186	1	0	0.0
1.8	274	7	0	0.0	238	0	0	0.0
1.9	249	4	0	0.0	236	1	0	0.0
2.0	315	2	0	0.0	229	14	0	0.0
2.1	357	15	0	0.0	287	47	0	0.0
2.2	393	30	0	0.0	315	45	0	0.0
C	311	82	1	0.3				

(Table 3 continued)

Experiment 3/91								
1.2	262	49	2	0.7	348	48	0	0.0
1.3	238	44	1	0.4	241	59	5	2.1
1.4	266	19	1	0.3	251	29	3	1.1
1.5	451	3	1	0.2	295	20	14	4.7
1.6	211	0	0	0.0	244	5	3	1.2
1.7	401	3	0	0.0	341	1	0	0.0
1.8	314	10	0	0.0	272	9	0	0.0
1.9	264	11	0	0.0	294	9	1	0.3
2.0	309	28	1	0.3	366	34	0	0.0
2.1	288	57	0	0.0	179	60	1	0.5
2.2	235	64	0	0.0	264	66	0	0.0
C	323	62	0	0.0				
Experiment 4/91								
1.2	358	18	3	0.8	296	14	0	0.0
1.3	328	16	3	0.9	245	21	1	0.4
1.4	235	9	4	1.7	290	15	6	2.0
1.5	329	10	11	3.3	339	29	26	7.6
1.6	280	5	4	1.4	287	14	6	2.1
1.7	342	5	1	0.3	300	6	6	2.0
1.8	444	7	1	0.2	503	1	1	0.2
1.9	325	2	0	0.0	367	3	0	0.0
2.0	296	5	0	0.0	252	14	1	0.4
2.1	254	24	0	0.0	306	42	0	0.0
2.2	300	32	0	0.0	265	48	0	0.0
C	526	62	0	0.0				

*) From total number of inseminated eggs

**) No-shock gynogenetic control

Table 4. Production (%) of 2n-gynogenetic larvae from total number of inseminated eggs in shock treated progenies (means of eight shock-treated progenies and five control groups). Values marked with asterisks are significantly different from their respective controls.

Meiotic gynogenesis		Mitotic gynogenesis	
Heat shock initiation (τ_0)	Mean ± S.E.	Heat shock initiation (τ_0)	Mean ± S.E.
0.05	1.6 ± 0.6*	1.2	0.5 ± 0.1
0.10	4.1 ± 1.6*	1.3	0.8 ± 0.2*
0.15	12.3 ± 5.0*	1.4	1.7 ± 0.5*
0.20	9.1 ± 3.5*	1.5	4.3 ± 1.1**
0.25	5.6 ± 1.6*	1.6	2.0 ± 0.8
0.30	1.9 ± 0.4**	1.7	0.6 ± 0.3
0.35	0.4 ± 0.2	1.8	0.1 ± 0.1
0.40	0.4 ± 0.2	1.9	0.1 ± 0.1
C	0.2 ± 0.2	C	0.1 ± 0.1

C = No-shock gynogenetic control.

* $p < 0.1$; ** $p < 0.05$

Table 5. Optimum heat shock timing observed in different experiments on induced gynogenesis in common carp

Temperature prior to shock (°C)	Duration of τ_0 (min)*	Optimum timing min	Optimum timing τ_0	2n-larvae (%)**	Reference
The 2nd meiotic division					
20	29.4	3.0-5.0	0.10-0.17	50 (1)	Hollebecq et al. 1986
		2.8	0.10	63 (2)	Gomelsky et al. 1989
		4.4-7.4	0.15-0.25	37 (3)	present paper
24	20.4	3.1-5.1	0.15-0.25	23(3)	present paper
25	19.0	2.0-4.0	0.10-0.21	100(3)	Sumantadinata et al. 1990
The 1st mitotic division					
20	29.4	47.6-53.2	1.6-1.8	9(2)	Gomelsky et al. 1989
		44.1-47.0	1.5-1.6	8(3)	present paper
22	24.1	40.0	1.6	11(3)	Nagy 1987
24	20.4	28.6-32.6	1.4-1.6	8(3)	present paper
		28.0-30.0	1.4-1.5	15(3)	Komen et al. 1991
25	19.0	23.4-30.6	1.4-1.5	15(3)	Cherfas et al. 1993
		40.0-45.0	2.1-2.3	-***	Sumantadinata et al. 1990
		55	2.9	-***	Sumantadinata et al. 1990

*) According to Ignatieva and Saat (pers. comm.).

**) in relation to: control (1), number of fertilized eggs (2), total number of inseminated eggs (3).

***) no data.

Fig. 1. Survival prior to hatching (a) and output of 2n-gynogenetic larvae (b) relative to total number of inseminated eggs in gynogenetic progenies heat shocked during the 2nd meiotic division, after incubation at 20°C or 24 °C. Curves are based on mean values of 3 experiments. C = no-shock gynogenetic control.

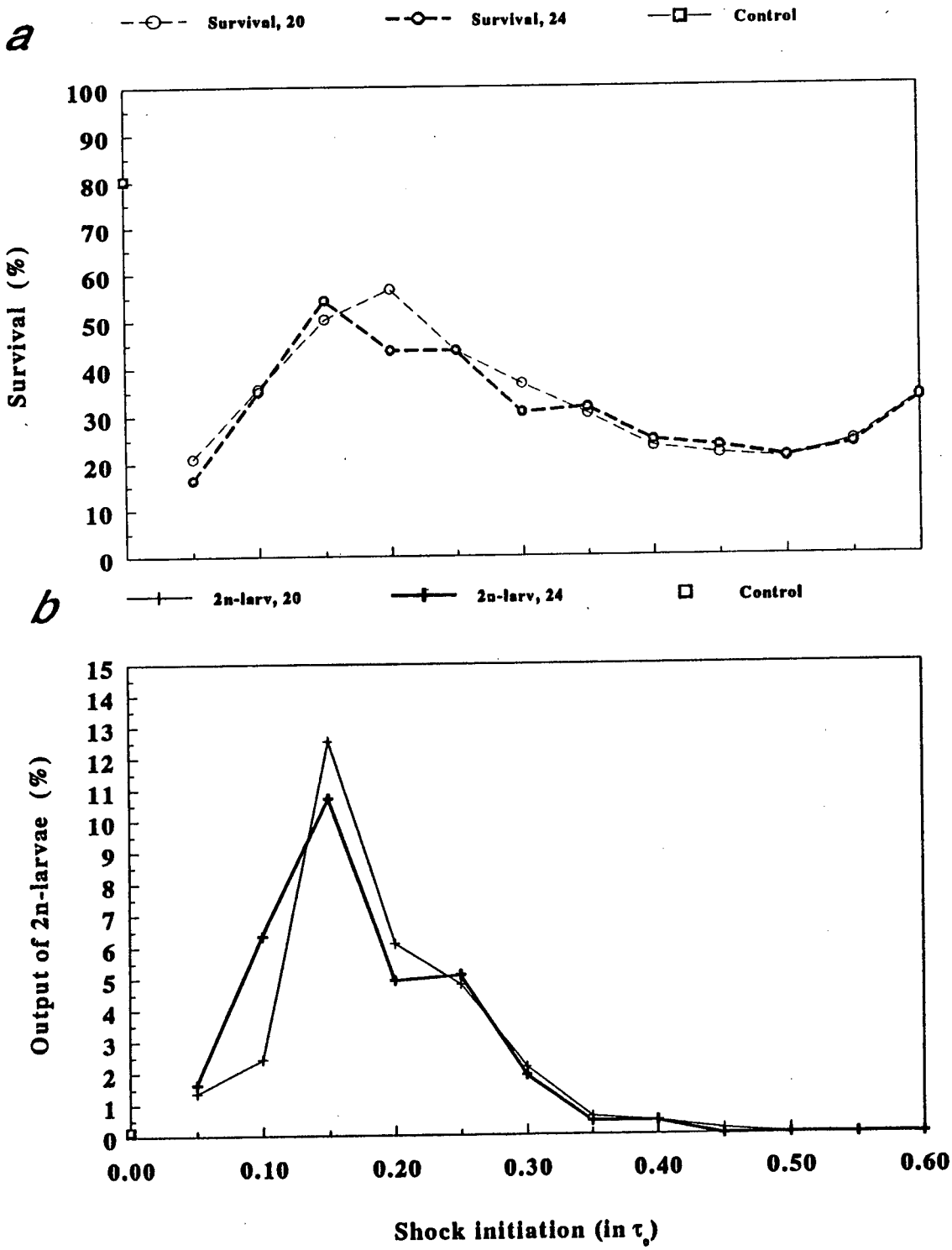
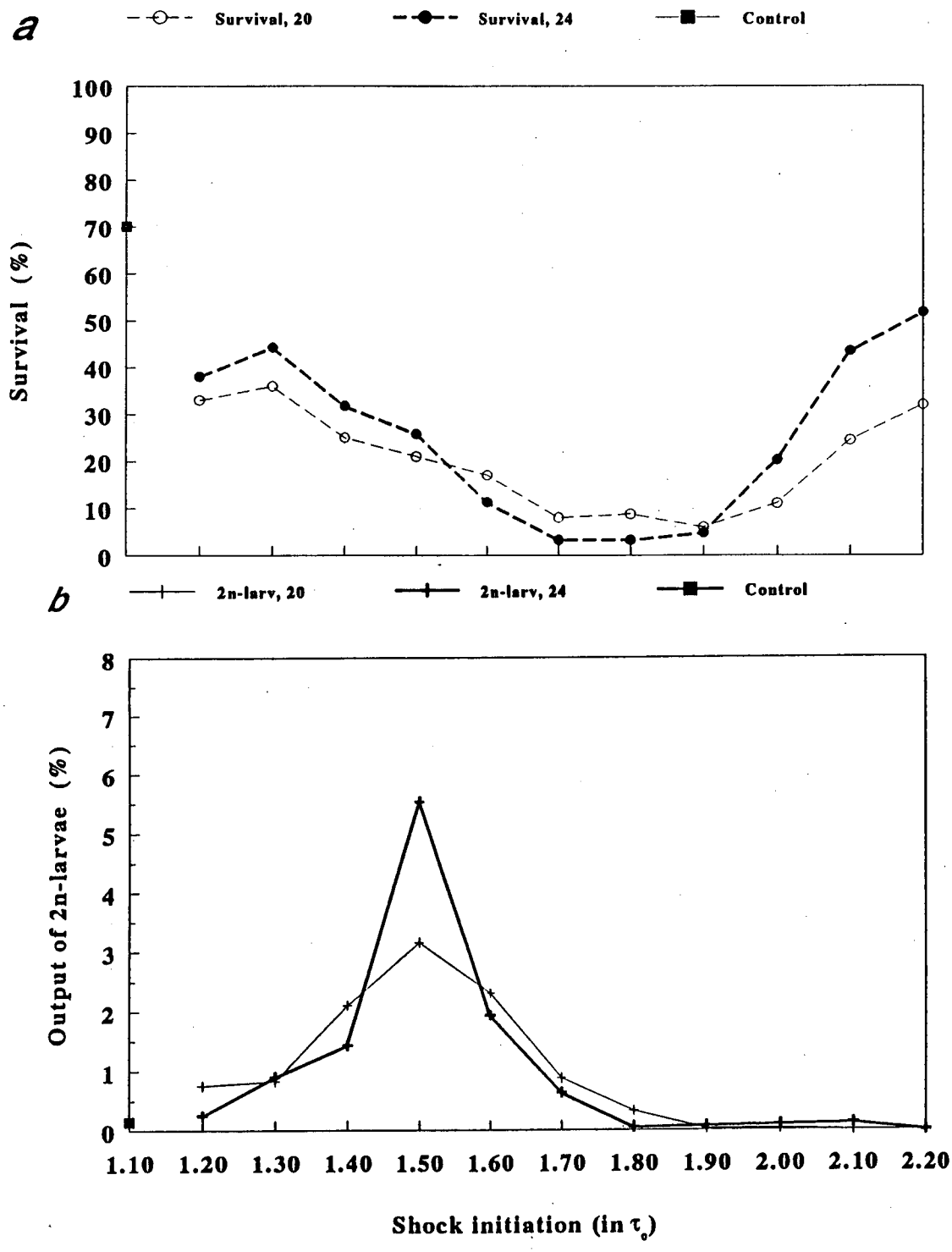


Fig.2 Survival prior to hatching (a) and output of 2n-gynogenetic larvae (b) relative to total number of inseminated eggs in gynogenetic progenies heat shocked during the 1st mitotic division, after incubation at 20°C or 24 °C. Curves are based on mean values of 4 experiments. C = no-shock gynogenetic control.



Induced diploid gynogenesis and polyploidy in ornamental (koi) carp, *Cyprinus carpio* L.

4. Comparative study on the effects of cold and heat temperature shocks

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Summary. A comparative study on the effects of cold and heat temperature shock on embryo development was conducted in investigations on induced gynogenesis in the common carp, *Cyprinus carpio* L. Temperature shocks were applied at different phases of the 2nd meiotic division within the limits of 0.05-0.60τ_0 (τ_0 = relative unit of embryological age). A remarkable difference in the effect of two temperature treatments applied at the same biological age after insemination (expressed in τ_0) was revealed. The curves of embryo survival and diploid gynogenetic larvae output showed bimodal response in cold-shocked gynogenetic progenies, with the highest level of diploid larvae output at the periods of 0.05-0.10τ_0 and 0.30-0.40τ_0 (after insemination), separated by a period of high sensitivity to cold shock (0.15-0.25τ_0). Contrary to this, the curves of embryo survival and diploid gynogenetic larvae output showed a single, narrow peak corresponding to 0.15-0.25τ_0 in heat-shocked gynogenetic progenies. The results obtained are in general accordance with those of previous experiments on induced gynogenesis and triploidy in common carp, in which either cold or heat shock were used. The data show different response to the two types of temperature treatments applied during the 2nd meiotic division.

Key words: Common carp - chromosome-set manipulations - heat shock - cold shock - induced gynogenesis and triploidy

Introduction

Temperature shocks are common agents used to induce suppression of cell division for chromosome-set manipulations in fish (e.g., Thorgaard 1983). Two types of temperature treatments, i.e. long cold shock and short heat shock are used for suppression of the 2nd meiotic division in eggs, in order to induce meiotic gynogenetic or triploid fish progenies. In spite of a large number of experiments on this subject, there is limited information concerning the comparative effect of these two types of temperature shock treatments on embryo development. The first indications of differential cold and heat shock effects were obtained already in the initial experiments on induced gynogenesis in fish carried out on loach *Misgurnus fossilis* L. (Romashov and Belyaeva 1965). Further data were obtained for other fish species, including the tilapia species *Oreochromis aureus* and *O. niloticus* (Don and Avtalion 1988; Hussain et al. 1991). Results of experiments on induced gynogenesis and triploidy in common carp have also indicated some differences in the effects of heat and cold shocks on embryo development, mainly concerning with the optimum shock timing at the 2nd meiotic division (Nagy et al. 1978; Taniguchi et al. 1986; Hollebecq at al. 1986; Linhart et al. 1987, 1991; Komen et al. 1988; Gomelsky et al. 1989; Cherfas et al. 1990, 1993), but a direct comparison of the effects of cold and heat temperature shock was not yet carried out in the common carp. The present investigation was aimed at obtaining accurate data on the comparative effects of cold and heat shocks applied to common carp eggs at the 2nd meiotic division, which are important for both applied chromosome-set manipulation studies and general understanding of the nature of temperature influences on cell structures.

Materials and methods

The experiments were conducted at the Fish and Aquaculture Research Station, Dor, during 1991-1992. The experimental procedures were identical to those described by Cherfas et al. (1993, submitted). Two sets of eggs, one subjected to cold shock and the other to heat shock were incubated in each experiment. All other experimental conditions were identical for both sets. Temperature shocks were initiated within the period of the 2nd meiotic division. The timing of shock initiation was expressed in τ_0 (Detlaff and Detlaff 1961; Cherfas et al. 1993, submitted). Temperature shocks were initiated (at $0.05\tau_0$ intervals) from 0.05 to $0.40\tau_0$ after insemination, in exp. 20/91, and from 0.05 to $0.60\tau_0$, in exp. 1/92 and 2/92. Absolute time for temperature shock initiation was calculated from the duration of one τ_0 - 29.4 min (according to Ignatieva and Saat, pers. comm.) at pre-shock water temperature of 20°C used in all experiments. The exact temperatures of the heat and cold shocks and their duration are given for each experiment in Table 1. Eggs were heated to 39.4-39.8°C for 1.8-2.5 min and cooled to 1.5-3.2°C for 30-45 min in heat and cold shock trials, respectively. After completion of temperature shock the eggs were returned to incubation at ambient water temperature of 20-23°C. Eggs were incubated in Petri dishes (250-350 eggs in each one) placed on trays within a circulated water system.

A control (no-shock) gynogenetic progeny was obtained in each experiment to examine egg quality, rate of fertilization and the level of spontaneous female chromosome-set diploidization. The results were analyzed according to embryo survival and production of diploid gynogenetic larvae in experimental progenies. Active, normally appearing gynogenetic larvae were considered as diploids.

Results

Control gynogenetic progenies

The rates of fertilization and embryo survival prior to hatching in control progenies varied in the ranges of 81-96% and 72-90%, respectively, indicating a good quality of the eggs used in the experiments (Table 1). A few diploid larvae were found in all control groups, and the rate of spontaneous diploidization was 0.5-0.9% from total number of inseminated eggs, i.e. somewhat higher than usual in common carp. All control embryos (as well as those obtained in shock treated progenies) had the recessive (transparent) body color, confirming their gynogenetic origin.

Shock-treated gynogenetic progenies

A clear dependence of the incubation results on shock timing was observed in all experiments. The dynamic of embryo survival was similar in all three experiments (Table 1) and the means of embryo survivals in cold and heat shocked progenies are presented in Figure 1. Embryo survival curves for cold shock treatments were characterized by two periods of high embryo survival (corresponding to $0.05\text{-}0.10\tau_0$ and $0.30\text{-}0.55\tau_0$). Embryo survival was similar within these two periods, but 15-30% lower than in control progenies. A drastic decrease in embryo survival have been registered in the period of $0.10\text{-}0.30\tau_0$ after insemination, indicating extremely high sensitivity to low temperature in this period.

For heat shock treatments, embryo survival curves were distinctively different from those of the, simultaneously applied, cold shock treatments (Table 1, Fig. 1). Embryo survival had a single peak within $0.15\text{-}0.25\tau_0$ after insemination, in which survival was close to that of the controls in

experiments 20/91 and 1/92. In experiment 2/92 embryo survival was very low, apparently due to a too high shock temperature.

The frequencies of diploid larvae have shown bimodal response to temperature treatment in all cold shocked gynogenetic progenies (Table 1, Figs. 2-3). According to the mean frequency of diploid larvae, the optimum timing for cold shock initiation corresponded to $0.05\tau_0$ and 0.30-$0.40\tau_0$. The number of gynogenetic diploids was extremely low (if any) when cold shock was initiated in other periods, including that of high survival (later than $0.40\ \tau_0$). Production of diploid larvae was similar in these two effective periods; it was 6.6% (at $0.05\tau_0$) and 5.3-5.6% (at 0.30-$0.35\tau_0$) when calculated from total number of inseminated eggs (Fig. 2), or 9.8% and 12.9-9.7% from the number of live embryos prior to hatching (Fig. 3). A single peak was observed in the curve of diploid gynogenetic larvae production in heat shock treatments (Figs. 2-3). The highest number of diploid larvae was obtained after heat shock initiated at 0.15-$0.25\tau_0$. The optimum timing corresponded to $0.20\tau_0$, when the mean frequency of diploid larvae was 16.2% from the total number of inseminated eggs (Fig. 2), or 26.3% from the number of live embryos prior to hatching (Fig. 3). Some variation existed between the different experiments (Table 1). Heat shock was more effective than cold shock (according to the output of diploid gynogenetic larvae) in exp. 20/91 and 1/92, and no difference was found between cold and heat shocks in exp. 2/92. Mean production of diploid larvae was higher in heat shock than in cold shock treatments (Figs. 2-3).

Discussion

It was shown (Boon-Niermmeijer 1991; Vachrameeva and Neyfakh 1965) that the sensitivity to high temperature shock at the initial embryological stages depends on the nuclear condition at the time of shock initiation. The results of the present experiments revealed differences between the

effects of cold and heat shocks on both embryo survival and chromosome-set diploidization, with a peak in the cold shock curve when a sharp drop occured in the heat shock curve, and vice versa. Thus, the data suggest that cold and heat shock affect different, specific intracellular processes.

The two types of temperature shock treatments were both efficient in producing diploid gynogenetic larvae, though heat shock gave higher output. The peaks of diploid gynogenetic common carp larvae were obtained at different optimal shock initiation timings within the 2nd meiotic division for cold and heat shocks. The features of the response of diploid larvae production to cold and heat shock treatments are independent of embryo survival, since the pattern does not change when it is calculated relative to the number of inseminated eggs or to that of live embryos prior to hatching (Fig. 2-3). Based on the data of Saat (1991) the optimum timing for cold shock corresponds to early anaphase II (the first peak) and telophase II (the second peak), while optimum timing for heat shock corresponds to middle-late anaphase II. The period from telophase II to separation of the 2nd polar body ($0.30\tau_0$ and $0.48\tau_0$ after insemination) is ineffective for both types of temperature shock treatments. These results are in agreement with previous published results. Bimodal response to cold shock of embryo survival and diploid gynogenetic (or triploid) larvae production was described by Nagy et al. (1978), Taniguchi et al. (1986) and Komen et al. (1988). According to our calculations, the optimal timing for cold shocks observed in these investigations have corresponded to $0.08\tau_0$ and $0.34\text{-}0.42\tau_0$ (Taniguchi et al. 1986), to $0.05\tau_0$ and $0.39\tau_0$ (Komen et al. 1988) and to $0.17\tau_0$ and $0.51\tau_0$ (Nagy et al. 1978). The results of first two studies coincide with those of the present study. Similar data were also obtained by Cherfas et al. (1990) when shock application shortly after insemination was examined. The highest number of diploid gynogenetic larvae was obtained within 0.03-

$0.07\tau_0$ after insemination, while a sharp decrease in embryo survival and diploid gynogenetic larvae production was observed when cold shock was applied later. The best results reported from other cold shock experiments on induced gynogenesis and triploidy in common carp (Gervai et al. 1980; Ueno 1984; Linhart et al. 1987, 1991) suggested shock initiation within a wide range of $0.06-0.27\tau_0$ after insemination, but a true optimum timing could not be calculated because cold shock was initiated at wide time intervals.

Only one effective period for shock initiation was usually estimated in experiments dealing with heat shock during the 2nd meiotic division (Hollebecq et al. 1986; Gomelsky et al. 1989; Sumantadinata et al. 1990; Cherfas et al. submitted), occurring within $0.10-0.25\tau_0$ after insemination (Cherfas et al. submitted, this study).

The dynamics of incubation has a clear opposing tendency in cold and heat shock treatments, with a peak for cold shock when a sharp drop occurs in the heat shock curve, and vice versa. In experiments on induced gynogenesis in loach (Romashov and Belyaeva 1965) the curves of diploid larvae production also had different optimal timing for cold and heat shock initiation. A pattern similar to that of the common carp was observed, and the effective interval after insemination approximately corresponded to the single heat shock and the second cold shock peaks of this study. In experiments on induced triploidy in tilapia (Don and Avtalion 1988) cold shock was effective within a long period after insemination, while only a narrow range therein was also effective for heat shock. Some indications of different shock timing for low and high temperatures (similar to those reported for the loach) were obtained in a further investigation on tilapia (Hussain et al. 1991). It may be safe to conclude, therefore, that cold and heat shocks applied during the 2nd meiotic division have different response curves.

Acknowledgments

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Table 1. Results of the experiments with temperature shocks applied at the 2nd meiotic division: embryo survival and diploid larvae production in gynogenetic progenies.

Shock initiation (τ_0)	Heat shock: No.of eggs	Heat shock: Survival prior to hatching (%*)	Heat shock: 2n- larvae (No.)	Heat shock: 2n- larvae (%*)	Cold shock: No.of eggs	Cold shock: Survival prior to hatching (%*)	Cold shock: 2n-larvae (No.)	Cold shock: 2n-larvae (%*)
a) Experiment 20/19		(39.4°C, 2.5 min				(1.4-2.6°C, 45 min)		
0.05	217	68	14	6.5	167	74	22	13.1
0.10	313	62	12	3.8	295	28	8	2.7
0.15	242	73	22	9.0	317	20	6	1.9
0.20	260	81	23	8.8	364	26	11	3.0
0.25	280	76	46	16.4	291	5	0	0.0
0.30	347	63	27	7.2	291	39	5	1.7
0.35	291	53	12	4.1	271	56	30	11.1
0.40	306	44	3	0.9	277	45	19	6.9
C**	325	90	1	0.3				
b) Experiment 1/92		(39.5°C, 1.8 min				(3.6-3.8°C, 30 min)		
0.05	236	38	9	3.8	272	49	6	2.2
0.10	287	39	18	6.3	407	53	2	0.4
0.15	206	81	55	26.7	398	19	2	0.7
0.20	300	77	101	33.7	463	12	1	0.2
0.25	260	61	35	13.5	339	3	0	0.0
0.30	285	49	9	3.2	371	27	15	4.0
0.35	272	42	2	0.7	269	77	9	3.3
0.40	293	23	2	0.7	312	73	1	0.3
0.45	280	22	0	0.0	446	83	0	0.0
0.50	291	26	1	0.3	246	72	0	0.0
0.55	267	37	0	0.0	288	68	3	1.0
0.60	353	33	0	0.0	189	53	0	0.0
C**	369	72	2	0.5				

c) Experiment 2/92

	(39.8°C, 2.0 min				(3.2-3.5°C, 30 min)			
0.05	257	11	0	0.0	316	63	14	4.4
0.10	205	20	1	0.5	358	28	1	0.3
0.15	171	16	4	2.3	247	2	2	0.8
0.20	231	25	14	6.0	254	2	0	0.0
0.25	283	11	3	1.1	268	17	5	1.9
0.30	197	14	0	0.0	236	52	24	10.2
0.35	237	4	0	0.0	207	49	5	2.4
0.40	213	2	0	0.0	256	52	5	1.9
0.45	229	3	0	0.0	273	53	1	0.4
0.50	225	6	0	0.0	304	66	2	0.7
0.55	219	5	0	0.0	308	62	0	0.0
0.60	229	7	0	0.0	190	47	1	0.5
C**	276	84	2	0.7				

*) From total number of inseminated eggs

**) No-shock gynogenetic control

Fig. 1. Survival (% from number of inseminated eggs) prior to hatching in heat and cold shock treated gynogenetic progenies (means of three experiments ± s.e.m.).

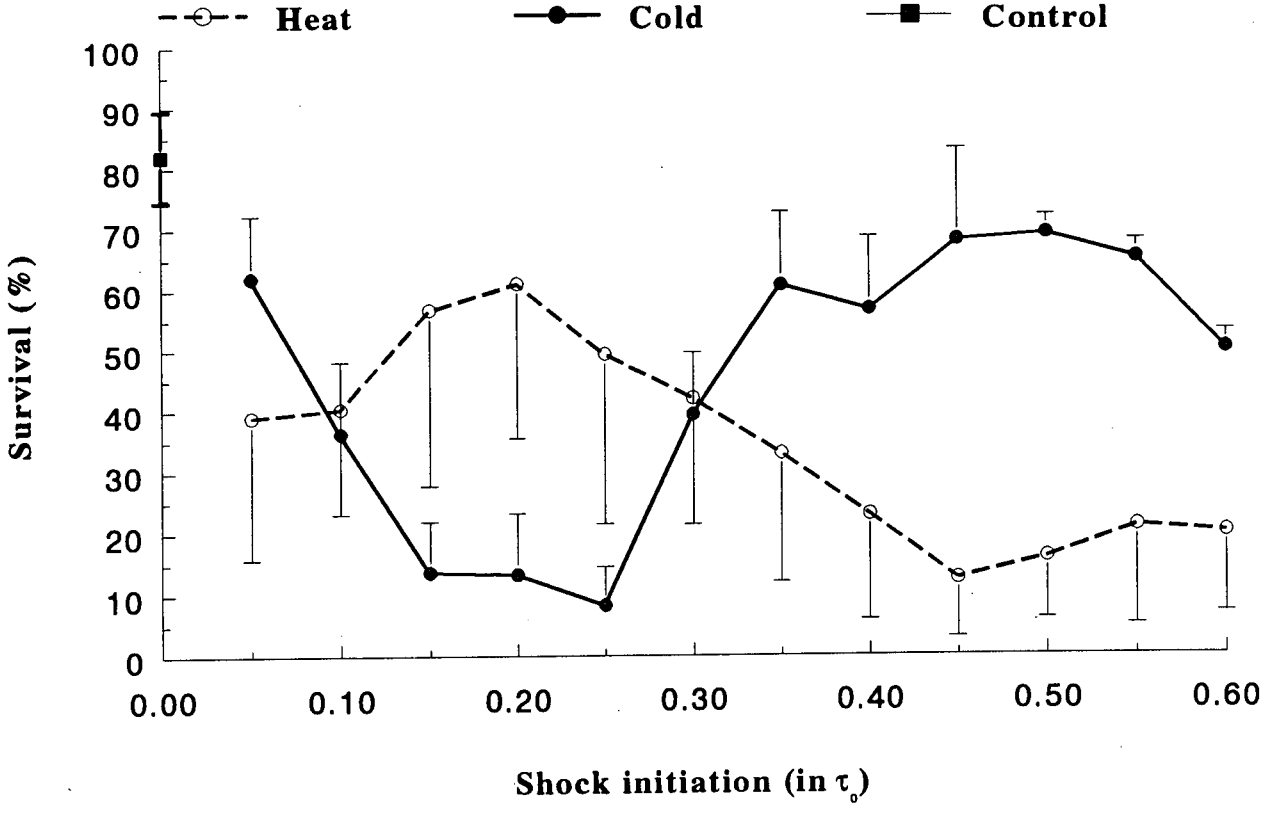


Fig. 2. Production of diploid larvae (% from number of inseminated eggs) in heat and cold shock treated gynogenetic progenies (means of three experiments ± s.e.m.).

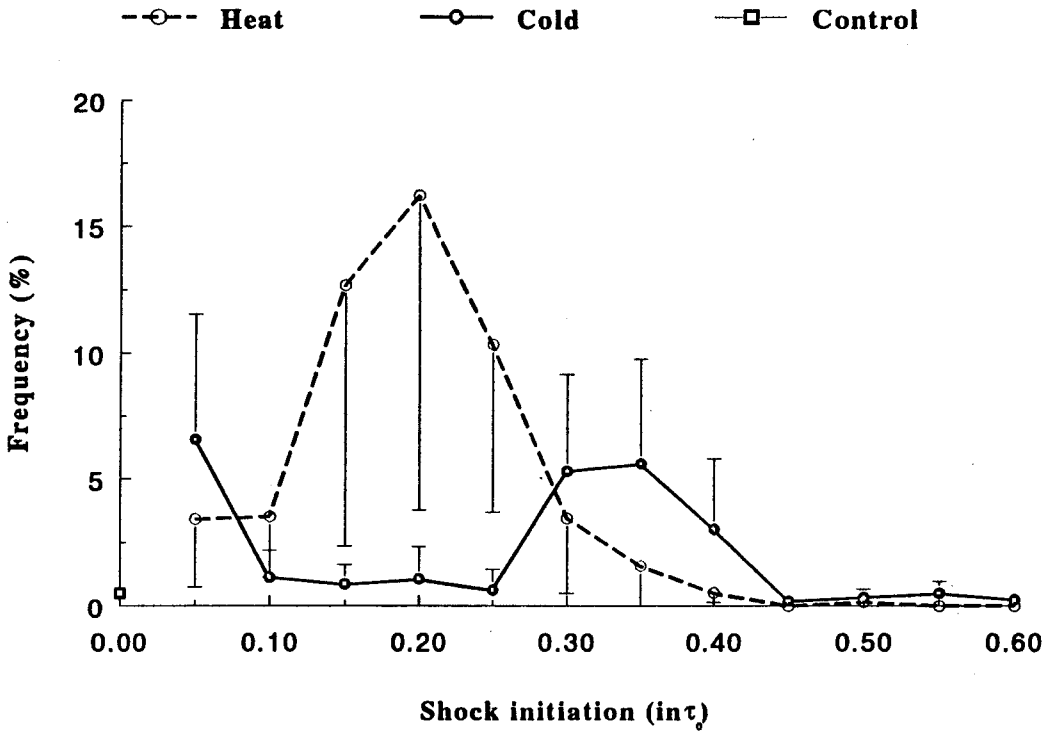
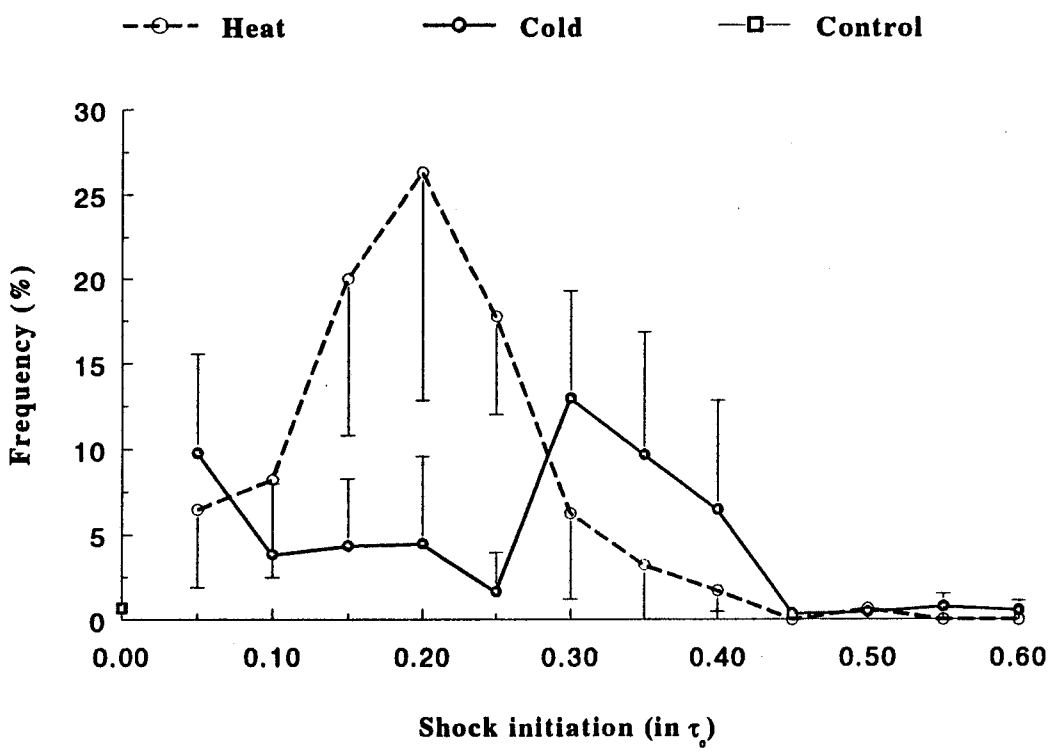


Fig. 3. Production of diploid larvae (% from number of live embryos prior to hatching) in heat and cold shock treated gynogenetic progenies (means of three experiments ± s.e.m.).



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INDUCED GYNOGENESIS AND POLYPLOIDY IN THE ISRAELI COMMON CARP LINE DOR-70*

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Abstract

Meiotic and mitotic diploid gynogenetic, triploid and tetraploid progenies were obtained in the Israeli common carp *(Cyprinus carpio* L.) line Dor-70 in laboratory experiments and in large scale production in Weiss incubation jars. Chromosome-set diploidization was induced by heat shock applied at the second meiotic division or at the first cleavage. A high rate of response to induction of diploid gynogenesis was observed. The best production was 29% meiotic gynogenetic larvae and 23% mitotic gynogenetic larvae (of fertilized eggs). About 10,000 meiotic gynogenetic larvae and 40,000 mitotic gynogenetic larvae were obtained from mass production experiments. Survival of gynogenetic progenies to age 2.5 months under pond conditions was 8% for meiotic gynogens and 3% for mitotic gynogens. A large number of mitotic gynogenetic fingerlings had morphological aberrations. The frequency of triploid larvae was 83-100% and that of tetraploid larvae 92-100%, in the most successful experiments, but heat shock resulted in markedly decreased larvae survival. Many tetraploid larvae were weak and/or deformed, and only two tetraploids were found (among 31 surviving fish) after two months rearing in ponds.

Abstract

Dor-70 is the most important commercial line of common carp *(Cyprinus carpio* L.) in Israel (Wohlfarth et al., 1980). The application of chromosome-set manipulation methods, including induced gynogenesis and polyploidy, has been planned as part of the program to further develop this line.

Induced diploid gynogenesis in fish may be achieved by insemination with genetically inactivated sperm, followed by suppression of the second meiotic division (meiotic gynogenesis) or the first cleavage (mitotic gynogenesis). X-rays, or more often high doses of UV, are used to genetically inactivate sperm, while

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chromosome-set diploidization is usually induced by temperature shock or hydrostatic pressure shock (Thorgaard, 1983; Chourrout, 1987). Meiotic gynogenetic progenies of common carp were obtained in a large number of laboratory experiments and in several large scale trials, while success in obtaining mitotic gynogenesis in common carp was limited (Hollebecq et al., 1986; Taniguchi et al., 1986; Wu et al., 1986; Linhart et al., 1987; Nagy, 1987; Komen et al., 1988, 1991; Gomelsky et al., 1989; Cherfas et al., 1990; Sumantadinata et al., 1990; Cherfas et al., 1993). The use of induced gynogenesis was proposed for obtaining inbred strains of Dor-70 for subsequent commercial hybridization with other common carp breeds (Wohlfarth et al., 1987).

Another component of the program is obtaining triploid progenies which may have advantages over diploids due to their sterility (Thorgaard, 1983). Triploid fish can be obtained either by supression of the second meiotic division in eggs fertilized with normal sperm or by producing tetraploids by suppressing the first cleavage (after normal fertilization) and crossing these tetraploid breeders with normal diploids (Thorgaard, 1983; Chourrout, 1987). The first method is considered less advantageous. Instead of producing all-triploid progeny it results in a mixture of diploids and triploids where the frequency of triploids varies depending on the efficiency of the induction. Furthermore, the methods used to suppress the second meiotic division (e.g., temperature shock) cause reduction of embryo survival. Obtaining triploid progenies by crossing tetraploids with diploids may be a better approach, but it depends on obtaining adult, reproductively competent, tetraploid fish. This has succeeded, so far, only in rainbow trout (Chourrout et al., 1986; Chourrout and Nakayama, 1987; Meyers and Hershberger, 1991).

Triploid common carp have been produced only by the direct induction method, by applying temperature shock (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; Recoubratsky et al., 1989, 1992; Cherfas et al., 1990) and hydrostatic pressure (Linhart et al., 1991). The frequency of triploids obtained varied. In the most successful studies it reached 100% (Gervai et al., 1980; Hollebecq et al., 1988; Recoubratsky et al., 1992). Tetraploid common carp larvae were produced by heat shock applied at the first cleavage (Recoubratsky et al., 1989; Cherfas et al., 1993). Cold shock and hydrostatic pressure shock were found less effective (Linhart et al., 1991). In most of these experiments small groups were obtained under laboratory conditions, except for Recoubratsky et al. (1989, 1992) who performed specific experiments on large scale production of triploid and tetraploid progenies.

No gynogenetic and triploid progenies of the Dor-70 line had been produced until recently. Results of the first experiments on chromosome-set manipulations in the Dor-70 line are presented in this paper. The main objective of the study reported here was to examine the response to induced gynogenesis and polyploidy in the Dor-70 common carp line and to develop protocols for mass production of gynogenetic and polyploid progenies in this breed.

Materials and Methods

The experiments were conducted at the Fish and Aquaculture Research Station, Dor, during 1992. Gynogenetic and polyploid progenies were obtained from two and three-year-old Dor-70 females. Eggs and sperm were obtained from breeders injected with calibrated carp pituitary extract (Yaron et al., 1984) using standard methods of artificial propagation (Rothbard, 1981).

Gynogenetic progenies were obtained in laboratory experiments (incubation in Petri dishes in a circulating water system) and in mass production experiments (incubation in Weiss jars). Polyploid progenies were obtained in Weiss jars only.

The technique of laboratory experiments using temperature shock was described in detail by Cherfas et al. (1993). The procedures for mass production in Weiss incubation jars, including heat shock treatment, degumming of eggs and incubation, were the same for gynogenetic and polyploid progenies and similar to those described by Recoubratsky et al. (1992). Sixty thousand to 130,000 eggs of

(90-200 g) were incubated in each 6 l Weiss jar. In gynogenesis experiments, 2.5-3 ml of irradiated sperm and in polyploidy experiments of intact sperm were used to inseminate each batch of eggs. Degumming of inseminated eggs was done with emulsion of milk in water (1 milk:9 water). Degumming was initiated 0.5 min after insemination and continued for 45-50 min (with a short break for heat shock application when the milk emulsion was drained away and substituted by heated water). Handling of eggs from insemination to completion of heat shock was carried out in a large bowl. Subsequently the eggs were transferred to Weiss jars for further degumming with milk emulsion and incubation to hatching. Larvae were kept in small net-cages for 3-4 days after hatching before being stocked into experimental ponds.

Induced gynogenesis. Irradiated sperm (UV, 800 j/m^2) of either Dor-70 or ornamental (koi) carp males was used to induce gynogenetic development (Cherfas et al., 1990). The koi is fully scaled while the Dor-70 has the recessive scattered (mirror) scale cover. The dominant S gene for scale pattern served as a marker to confirm gynogenetic origin in the progenies. Duplication of the female chromosome-set was induced by heat shock applied to the eggs at the second meiotic division (to induce diploid meiotic gynogenesis) or at the first cleavage (to induce diploid mitotic gynogenesis). The optimal shock timing was investigated. The time of heat shock initiation was expressed in the relative unit of embryological age τ_0. The absolute time for heat shock initiation was calculated using Ignatieva and Saat's data (unpubl.), showing that the duration of one τ_0 unit equals 29.4 min at a pre-incubation water temperature of 20°C and 20.4 min at a pre-incubation water temperature of 24°C.

Two experiments on meiotic gynogenesis (experiments 8/92 and 13/92) and one experiment on mitotic gynogenesis (experiment 6/92, two replicates) were conducted in Petri dishes to examine the optimum timing of heat shock. Eggs of several females (mixed) were used in experiments 8/92 and 13/92 and of one female in experiment 6/92. Mixed irradiated sperm of four or five Dor-70 males was used in all cases. Time periods after insemination for heat shock application, shock temperature and duration were based on the results of our experiments on induced gynogenesis in koi (Cherfas et al., in prep.). In experiments on meiotic gynogenesis, heat shock was applied successively to small samples (200-250) of inseminated eggs at 0.05 τ_0 intervals, within the range of 0.05-0.40 τ_0 after insemination. Heat shock temperature was 39.0-39.3°C and shock duration was 2 min. In the experiment on mitotic gynogenesis, heat shock was applied at 0.10τ_0 intervals from 1.2 to 2.2τ_0 after insemination. Heat shock temperature was 39.9-40.0°C, and shock duration was 1.8 min.

Embryo survival to hatching and output of diploid larvae were examined in shock treated progenies. The number of diploid larvae was counted 2-3 days after hatching. Active, normal appearing, larvae were considered diploid. No-shock gynogenetic progenies of the same females were used as controls for examination of the fertilization rate and frequency of spontaneous diploidization. The fertilization rate was estimated as the proportion of either normally cleaving eggs at the morula stage or live embryos 24 hours after insemination.

One experiment on mass production of meiotic gynogenetic progenies (15/92) and two of mitotic (21/92, 21a/92) were conducted in Weiss jars. Eggs from one female were used in experiment 21/92 and from four and three females, respectively, in experiments 15/92 and 21a/92. Irradiated sperm of koi males was used in these experiments. In experiment 15/92, heat shock was initiated at 0.2 τ_0 after insemination; shock temperature was 39.3°C and shock duration was 2 min. In experiments 21/92 and 21a/92, heat shock was applied at 1.4-1.5 τ_0 after insemination; shock temperature was 39.4-39.5°C and shock duration 2.4-2.5 min.

Samples of inseminated eggs had been taken prior to heat shock in each experiment and were incubated in Petri dishes. These no-shock progenies served as controls, as in the laboratory experiment.

Embryo survival during the first 24 hours and output of diploid larvae were estimated.

The number of active, i.e. diploid gynogenetic larvae, was calculated volumetrically upon releasing them into ponds.

Induced polyploidy. Polyploidy was induced by heat shock applied at the second meiotic division (to obtain triploid progenies) or at the first cleavage (to obtain tetraploid progenies). Males and females were of the Dor-70 line. Eggs of several females and non-treated sperm of several males were used in each experiment. The mass production procedure was used. Heat shock temperatures were 38.5-39.6°C and 39.5-39.9°C in experiments on induced triploidy and tetraploidy, respectively. Shock duration was 2-2.5 min. Heat shock was initiated at 0.1-0.2 τ_0 after insemination for suppression of the second meiotic division and at 1.5 τ_0 for suppression of the first cleavage. The absolute time for heat shock initiation was calculated as described above.

The embryo survival and output of larvae were examined in shock-treated offspring. The fertilization rate was estimated in samples taken before heat shock initiation in each experimental progeny. The number of larvae obtained was estimated volumetrically 4-5 days after hatching, upon releasing them into ponds.

Evaluation of ploidy level. The ploidy level was examined by three conventional methods: counting the number of nucleoli per nucleus (in larvae), measuring the erythrocyte size and measuring the DNA quantity in erythrocyte nuclei (in fingerlings). The data obtained were compared with those of control (diploid) fish. Nucleoli were counted in interphase nuclei of epithelium cells of one or two-day-old larvae using the standard aceto-carmine method. The ploidy level was estimated based on the knowledge that, in the common carp, the mean number of visible nucleoli per genome usually varies within the range of 0.85-0.9 (Cherfas et al., 1990, 1991). This method provides an early and almost immediate estimate of ploidy level.

Blood smears stained with Giemsa were prepared for determination of the sizes of the erythrocytes and their nucleus. Relative amounts of DNA quantities per nucleus were estimated by flow cytometry using a modification of the method described by Thorgaard et al. (1982). We used propidium iodide instead of diamidino-2-phenylindole (DAPI; Moav and Hagani, in prep.). The fluorescent activated cell sorter, FACS IV (Becton-Dickinson) at the Tel Aviv University, was used with an Argon laser 488nm and emission was collected after LP 570nm filter.

Rearing of gynogenetic progenies. Gynogenetic larvae obtained in Weiss jars were raised in three experimental ponds (each of 0.04 ha) to the age of 2.5 months.

Results

The eggs used in all the experiments were of good quality, as judged from data on the control samples. The mean fertilization rate was 85%; embryo survival prior to hatching 92% and output of normal larvae 90% among controls in the polyploidy experiments. The mean fertilization rate was 82%, showing good quality of the irradiated sperm, and embryo survival prior to hatching was 83-97% among controls in the induced gynogenesis experiments. The frequency of spontaneous gynogenetic diploids was 0.13±0.09% (mean of six control progenies), which is within the usual range for common carp.

Meiotic gynogenesis. The results of the two laboratory experiments were similar (Table 1). The survival in shock treated progenies was high, though decreased survival was observed when shock was applied early, especially in experiment 8/92. Chromosome-set diploidization was induced in most treatments, but heat shock was especially effective when initiated within 0.05-0.15 τ_0 after insemination.

The results of the large scale experiment 15/92 are given in Table 2. The output of diploid larvae in shock treated gynogenetic offspring was 7-8% of the total number of fertilized eggs (50,000-70,000 per jar), which is similar to that obtained in the small-scale experiments among eggs subjected to heat shock at 0.2 τ_0. About 10,000 diploid larvae were produced in two Weiss jars. No diploid larvae were found among 200 embryos in the no-shock control sample.

Mitotic gynogenesis. Results of the two replicates of experiment 6/92 are presented in

Table 1. The output of diploid larvae in meiotic gynogenetic progenies shocked at different t_0 after insemination.

Heat shock timing (τ_0)	*No. of fertilized eggs*	*Survival prior to hatching (%)*	*Diploid larvae (no.)*	*(%)*
Experiment 8/92: Heat shock of 39.1-39.3°C for 2 min				
0.05	143	51	27	18.8
0.10	160	41	30	18.7
0.15	123	43	18	14.6
0.20	173	76	11	6.3
0.25	168	85	3	1.7
0.30	218	84	6	2.7
0.35	200	83	0	0.0
C	182	96	0	0.0
Experiment 13/92: Heat shock of 39.0-39.1°C for 2 min				
0.05	199	51	37	18.5
0.10	217	75	64	29.4
0.15	216	52	30	13.8
0.20	362	64	19	5.2
0.25	221	86	24	10.8
0.30	174	81	6	3.4
0.35	208	81	8	3.8
0.40	208	74	1	0.4
C	242	97	1	0.4

C = no shock gynogenetic control

Table 3. Embryo survival was very low in all shock-treated progenies. Heat shock initiated at 1.7 τ_0 after insemination or later resulted in complete mortality of the embryos before hatching. Diploid larvae were obtained when shock initiation was within 1.2-1.6 τ_0, with the highest output at 1.4-1.5τ_0.

The results of the mass production experiments are presented in Table 2. Results obtained in experiment 21/92 were similar to those in Petri dishes. Diploid gynogenetic larvae output was about 7% of the total number of fertilized eggs (80,000-90,000 per jar) with a maximum of 8% when heat shock was initiated at 1.5 τ_0 after insemination. The output of diploid larvae in progeny 21a/92 was 23% of approximately 130,000 fertilized eggs. About 40,000 mitotic gynogenetic larvae were obtained in the two experiments. Two diploid embryos were found among 419 embryos in

Table 2. Mass production of diploid gynogenetic larvae in Weiss jars.

Jar	No. fertilized eggs[1]	Shock conditions			Embryo survival (%)	Diploid larvae	
		τ_0[2]	Temperature (°C)[3]	min		(no.)	(%)
Meiotic gynogenesis							
Experiment 15/92							
4	71,175	0.2	39.3	2.0	56	5,760	8.0
5	55,575	0.2	39.3	2.0	70	4,089	7.3
Total:	126,750					9,849	7.7
Mitotic gynogenesis							
Experiment 21/92							
7	81,120	1.4	39.5	2.5	58	4,658	5.7
8	92,625	1.5	39.5	2.5	28	7,467	8.0
Total:	173,745					12,125	6.9
Experiment 21a/92							
	128,700	1.5	39.4	2.4	49	29,870	23.2

1 The number of fertilized eggs was calculated as the total weight of the eggs multiplied by 650 (the mean of eggs in 1 g) and the rate of fertilization.
2 After insemination
3 Final heat shock temperatures

the no-shock control samples of experiment 21/92 and none among 224 embryos in the control of experiment 21a/92.

Rearing of gynogenetic progenies. The results of rearing gynogenetic larvae in ponds are given in Table 4. All but one of 1236 fingerlings from experiment 15/92, and all 1168 fingerlings from experiments 21/92 and 21a/92, had scattered (mirror) scale cover, as expected. Survival in all gynogenetic progenies was low. Most of the progeny in experiment 15/92 perished during the first 30 days of rearing in ponds. About 130 meiotic gynogenetic common carp (mean weight 0.5 kg) and 500 mitotic gynogenetic common carp (mean weight 0.2 kg) survived to the end of the first summer. A large number of abnormal fingerlings was found in the two mitotic gynogenetic progenies, though it was higher in progeny 21/92 which were obtained from a single female (25% vs. 7%). Almost all abnormal fish had the same type of deformity, i.e. bent caudal part of body. No abnormal fish were found in meiotic gynogenetic progeny 15/92.

Table 3. Survival and output of diploid larvae in mitotic gynogenetic progenies shocked at different τ_0 after insemination.

Heat shock timing (τ_0)	*No. of fertilized eggs*	*Survival prior to hatching (%)*	*Diploid larvae (no.)*	*(%)*
Experiment 6/92: Heat shock of 39.9-40.0°C for 1.8 min				
1.2	175	14	2	1.1
1.3	138	10	2	1.4
1.4	139	19	9	6.4
1.5	90	22	6	6.6
1.6	120	8	2	1.6
1.7	136	0	0	0.0
1.8	168	0	0	0.0
1.9	115	0	0	0.0
2.0	138	0	0	0.0
2.1	129	0	0	0.0
2.2	100	0	0	0.0
C	95	94	0	0.0

C = no-shock gynogenetic control.

Table 4. Rearing of gynogenetic progenies in ponds for 2.5 months.

Progeny	*Stocked*		*Survival*			
	Date	*No.*	*Date*	*No.*	*%*	*Mean weight (g)*
Meiotic gynogenesis						
Experiment 15/92						
	May 29	9,849	June 28	1,236	12.5	10
	June 28	218*	August 10	142	65.1	210
Mitotic gyngogenesis						
Experiment 21/92						
	June 28	12,125	September 8	389	3.2	70
Experiment 21a/92						
	June 28	29,870	September 7	779	2.6	51

* Selected for further rearing out of the 1,236 surviving fish.

Table 5. Experiments on mass production of triploidy (60,000-85,000 fertilized eggs per batch).

Experiment (no. of females)	Shock conditions			Results			
	τ_0*	Temperature (°C)	min	Embryo survival (%)	Normal larvae (%)	No. of larvae examined	3n (%)
7/92(3)	0.15	39.6	2.5	18	1.4	16	81.3
	0.20	39.6	2.5	16	2.4	4	50.0
	C	-	-	99	95.1	-	-
9/92(6)	0.10	37.0	2.0	10	2.3	9	44.4
	0.15	37.0	2.0	29	16.0	14	28.6
	0.20	37.0	2.0	50	34.4	16	18.8
	C	-	-	91	83.9	-	-
11/92(3)	0.20	39.6	2.0	32	14.1	33	81.8
	C	-	-	98	93.6	-	-
12/92(3)	0.20	38.6	2.0	56	n.d.	7	14.2
	0.20	39.0	2.0	59	n.d.	13	38.4
	0.20	39.6	2.0	48	17.5	4	74.4
	C	-	-	75	n.d.	-	-
19/92(3)	0.15	39.1	2.0	36	12.1	15	86.6
	0.15	39.2	2.0	38	17.7	12	83.3
	C	-	-	94	n.d.	-	-
20/92(3)	0.15	39.1	2.25	40	14.0	12	83.3
	0.15	39.4	2.5	63	17.2	9	100
	C	-	-	99	n.d.	-	-

C no-shock gynogenetic control
* After insemination
n.d. No data

Table 6. Experiments on mass production of tetraploidy (71,000-87,000 fertilized eggs per batch).

Experiment (no. of females)	*Shock conditions*			*Results*			
	τ_0[1]	*Temperature (°C)*	*min*	*Embryo survival (%)*[2]	*Normal larvae (%)*	*No. of larvae examined*	*4n (%)*
17/92(1)	1.5	39.5	2.5	76	30.6	16	100
	1.5	39.8	2.5	54	20.8	14	92.8
	1.5	39.9	2.5	39	10.3	15	93.3
	C	-	-	89	n.d.	-	-
19/92(1)	1.5	39.6	2.5	16	4.3	4	100

1 After insemination.

2 After 24 hours in experiment 17/92 and before hatching in 19/92.

n.d. No data

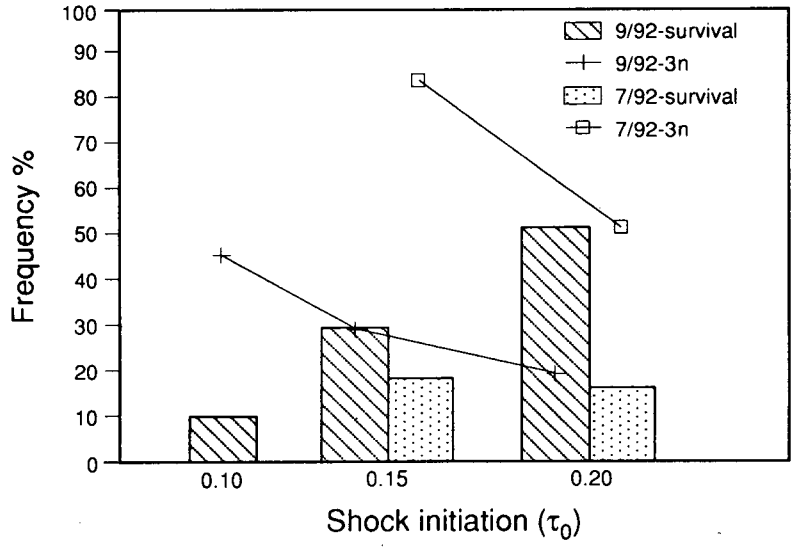

Fig. 1. Embryo survival and frequency of triploid larvae from batches of eggs subjected to heat shock at three embryological ages. Bar markings refer to different experiments as indicated.

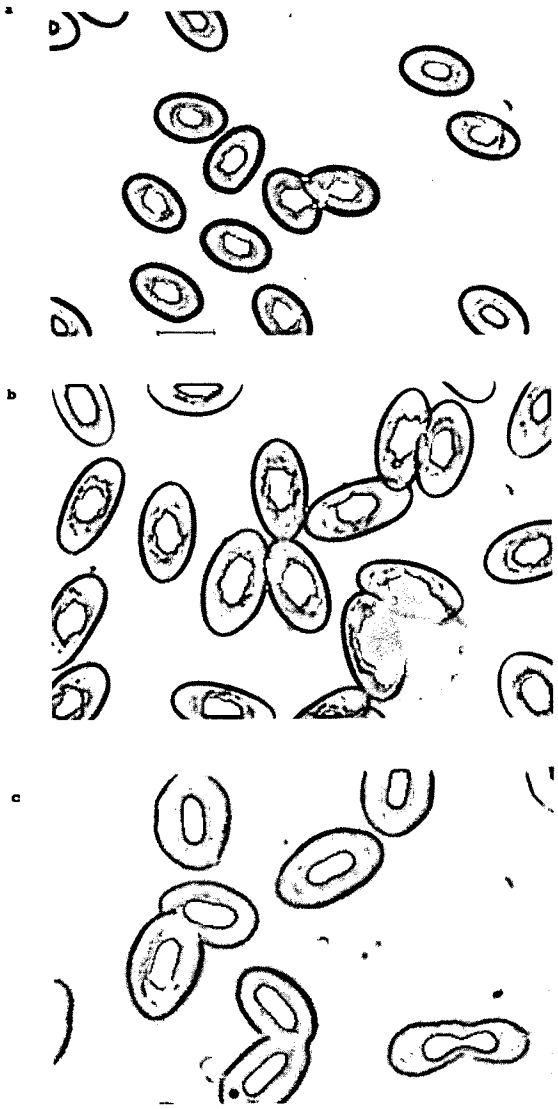

Fig. 2. Erythrocyte from a) diploid (17/92), b) triploid (20/92), and c) tetrapolid (17/92) common carp. Bar equals 10μ.

Induced triploidy. Triploidy was induced in 6 experiments in which 13 shock treated groups were obtained (Table 5). Almost all of them contained both triploid and diploid-aneuploid larvae in different proportions. The results of the first two experiments (7/92 and 9/92) show that the frequency of triploid larvae was higher (but embryo survival lower) when heat shock was initiated earlier than 0.2 τ_0 after insemination (Fig. 1). This is in good agreement with results on induced gynogenesis reported above. Even when a "low" shock temperature of 37°C was used (mistakenly,in experiment 9/92), embryo survival was only 10% and 29% at 0.10 τ_0 and 0.15 τ_0, respectively, compared to 50% when shocked at 0.2 τ_0, and 91% in the no-shock control. Thus, initiation of heat shock at 0.10 τ_0 is too early. Different heat shock temperatures were studied thereafter, when shock was applied at either 0.2 or 0.15 τ_0. When heat shock was applied at 0.2 τ_0, the frequency of triploids increased with the increase in shock temperature and was highest (50-82% in different experiments) at 39.6°C. The best results were obtained at 0.15 τ_0 after insemination (experiment 19/92 and 20/92). At a shock temperature of 39.1-39.4°C, triploid frequencies were higher than 80% in all progenies. The mean frequency (± S.E.) of triploids obtained in four progenies (experiments 19/92 and 20/92) was 88% (± 5%), with an embryo survival of 44% (± 7%) of which 15% (± 2%) were active, normal looking larvae. A similar triploid frequency was obtained in progeny 7/92 which was also shocked at 0.15 τ_0 (shock temperature of 39.6°), but very low embryo survival was observed in this experiment.

Larvae obtained in experiments 12/92 (about 13,700), 19/92 (7,400) and 20/92 (7,000) were stocked in ponds for further rearing and investigations (in prep.).

Induced tetraploidy. Tetraploidy was induced in two experiments (four groups of shock treated offspring), both of them were successful (Table 6). In experiment 17/92 embryo survival for the first 24 hours only was estimated, because hatching occurred earlier than usual. The output of active larvae was 10.3-30.6% of the total number of fertilized eggs. Increasing the shock temperature from 39.5°C to 39.9°C tended to decrease embryo survival. The mean frequency of tetraploid larvae, revealed in samples by nucleoli counts, was 95.4% (± 2.3%). A large number of weak larvae was found in all shock treated progenies.

About 60,000 larvae, obtained in experiments 17/92 and 19/92, were stocked into two

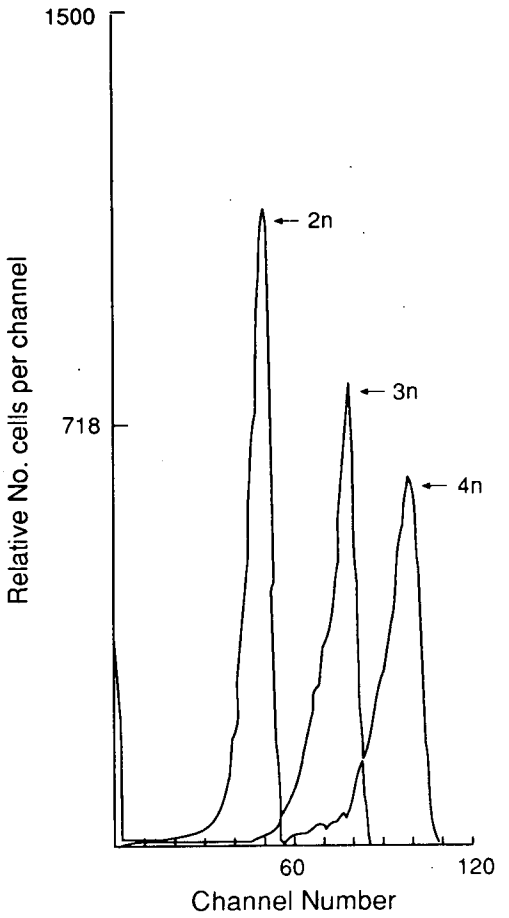

Fig. 3. Typical flow cytometric diagram of DNA fluorescence in diploid (control, exp. 20/92), triploid (shocked, exp. 20/92) and tetraploid (shocked, exp. 17/92) common carp erythrocytes. 5µl of heparinized blood were used for each sample and analysed separately at the same time.

ponds. Two tetraploid fish (one in each progeny) were found among 31 surviving fish after 45-60 days of rearing in ponds (the rest were diploids). One tetraploid (identified by both flow cytometry and erythrocyte size, see Figs. 2 and 3) weighed 74 g and was normal in appearance and is now being reared under laboratory conditions. The other (identified only by erythrocyte size) weighed 86 g, had morphological aberrations and died later.

Discussion

Induced gynogenesis. The mirror scale cover in fish obtained in the Weiss jars experiments confirms the gynogenetic origin of all experimental progenies.

The optimum shock times for obtaining meiotic gynogenesis and mitotic gynogenesis in the Dor-70 line, according to the results of our laboratory experiments, are 0.05-0.15 τ_0 and 1.4-1.5 τ_0, respectively, after insemination. This is somewhat earlier for suppression of the second meiotic division and the same for the first cleavage, as results obtained in experiments with koi (Cherfas et al., in prep.).

Production of diploid meiotic gynogenetic larvae was high (19% and 29% of fertilized eggs at 0.05-0.10 τ_0) in both laboratory experiments. Heat shock was less effective in meiotic gynogenetic progeny 15/92 in Weiss jars, possibly because heat shock initiation (at 0.2 τ_0) was too late. The frequencies of diploid mitotic gynogenetic larvae were within the common range for common carp in progenies 6/92 and 21/92, but unexpectedly high in progeny 21a/92 in which 23% diploid larvae were obtained. We cannot exclude the possibility that these include gynogenetic fish of meiotic origin produced by spontaneous diploidization, as was recently shown by N. Taniguchi (pers. comm.). However, the number of meiotic gynogens could only have been very small, according to the level of spontaneous diploidization in control progenies.

The success in mass production of mitotic gynogenetic progenies is the most important result of these experiments. Further progress will depend on the reproductive ability of gynogenetic females, which is sometimes less than optimal (Gomelsky et al., 1979; Komen et al., 1992). If fertile gynogenetic females will be obtained, mitotic gynogenetic Dor-70 progenies could be used as founders of clones. These could be utilized in selection work and for specific scientific investigations in which completely homogeneous groups are required.

Induced polyploidy. Cytometric and flow cytometry analyses performed on fingerlings supported the conclusion reached after nucleoli count in larvae regarding the polyploid nature of the progenies.

Based on the results, the following heat shock parameters can be recommended for mass production of polyploid or predominantly polyploid progenies:

- to induce triploidy, shock initiation at 0.15 τ_0 after insemination, shock temperature of 39.0-39.5°C and shock duration of 2-2.5 min. The expected frequency of triploids is 80-100% and output of larvae 15-20% of that obtained in no-shock control groups.

- to induce tetraploidy, shock initiation at 1.5 τ_0 after insemination, shock temperature of 39.5-40.0°C and shock duration of 2-2.5 min. The expected frequency of tetraploid larvae is 90-100% and output of larvae 15-20% of that obtained in no-shock control groups.

These optimum heat shock parameters are similar to those for obtaining diploid meiotic and mitotic gynogenesis, respectively.

In their most successful mass production experiments, Recoubratsky et al. (1989, 1992) also obtained 80-100% triploids and the output of larvae in shock treated progenies was 50-70% of controls. The frequency of tetraploid larvae in investigated samples varied (41-100%) and the output of normal larvae 0-11% in different shock treated progenies. A single tetraploid fingerling was found at the end of the growing season (Gomelsky, pers. comm.). The Dor-70 line seems to be more sensitive to heat shock (especially during the second meiotic division) than the breed used by Recoubratsky et al., as can be seen from the higher embryo mortality rate in our mass induction experiments, even though we used lower shock temperatures than those used in Recoubratsky et al. (1992), 40-41°C. The death of a large proportion of the embryos was observed immediately after shock application, probably caused by cytoplasmic damage in shock treated eggs. The frequency of chromosome-set diploidization, however, was similar in both investigations. The results confirm that tetraploid common carps have an extremely low survival rate, similar to tetraploid fish of other species. It is possible that low survival in tetraploid fish is caused by the large number of homologous chromosomes, which interfere with important vital functions. This may be even more of a problem in species of tetraploid origin, like the common carp.

Acknowledgements

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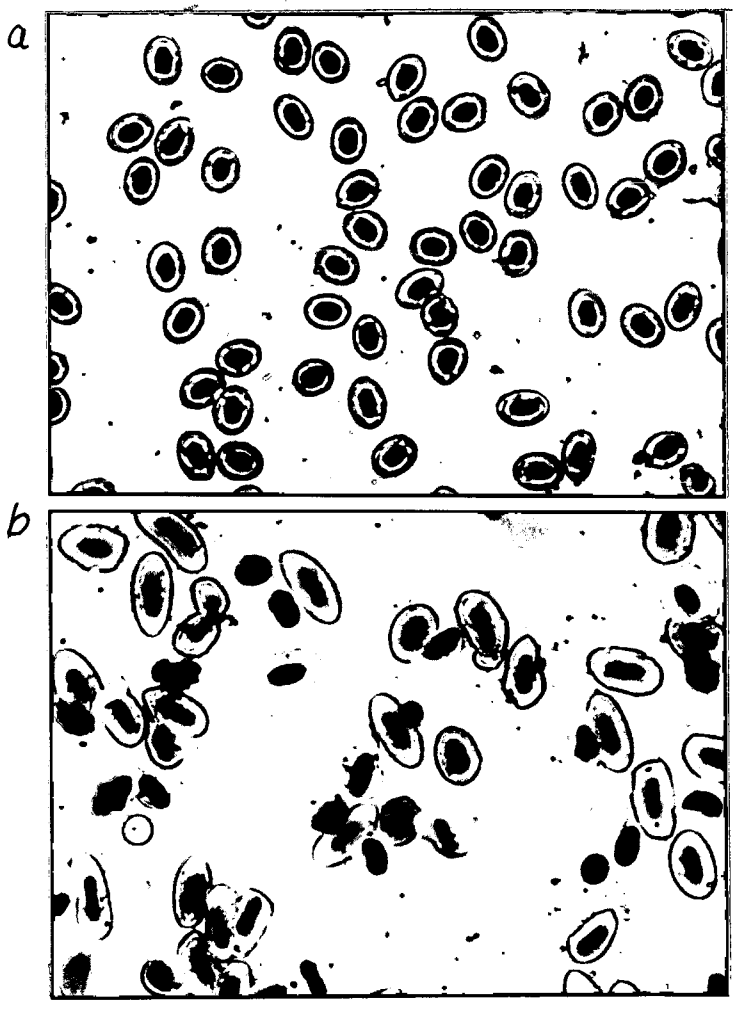
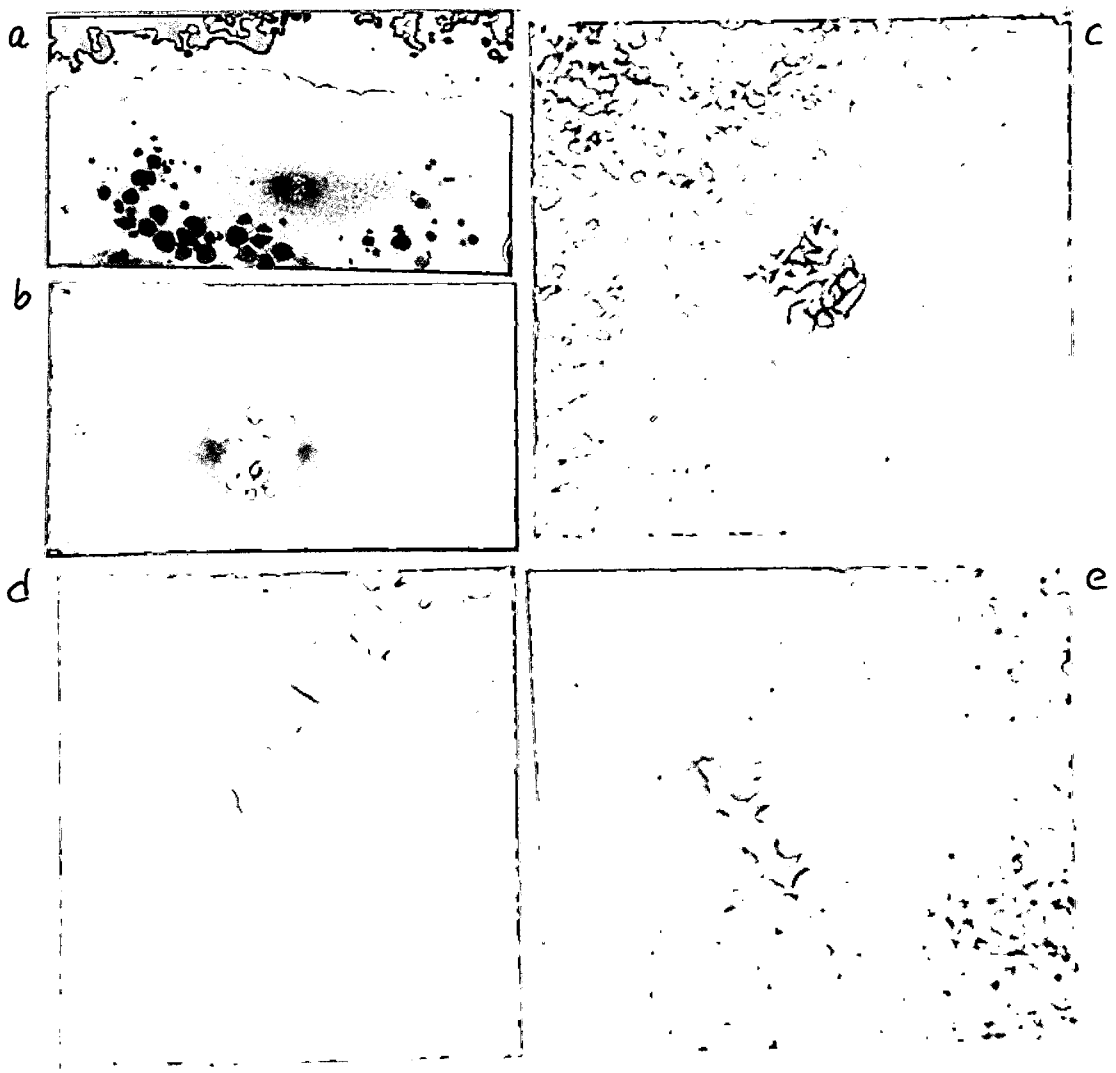


Suppl. 2. Ploidy level estimation by erythrocyte size: diploid (a) and tetraploid (b) fish. Blood smears stained with Gimsa.



Suppl. 3. Timing of the 1st mitotic division stages (τ_0 , after insemination):
a, b - aproaching of female and male pronuclei ($1.2\,\tau_0$), c - formation of the metaphase spindle ($1.4\,\tau_0$), d, e - metaphase of the 1st mitotic division ($1.5\text{-}1.6\,\tau_0$).
Series sections stained with hematoxylin.

1 - A circulated water system for incubation of small egg samples in Petri dishes;
2 - Weiss jars used for egg incubation in a large-scale experiments (milk emulsion for degumming of eggs in jars # 7 and 8);
3 - Egg treatment by malachite green against fungus.

Pressure/Thermal shock

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Thermal shock experiments were conduced primarily in Israel, but techniques were applied in the course of the U.S. studies to produce gynogens for sex reversal. Attempts to produce sufficient numbers of gynogens for large-scale sex reversal experiments were not successful. As previously indicated, the different fish stocks may have been a factor.

Hydrostatic pressure to induce second polar-body retention or interfere with first mitosis has not been widely tested in carps. The equipment is expensive compared to materials needed for thermal shock and experimental procedures are more difficult to accomplish. Cassani and Caton (1986) and Cassani et al. (1990) used pressure shock for early and late period manipulation of grass carp, *Ctenopharyngodon idella*. The pressure chamber that they used was somewhat smaller (0.8 L) than the one we developed. Linhart et al. (1991) is the only published account of pressure-induced chromosome manipulation in the common carp.

Heat Shock: During several trials, attempts to produce polar body gynogens were unsuccessful. Eggs from local stocks of mirror carp were activated with U.V. treated (800 joules) milt of fully-scaled common carp. Thermal shock of 39.0°C was applied at three different post-activation intervals from an ambient temperature of 20°C. The time of shock was based on the biological age as reported by Detlaff and Detlaff (1961) with a duration of 1.5 minutes. The shock was applied at 0.15, 0.20 and $0.25\tau_0$ according to the results of the earlier Israeli studies (Cherfas et al. 1993).

Pressure Shock: The cylinder used in our experiments was constructed from stainless steel with a total volume of 1.04 l, 0.92 l of which is usable. In experiments with non adhesive or degummed eggs, the workable volume will permit treatment of over one half million carp eggs. The cylinder is 23 cm in diameter and 7.6 cm deep, which will accomodate 7-8 small (5 cm) petri dishes for experimental work. In replicated treatments, the small petri dish will permit sticking 100-175 eggs without undue crowding. Eggs of Koi females (mirror) were fertilized or activated with milt from fully-scaled common carp. For gynogenesis, the milt was U.V. treated as described. Eggs were activated in

small petri dishes maintained at 20°C until shocked. Pressure was applied at time intervals corresponding to $0.5\tau_0$ units up to 0.30 τ_0. Late shock for mitotic gynogenesis was included in one series; shock time was initiated at $1.2\tau_0$ and increased to $1.7\tau_0$ in $0.1\tau_0$ units. About 45 seconds were required to attain selected pressures. Trials were tested at 7,000, 7,500, 8,000 and 8,500 PSI, each of which was applied for 1.0 minutes.

Results: No diploid gynogens were produced in the heat shock treatments. In some treatments, 35-54% of the developing treated embryos were viable and the control had 60-96% viable embryo development, most of which hatched (Table 1a).

Pressure-shocks for polar-body induction were conducted in a series of three trials. One produced no viable diploids, but the control group was also inviable. The other two series produced viable embryos (Table 1b) and viable gynogens (Table 1c). The highest development rate was in replicates treated at 0.25 and $0.30\tau_0$ and the greatest yield of gynogens corresponded to these shock times. The best pressure appeared to be in the 7,500 to 8,000 PSI range, as yield was lower at 7,000 and 8,500. All gynogens were light colored as would be expected with koi females.

Late shock for mitotic interference had relatively low levels of embryonic development and no gynogens hatched (Tables 1b & c). The control replicate in this treatment had about a 65% hatch rate.

The optimum time of shock for pressure induction of polar- body gynogens appears to similar to the effective period for thermal shock induction in koi (Cherfas et al. 1990) and common carp (Cherfas et al. 1993). Compared to thermal shock protocol, pressure shock induction is both more expensive to initiate and more complicated to administer. Effectiveness of late shock for Chinese carps (Cassani et al. 1990) and more efficient production of triploid Chinese carp (Cassani and Caton 1986) may suggest selection of pressure treatment over thermal shock, but more experimentation will be required to demonstrate this advantage in common carp.

Table 1A. Percent viable embryos in thermal* induced polar body gynogenesis in common carp.

Shock	τ_0						
(°C)	O.05 (1.2)	0.10 (1.3)	0.15 (1.4)	0.20 (1.5)	0.25 (1.6)	0.30 (1.7)	Control
35.0	-	-	-	54.0	35.0	-	69.0
38.0	-	-	-	2.0	10.0	-	96.0
39.0	-	-	-	39.6	39.7	-	60.0

Table 1B. Percent viable embryos in pressure-induced polar body or endomitotic gynogenesis in koi carp.

(PSI)							
7000	10.1	29.8	6.9	2.9	48.4	41.1	88.7
7500	12.6	23.8	35.2	0	58.8	48.8	74.7
8000	13.3	25.5	18.9	14.4	62.0	61.9	51.4
8500	26.9	34.1	16.2	25.4	28.7	19.2	65.3
	(0.8)	(5.9)	(26.1)	(12.3)	(0)	(15.4)	65.3

Table 1C. Diploid gynogens from polar-body and endomitotic pressure shock** induction in koi carp.

(PSI)							
7000	0	0	0	0	5	7	136
7500	0	0	0	0	10	1	110
8000	0	0	0	0	13	1	66
8500	0	1	4	2	5	3	31
	(0)	(0)	(0)	(0)	(0)	(0)***	31

*) Thermal shock duration = 1.5 min; Pressure shock duration = 1.0 min at full pressure + 0.45 sec to reach.

**) No gynogens produced in PB thermal shock treatments, hatch among controls was proportional to percent viable embryos.

***) No gynogens were produced in endomitotic pressure shock.

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2. Investigations on spontaneous diploidization in koi

EVIDENCE FOR THE HERITABLE NATURE OF SPONTANEOUS DIPLOIDIZATION IN COMMON CARP EGGS (short communication).

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Spontaneous diploidization of maternal chromosomes (SDM) is a well known phenomenon in fish. It was detected in experiments on artificial gynogenesis by the appearance of diploid descendants among haploid embryos when no treatment inducing female chromosome diploidization was applied. The occurrence of triploid embryos in usual crossing or the detection of triploid adult fish in cultivated stocks or natural populations of bisexual fish species were considered as further indications of SDM (reviewed by Cherfas 1981, Thorgaard 1993, Benfey 1989). Judging from the output of spontaneous gynogenetic diploids or triploids in usual progenies, SDM is a relatively rare event, the frequency of which doesn't normally exceeds 1-1.5%(Cherfas 1981). That is why the cases of high SDM frequency in fish are of special interest. Among bisexual fish species, high SDM frequency was recorded in rainbow trout *Oncorhynchus mykiss* and tench *Tinca tinca* based on the occurance of high proportions of triploid fish in hatchery stocks (Thorgaard & Gall 1979; Flajshans, Kvasnicka & Rab 1993) and in ornamental (koi) common carp *Cyprinus carpio* L. according to output of spontaneous diploid embryos in gynogenetic progeny (Cherfas, Rothbard, Hulata & Kozinsky 1991). So far, our understanding of the cause of SDM in fish is restricted to general considerations. Some accidental internal or environmental factors (e.g. over-maturation of eggs, sudden temperature changes, etc.) affectingthe processes of egg fertilization are usually believed to be possible causes of SDM in fish. For some cases, genetic predisposition to SDM was suggested, but not proved (Cuellar & Ueno 1972, Thorgaard & Gall 1979, Flajshans et al. 1992). No specific investigations related to this problem are known to us. The present experiment is, apparently, the first attempt of such investigation in which data confirming hereditary nature of the SDM phenomenon in common carp were obtained. An unexpected high SDM frequency was recorded for one koi female in our experiments on induced gynogenesis in

common carp (Cherfas et al. 1991). The proportion of spontaneous diploids in a gynogenetic progeny (intact eggs x genetically inactivated sperm) and of amphimictic triploid embryos in a control progeny (intact eggs x intact sperm) obtained from this female were 19% and 36%, respectively, from the total number of fertilized eggs. Some of the gynogens obtained in this experiment were reared to maturity for further investigations, the most important of which was the examination of a possible hereditary control of the SDM phenomenon. For this reason a second successive gynogenetic generation was obtained and analyzed.

The experiment aimed at producing the second gynogenetic progeny was carried out at the Dor Station in summer 1993. A gynogenetic progeny was obtained from one four-year-old gynogenetic female (generation of 1989) by inseminating intact eggs with genetically inactivated sperm of edible common carp males, according to following scheme: koi female (orange color, genotype $b_1b_1b_2b_2$) x males of edible common carp (wildtype color, genotype $B_1B_1B_2B_2$). Sperm genetic inactivation was achieved by irradiation of sperm with UV ($800J/m^2$). The dominant color alleles B_1 and B_2 served as genetic markers for confirming sperm genetic inactivation, as in our previous investigations (e.g. Cherfas, Kozinsky, Rothbard & Hulata 1990, Cherfas, Hulata & Kozinsky 1993). No shock treatments was used for inducing chromosome-set diploidization in the eggs. Eggs and sperm were obtained after injection of breeders with a calibrated carp pituitary extract (Yaron, Bogomolnaya & Levavi 1984). About 40,000 eggs (60g) of usual size and color were obtained from the koi female. Inseminated eggs were placed in a Weiss Jar for incubation up to hatching. For obtaining more precise data, samples of 100 fertilized eggs were also incubated in a Petri-dish placed in a circulated water system. After hatching, the larvae were kept in small net cages in running water. The number of 2n-gynogenetic larvae obtained was estimated 4 days after hatching. All normal in appearance, active larvae were considered as diploid ones. A small number of control embryos (intact eggs x intact sperm) were simultaneously incubated in a Petri-dish, but due to a technical problem no data are available.

The results of incubation of gynogenetic progeny are given in Table 1. The proportion of live embryos after 24 hours was 50%, from the total number of inseminated eggs. Since this index usually corresponds to fertilization rate, the number of eggs in Weiss Jar that started gynogenetic development can be estimated at about 20,000. The relative output of 2n-gynogenetic larvae was the

same in the Weiss Jar and in the Petri-dish, i.e. 34-35% from the total initial number of gynogenetic embryos. Apparently, the frequency of SDM *per se* was even higher, if we consider embryo mortality prior to hatching. All larvae had typical "transparent" color, and all fingerling developed orange color, confirming their gynogenetic origin, whereas all control larvae had the dominant wildtype pigmentation. Most of the gynogenetic larvae obtained were stocked into ponds for further rearing, and 500 gynogenetic larvae were kept in a laboratory tank. After one month the survival of fish in the tank was 90%, showing high viability in this gynogenetic progeny.

Thus, the high SDM revealed in the koi female in the 1989 experiment was retained in her gynogenetic daughter. According to the production of spontaneous 2n-gynogenetic larvae (and of spontaneous triploids in the 1989 experiment), SDM frequency was nearly the same in the first and the second gynogenetic generations or even somewhat higher in the second one. These results allow to conclude that the SDM phenomenon in koi has a genetic control.

Based on published literature on natural and induced gynogenesis in fish (reviewed by Cherfas 1981, Thorgaard 1983, Chourrout 1987), three types of cytological transformations of meiosis may be responsible for obtaining progenies with unreduced maternal chromosome number: premeiotic endoreduplication of chromosome set followed by two meiotic divisions, suppression of the first meiotic division, and suppression of the second meiotic division. The first two types result in production of unreduced and genetically uniform eggs. They are known to occur in natural gynogenetic forms of fish, and the first type also in some interspecific fish hybrids obtained under laboratory conditions (Emelyanova 1984). Suppression of the second meiotic division is usually induced in experiments on artificial gynogenesis (or triploidy) for restoration of the diploid chromosome number in haploid eggs. As was shown in a large nubmer of investigations, this transformation provide genetic variability of maternal chromosome sets. As far as the SDM phenomenon is concerned, it is also believed to result from suppression of the second meiotic division, induced by some accidental factors. This hypothesis was confirmed by genetic analysis of experiments on plaice *Pleuronectes platesa* (Thompson, Purdom & Jones 1981) and common carp (Cherfas, unpubl.). In both experiments, the segregation of marker genes was the same in gynogenetic progeny resulting from SDM and from induced diploidization of maternal chromosome set (by suppression of the second meiotic division).

In order to validate what type of meiosis transformation is responsible for SDM in koi, females of the first gynogenetic generation (three surviving fish only, including the female giving eggs) and fingerlings of the second gynogenetic generation were investigated by electrophoresis. Five polymorphic proteins (TF, LDH, MDH, PGM, GPI) were analyzed, and following results obtained (Ben-Dom, Cherfas & Hulata, unpubl.): 1. females of the first gynogenetic generation have similar electrophoretic phenotype and are heterozygotes according to Ldh, Mdh, Pgm and Gpi-2 genes; 2. no segregation in heterozygous genes was observed in the second gynogenetic generation (n= 13 - 33 fingerlings were used for testing the different protein genes); 3. all fish of the second gynogenetic generation have the same electrophoretic phenotype, which is identical to that of their mother. The genotypic identity of all investigated gynogenetic fish according to the gene markers tested shows that SDM may have resulted from endoreduplication of the chromosome-set in the early meiosis, or from some transformation of the first meiotic division excluding gene segregation. These hypotheses will be further examined cytologically.

The results of the present experiment are believed to be the first experimental evidence of the SDM inheritance in fish. The data obtained are of general interest for different fields of fish cytogenetics, and, in particular, for further understanding of natural polyploidy in fish.

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Table 1. Results of incubation of gynogenetic progenies

Condidions	No. of fertilized eggs	Normal larvae obtained (no.)	(%)	Wild-type (%)
Weiss Jar	20,000*	7,000*	35	0
Petri-dish	100	34	34	0

*) Estimated from a standard of 1000 larvae

3. Investigation of triploid progenies.

Assessment of triploid common carp for culture

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Abstract

Results of rearing triploid common carp to sexual maturity in separate and communal ponds are presented. The majority of the one-year-old triploid fish had undeveloped gonads and were sterile. Considerable excess of males was observed in one triploid group. Triploid fish had lower survival (about 70% of diploid control), and their growth rate was about 1.3-1.7 times lower than that of the diploid control fish. No dependence of fish weight on gonad state was found. No advantage of culturing triploid carp over diploid carp was observed.

Introduction

Commercial interest in obtaining triploid fish is based on the assumption that triploids have some advantage over diploids. Investigations of this subject, carried out on several fish species, gave different results depending, apparently, on the biological features of the species and on experimental conditions, including the method of obtaining the triploid progenies (Lincoln, 1981; Wolters et al., 1982; Benfey and Sutterlin, 1984; Solar et al., 1984; Krasznai and Marian, 1986; Thorgaard, 1986; Ihssen et al., 1991; Sugama et al., 1992). The advantage of triploids may primarily arise from their sterility. Experiments on several fish species, i.e., plaice (Pleuronectes platessa) x flounder (Platichthys flesus) hybrid, channel catfish (Ictalurus punctatus), loach (Misgurnus anguillicaudatus) and -

rainbow trout (Oncorhynchus mykiss) confirmed that triploid fish have better growth beyond the age of sexual maturity when somatic growth is usually supressed by reproductive processes (Purdom, 1976; Wolters et al., 1982; Suzuki et al., 1985; Thorgaard, 1986; Ihssen et al., 1991). Rearing of sterile triploids may prevent uncontrolled fish reproduction. Thus, utilization of triploids in aquaculture may be especially beneficial when cultivation period extends beyond sexual maturation. Several investigations (most of which were limited to observations on fingerling) gave fragmentary data concerning aquaculture assessment of triploid common carp. No difference in growth was observed, in the short-term experiments of Gervai et al. (1980), between diploid and triploid carp fingerlings under the conditions of communal rearing in tanks, while Wu et al. (1979; cited by Wu, 1990) found that the average weight of 5-month-old triploids was more than twice that of diploids reared in the same pond. Different results were obtained in two experiments of Taniguchi et al. (1986) when fish were reared to adult stages in communal ponds. Triploids grew slower than diploid controls in crossbred offspring of Japanese strain (female) x European strain (male) of common carp, but better growth of triploids was observed in offspring of the pure Japanese strain. Recoubratsky et al. (1990) reported that total fish production during the first summer in ponds stocked with either triploid and diploid common carp was similar. Although promising, these data are insufficient for evaluating triploid common carp's potential for aquaculture.

The main purpose of the present study was to investigate survival, growth rate and gonad state in triploid common carp up to the age of sexual maturity. The problem is of special interest for Israeli pond aquaculture where common carp reaches sexual marturation during the first summer, but it is marketed after a 2-year culture cycle.

Materials and methods

The experiments were conducted at the Fish and Aquaculture research station Dor (Israel) during 1992-1993. Triploid fish were obtained from the Israeli Dor-70 common carp line in the 1992 spawning season. Triploidy was induced by applying heat shock to fertilized eggs at meiosis II (Cherfas et al., 1993). Two shock treated progenies (STP),

i.e., 12/92 and 20/92, and thier corresponding control progenies (CP), obtained from the same parents without any treatment, were used. The proportions of triploid fish in STP were 75-80% (12/92) and about 90% (20/92). The larvae were stocked into separate ponds for primary nursing. Growth comparison started with 30-day-old fry (12/92) and 52-day-old fingerlings (20/92). Mean weights of both 12/92 STP and CP groups was about 2g and that of 20/92 STP and CP groups was 49g and 23g, respectively. The difference between 20/92 STP and 20/92 CP arose from lower fish density in the STP nursery pond, resulting from lower survival. STP and CP groups were stocked into separate and communal 0.04 ha ponds, with differential marking in the latter. Fish growth was monitored every 7-10 days by sampling the ponds. Additional data of the culture conditions are given in Table 1. In December 1992 the ponds were drained, and survival and mean fish weight were estimated. The fish were transferred to a set of new ponds, and the numbers of fish in STP and CP groups were equalized in the separate ponds again. Some fish from each STP group were identified by ploidy level to estimate the proportions of triploids at the end of the first rearing period.

In June 1993 all the fish in the STP groups were classified by ploidy level, according to erythrocyte size. All STP and CP fish were weighted individually. Gonads of adults were inspected macroscopically and weighted. Ovary maturation stages were estimated according to Horvath (1985) and those of testes according to Gupta (1975). The Gonado-somatic index (GSI) was calculated as gonad weight/ body weight (in %). Corrected mean weights for STP and CP groups in communal ponds were calculated according to Wohlfath and Milstein (1987) - correction factors used were 3.2 and 4.6 for communal pond 12 and 30, respectively (see Table 1). Mean daily weight gains (DWG) were calculated as: (final weight-initial weight)/days of rearing.

Results

The results of the growth comparison of the STP and CP groups are presented in Table 1. During the first period, survival of STP groups was lower than that of the corresponding CP groups. Mean survivals (±S.E.) were 59.1±3.7% and 83.2± 3.7% in STP and CP, respectively. The proportions of triploid fish in STP (in samples taken in December 1992) was close to that estimated on larvae, i.e. 75% (STP 12/92) and

85% (STP 20/92). By the end of the first period the mean weight of the fish in the 12/92 STP group was similar to that in the 12/92 CP group, in spite of the considerably lower fish density in pond 19. The 20/92 STP group has maintained its initial weight advantage under the lower density in pond 18, but the mean weight of the 20/92 STP group was similar or even lower than that of CP in communal ponds (ponds 12 and 30, respectively). The corrected final mean weights indicate a large difference in favour of 20/92 CP group in both communal ponds, in spite of its initial lower weight.

During the second period (Table 1), fish survival was similar in the STP and CP groups, except in (communal) pond 14 where the survival of CP was lower. The final mean weight of STP groups was lower than that of the corresponding CP groups in all ponds, independently of the former's higher initial mean weight (i.e., final weight for the 1st period) and rearing conditions. DWG in CP was about 1.3-1.7 times higher than in STP (Table 1).

Mean weights of triploid (and the few diploid) females and males in STP groups and those of diploid females and males from the corresponding CP groups by the end of the experiment are presented in Table 2. Triploid fish were less heavier than CP ones, and this deviation was more pronounced in males (Fig.1). Most of the diploid fish found in the STP groups were also larger than the triploids in the same groups (Table 2). No dependence of fish weight on gonadal state was found. Variability of weight was greater among triploids than among diploids.

The proportions of females and males in both CP groups were close to the expected 1:1 ratio. The sex ratio among triploids was 1:1 in the 12/92 STP group, while excess of males was found among triploids in the 20/92 STP group (Table 2).

Most CP males and CP 12/92 females were already mature by the end of the experiment, while most of the CP 20/92 females had well developed gonads in stages 3, 3-4. The majority of triploid females and males had small gonads in stages 2, 2-3 (females) and 3-4 (males). GSI in triploid fish was 5-8 times lower than in diploids (Table 3). Some triploid fish (4.8% in STP 12/92 and 3.3% in STP 20/92) had completely undeveloped gonads. A few triploid males released a small amount of sperm by stripping, but the semen didn't look normal in consistency and

color. Almost all triploid fish were sterile. The presence of mature females among triploids in the STP 12/92 group was unexpected. These females had developed gonads in stage 4 and a GSI of 4-10%.

Discussion.

Triploid common carp were found to have lower survival during the first several months and it did not differ from that of control fish later. Growth rate was lower than that of diploids, both prior to and after attaining sexual maturity. The results obtained in separate and communal ponds were similar. Thus, competition between triploid and diploid control fish (if any) didn't have a considerable effect on growth rate in communal ponds. The decreased survival in STP could have resulted from the heat shock treatment (since no selective mortality of triploids was observed during period of fish rearing), while the lower growth rate of triploids resulted, apparently, from negative physiological effects of triploidy.

The heat shock treatment used to induce triploidy seems to cause changes in the sex ratio in favor of males. An excess of males (1:1.5) was also found among adult triploid common carp in another STP group reared at the Dor Station in 1993 (Cherfas et al., unpubl.). Solar et al. (1984) found excess of males (1:1.4) among triploid rainbow trout. These sex ratio deviations could, possibly, result from lower survival in triploid females than in triploid males.

Triploidy resulted in almost complete sterility, though some triploid males and females developed gonads which looked normal although their GSI was considerably lower than in diploids. An earlier study (Cherfas et al., unpubl.) has, however, shown that although triploid males can spermiate they are functionally sterile and any embryo developing from their matings die soon after hatching. The reproductive competence of the apparently mature triploid females needs to be evaluated.

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Table 1. Results of the growth comparison of diploid (CP) and shock treated (STP) groups.

Indices	Separate rearing Exp. 12/92		Separate rearing Exp. 20/92		Communal rearing Exp. 20/92			
	CP	STP	CP	STP	CP	STP	CP	STP
a) Rearing period: from June and August 1992 to December 1992								
Rearing period (days)	176	176	128	128	142		130	
Pond No.	23	19	26	18	12		30	
Initial no. of fish	170	170	170	170	85	85	33	33
No. of fish at harvest	125	85	153	113	69	48	29	21
Survival (%)	73.3	50.0	90.0	66.4	81.8	56.4	87.8	63.6
Initial mean weight (g)	2.4	1.5	22.9	52.3	22.3	49.4	20.2	35.4
Final mean weight (g)	1012	1047	495	577	436	441	529	485
Corr. mean weight, g	-	-	-	-	479	398	564	450
DWG (g)	5.7	5.9	3.7	4.1	2.9	2.8	3.9	3.5
b) Rearing period: from December 1992 to June 1993								
Rearing period (days)	165	165	113	113	155		154	
Pond No.	33	34	25	23	26		14	
Initial no. of fish	85	85	113	113	69	43	27	21
No. of fish at harvest	79	78	107	103	51	31	20	19
Survival (%)	92.9	91.8	94.7	91.1	73.9	72.1	74.1	90.5
Final mean weight(g)	1590	1400	1110	980	990	830	1200	990
DWG (g)	3.5	2.1	5.4	3.6	3.6	2.5	4.4	3.3
3N fish in STP (%)	-	79.5	-	92.0	-	87.1	-	94.7

Table 2. Final mean weight (kg) in triploid and diploid fish

Groups (pond #)	Females			Males		
	Mean ± S.E.	CV	n	Mean ± S.E.	CV	n
Separate rearing						
12/92 3n (34)	1.50±0.08	29.1	30	1.28±0.07	28.1	29
2n* (33)	1.63±0.06	22.0	31	1.54±0.05	16.2	27
2n** (34)	1.54±0.22	35.1	7	1.48±0.19	39.2	9
20/92 3n (23)	1.03±0.05	25.2	27	0.94±0.04	26.5	41
2n* (25)	1.18±0.04	23.7	57	1.04±0.03	18.2	50
2n** (23)	0.80±0.29	51.2	2	1.25±0.28	44.0	4
Communal rearing						
20/92 3n (26)	0.90±0.08	25.9	9	0.71±0.08	46.2	17
2n* (26)	1.05±0.04	20.6	29	0.92±0.04	20.7	22
2n** (26)	1.26±0.43	48.8	2	1.09±0.32	41.5	2
20/92 3n (14)	1.03±0.05	16.1	9	0.92±0.09	31.1	9
2n* (14)	1.20±0.08	20.2	10	1.20±0.06	15.9	10
2n** (14)	-	-	0	1.19	-	1

*) Diploid fish from CP

**) Diploid fish from STP

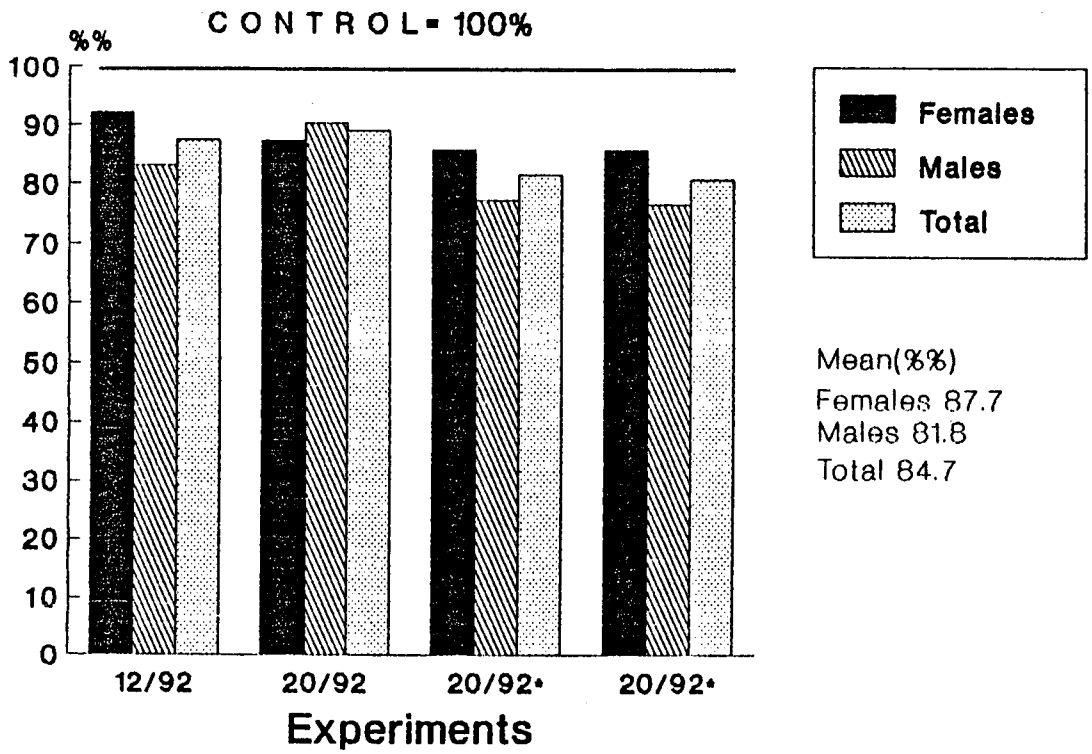
Table 3. Gonad state and GSI in one-year-old CP and STP fish*

Exp.	Ploidy	Females: GSI Mean±S.E.	Females: GSI n	Females: Mature (%)**	Males: GSI Mean±S.E.	Males: GSI n	Males: Mature (%)**
12/92	3n	1.4±0.5	30	17	1.1±0.1	29	3
	2n	7.5±1.0	31	68	5.4±0.5	27	81
20/92	3n	0.3±0.1	27	0	0.7±0.1	41	7
	2n	2.4±0.4	57	16	4.3±0.3	50	82

*) The results are given for separate ponds; similar data were obtained in the communal ponds.

**) Females having gonads in the 4th stage and males releasing sperm by stripping.

Figure 1. Mean weight of triploid fish from STP and corresponding control diploid fish in separate and communal (*) ponds.



The reproductive ability of common carp (Cyprinus carpio L.) triploid males

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ABSTRACT

The results of crossing a diploid female with triploid males of common carp, Cyprinus carpio L., are presented. The sperm obtained was functional and active, and true amphimixis took place. The fertilization rate was better, and embryo survival was similar to the control (o 2n x o 2n). However all larvae died soon after hatching. According to nucleoli count, the ploidy level of the larvae was close to 2.5n in two progenies and a diploid level was revealed in a third one.

INTRODUCTION

The reproductive ability of triploid fish is a crucial factor in programs attempting the use of triploids in aquaculture. It may be more advantageous to use triploids rather than the usual diploid fish because of the triploids' sterility. Although triploid common carps were obtained in a number of experiments (see Gomelsky and Grunina, 1988) there are only a few investigations concerning their reproductive capacity. Gervai et al. (1980) and Taniguchi et al. (1986) described undeveloped gonads in the artificially produced triploid common carps.

Sperm production by triploids has been reported for several fish species including plaice, Pleuronectes platessa (Lincoln, 1981), rainbow trout, Oncorhynchus mykiss (Benfey et al., 1986; Lincoln and Scott, 1984), grass carp, Ctenopharyngodon idella (Allen et al., 1986; Van Eenennaam et al., 1991), and tilapia Oreochromis aureus (Pennman et al., 1987). Sperm was also obtained from triploid males of crucian carp Carassius auratus gibelio Bloch produced by phenotypic sex inversion of triploid gynogenetic females (Gomelsky and Cherfas, 1985). In all cases triploid males were shown to produce actively motile sperm, but progeny obtained

from crossing such males with diploid females were inviable. The only exception was the case of grass carp, in which a few fingerlings were produced using the sperm from triploid males and all of them were diploids (Van Eenennaam et al., 1991).

The quality of sperm from triploid common carp males has not been previously investigated. The experiments reported here were conducted to examine the competence of spermatozoa produced by triploid carp males.

MATERIAL AND METHODS

Triploid fish have been produced from crossing one female of ornamental (koi) common carp with several males of edible common carp in our experiments on induced gynogenesis carried out at the Fish and Aquaculture research station Dor (Israel) in 1989 (Cherfas et al., 1991). Triploid descendants have resulted from spontaneous diploidization of female chromosome set in a part of eggs. Female of orange colour (genotype $b_1b_1b_2b_2$, recessive type) and males of wild color (genotype $B_1B_1B_2B_2$, dominant type) were used. According to this triploid fish were of $B_1b_1b_1B_2b_2b_2$ genotype and had wildtype coloration.

During 1989-1991 the fish were kept under standard pond conditions. In 1991 two-year-old fish (by approximate body weight 2kg) were tested according to erythrocyte size, and 18 males were identified as triploid ones.

The reproductive ability of these triploids was examined in March 1991, when all their diploid sibs had reached sexual maturity. Fifteen of the 18 triploids were completely sterile. Three triploid males each produced 1.5-2 ml of semen by stripping, after hormonal injection by calibrated carp pituitary extract (Yaron et al., 1984). The colour of the semen was gray, resembling diluted semen of diploid males. Sperm quality was evaluated by crossing triploid males with diploid koi female. Eggs obtained from the same female were used in crossing with triploid males. Quality of the eggs was tested by the control progeny obtained from crossing this females with diploid males (mixed sperm from 3 diploid males was used).

Eggs were incubated in Petri dishes placed on trays within a

circulating water system. Number of nucleoli per nuclei was used as an index of ploidy level. It was estimated in one-day-old larvae using the standard aceto-carmine method (e.g., Cherfas et al., 1990, 1991). The individual ploidy level was estimated as mean from counting nucleoli in nuclei of 100 epithelial cells. The mean number for a given progeny was calculated from individual values. About 25 larvae were analyzed in each of triploid males progenies and 17 larvae was analysed in the control..

RESULTS AND DISCUSSION

Fertilization rate was high in all groups fertilized with sperm of triploid males Table 1). Embryo survival until hatching did not differ in the experimental and control progeny. The early expression of the color genes enabled examination of progeny composition at the embryo-larvae stages. Segregation into wildtype pigmented (i.e., dominant) and non-pigmented (i.e., recessive) types was observed in all experimental progeny. This was the result of gene segregation in triploid heterozygous males and showed that true amphimixis took place in the course of fertilization. A high frequency of abnormal larvae was observed in the progeny obtained from triploid males (Table 1). These abnormalities were not specific, mainly expressed in different body shapes. All larvae (including morphologically normal) died during the first 6-8 days after hatching.

The results of nucleoli number analysis are given in Table 2. The progenies of triploid males #1 and #2 have clearly differed from the control group. As it was expected, the individual counts of nucleoli have ranged between diploid and triploid levels. The high variability in nucleoli counts has resulted from irregular separation of trivalents in the triploid meiosis. The progeny of male #3 does not differ from the control group neither by the mean count nor by the variability of the individual counts. This allow to consider all larvae obtained from male #3 as diploids. It should be noted that nucleoli count does not enable estimation of precise chromosome number. Apparently, the larvae obtained from triploid male #3 were not true diploids but aneuploids with chromosome numbers close to the diploid one.

The results obtained have shown that some triploid males of

common carp produce sperm after hormonal injection. Although the spermatozoa are active, and true amphimixis takes place in the course of fertilization, progeny obtained from triploid males is inviable. This allow to conclude that triploid common carp males are functionally sterile.

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TABLE 1

Results of incubation of the progeny obtained from a diploid female and triploid males of common carp.

Progeny (male no.)	No. of fertilized eggs	Fertilization rate (%)	Embryo survival prior to hatching (%)[a]	Total larvae obtained		Normal larvae obtained	
				(no.)	(%)[a]	(no.)	(%)[b]
1	564	88	93	234	42	124	53
2	484	76	78	103	21	53	51
3	487	75	87	171	35	77	45
Control[c]	291	60	82	136	47	112	82

[a]) Relative to number of fertilized eggs.

[b]) Relative to total number of larvae obtained.

[c]) o 2n x o 2n

TABLE 2

Results of analysis the progenies by nucleoli number (NN)

Progeny (male no.	Individual NN, range	Mean NN ± S.E.	CV	Ploidy level*	No. of larvae
1	1.6 - 3.1	2.2 ± 0.1	15.5	2n-3n	29
2	1.7 - 2.5	2.1 ± 0.1	13.5	2n-3n	26
3	1.6 - 1.9	1.8 ± 0.0	3.7	2n	24
Control	1.7 - 1.8	1.8 ± 0.1	2.5	2n	17

* Interpreted according to haploid number of 0.9 in the control progeny.

4. Investigations on sex reversal.

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Sex reversal in carps has had differential success. Nagy et al. (1981) reported a wide range of treatments with methyltestosterone at 100 mg/kg of feed which were successful. Some treatments started early posthatching while others were initiated as late as 98 days of age, with a comparable size range. Komen et al. (1989) were most successful using 50 mg/kg and beginning at 42 days of age; like the earlier studies, duration was about 5 weeks. Komen found that sex-reversal success was lower if treatment started earlier, and sterility rather than sex reversal often resulted. Komen et al. (1989) also achieved sex reversal when treatment started as late as 70 days with fish of 12 g and approximately 70-75 mm long. These results are similar to those of Gomelsky (1986) who initiated treatment with 9g and 12g fish where the number of males was significantly increased.

If information on gonadal development is related to the successful sex-reversal treatments, a correlation is not always clear. One factor which has not been adequately considered is the interaction of growth rate and gonadal differentiation. Parmentier and Timmermans (1985) reported that cytological differentiation occurred in common carp at 17-20 weeks of age and a size larger than about 55 mm (TL) or about 5 g. But in the various sex-reversal treatments, different growth rates have been reported; while treatments may have been applied on similar time schedules, the size ranges have been quite different. Nagy et al. (1981) indicated success within a size range starting at 3 mg to 1.3 g and ending at 0.7 to 7.1 g., while Komen et al. (1989) had low success in the smaller size range. Both Komen et al. (1989) and Gomelsky (1986) were successful in treatments initiated in fish of 6-10 weeks but similar in size to 17-week-old fish of Parmentier and Timmermans (1985).

During the progress of the current studies in the U.S., we investigated the effect of growth rate on gonadal differentiation. This question seemed basic to the development of a sex-reversal protocol which could be more uniformly applied. In order to examine the relationship, different growth conditions were established by manipulating the population density. Using density-dependent growth effects, populations were developed which were of the same age but had different growth rates. The growth dynamics will be discussed initially, then the results of the

histological studies of gonadal development within these populations will be described, and finally the hormone treatments will be related to these two areas.

Nursery-Pond Population Dynamics

Density-dependent growth is discussed by Backiel and LeCren (1978) and Hepher (1978) and specific examples for carp are provided by Moav and Wohlfarth (1974), Wohlfarth (1977), and Hulata et al. (1976). Growth control was the mechanism used to regulate hormone delivery in sex reversal of grass carp (Shelton 1986b).

During the three-year study, various stocking regimes were established. A two-pond series was used in the first year to examine gonadal development in relation to these population growth patterns. Two-day-old carp were stocked at 177 and 286/m^2 and samples were taken weekly over the 160-d growth period. In the second year, a ten-pond series was used to examine a wider range of growth patterns and gonadal relationships. Stocking rate ranged from 12.5 to 125/m^2; the growth period was about 150 d (133-168). As in year one, periodic samples were taken for growth, and fish were preserved for later histological studies. Growth trajectories in each of the ponds was related to stocking density, but the rates were more controlled by the effect of early mortality. Usually, the growth pattern was well established by two months and often was nearly asmyptotic. Because of the unknown level of early mortality, density-dependent growth was reported in relation to the final population density or draining density. In the final year, a six-pond series was developed with stocking rates of 75-207/m^2. The growth period was only 68 days, as the pattern was well established in this interval.

Results: The growth trajectories for the two-pond series is depicted in Figure 1. Growth rate differences were established within the first 18 days following stocking. The growth trajectories based on mean growth rates maintained distinct differences throughout the 160-day study period. The final mean size of the higher density population was 43.5 mm (TL) compared to the mean size of 52.4 mm in the lower density population. Survival from stocking was about 18 and 10% and density at draining was 39 and 8.4/m^2, respectively (Table 2). One of the primary questions to be addressed during this investigation was the relative effects of size and chronological age on the physiological process of gonadal differentiation; these results will be discussed in a subsequent section.

During the second year, the ten-pond series provided more information on density-dependent growth during first-season stocking. The growth trajectories were inversely related to population density at draining (Figure 2). As suggested from the two-pond study, growth-rate pattern was generally established within ponds early in the season, probably affected by early survival. The greatest growth rate was in the pond which had been stocked at one of the highest rates but had the highest mortality, while the slowest growth was in the pond with highest population density at draining, but which was also originally stocked with one of the higher numbers of fry (survival of O.2 and 15.7%, respectively) (Table 2). Population density at draining ranged from 0.24 to 19.6/m^2 with respective mean sizes of 254.9 and 48.7 mm. With respect to the impact of mortality on the growth dynamics, it is interesting to note that a water-quality caused mortality occurred on day 36 in the pond with the highest final growth rate; prior to the die-off, the growth pattern was at a much lower rate (Figure 2).

The growth profiles of the populations in the various ponds were similar among relatively similar densities. The early growth rate reached a level within the initial period and thereafter the mean size of the fish changed little. In the two ponds with the highest populations, the growth trajectories were essentially asymptotic after 4-6 weeks; the final size was, nevertheless, inversely related to the two population sizes (Table 2). The populations with relatively intermediate levels (1.9-3.2/m^2) had similar growth profiles. Fish in the populations with the lowest survival (final densities of 0.24-0.65/m^2) had the highest growth rates, but the growth profiles were not as rapidly stabilized; growth did not slow until after about 10 or 12 weeks. Gonadal differentiation in the various population-growth patterns established in year two, will be discussed subsequently.

The growth studies in the nursing ponds for the third year, were not continued throughout the entire growing season. Once the growth patterns were evident based on the sampling by day 36, the population to be used in sex reversal studies was identified and the nursing was terminated. It can be seen that the growth rates were relatively rapid for the two populations with low survival (< 1%) and resultant low density (Figure 3). As in the ten-pond study, growth patterns were similar among ponds with higher population densities (> 11/m^2); it appears that growth in these populations was near asymptotic and that average size would not have been expected to exceed 50-75 mm. The overall pattern of density-dependent growth can be visualized by comparing the final size in relation to the final density (Figure 4). For the third-year pond studies which were

terminated mid-way through the season, the relationship was similar to the earlier studies where the entire growing season was used. In other words, the growth pattern was established early and only a relatively low growth rate would have been expected in the final portion of the season which would bring the final sizes into alignment with the ultimate first-year sizes seen in the earlier pond studies.

Gonadal Differentiation

The pattern of gonadal differentiation is a primary consideration for effective sex-reversal treatment. Published descriptions of gonadal differentiation in common carp are summarized in Table 3. Since growth conditions for these previous studies were different from the growth environment when post-hatching fry are nursed in ponds, and with the knowledge that density-dependent growth dramatically changes the age/size relationship, we felt it was important to more clearly define stages of development which are pertinent to hormone-induced sex reversal within the context of managed growth. The developmental stages of most interest to the efficacy of functional sex reversal are the phases of anatomical and cytological differentiation.

The conditions for studying effects of density-dependent growth on gonadal development were established in the nursery pond experiments described in the previous section. The populations were sampled periodically by seining, growth information was recorded and representative samples were preserved in Bouin's fixative. Fish were initially screened for gonadal development using the gonadal-squash technique (Guerrero and Shelton 1974). Fish which had not undergone cytological differentiation were processed further. A specimen of gonadal tissue was removed from the segment adjacent to the connection of the pneumatic duct to the swim bladder. Standard embedding, sectioning and staining techniques were used (Humason 1967). Developmental stages were defined based on the description of gonadal differentiation in the common carp (Parmentier and Timmermans 1985).

Results: In the two-pond study, our sampling was more frequent that in the ten-pond study; therefore, we feel more confident with the discriminatory precision in the former series. The data for staging were based on the earliest time and the smallest size at which the transition occurred under the growth conditions in the two ponds.

Under the conditions of relatively rapid growth (low density), the anatomical differentiation of females was recognizable at about 70 days of age in contrast to the age of 84 days in the slower growing population (Table 4). The sizes associated with this developmental period was 37 mm (TL) versus 33 mm in the two respective (low and high density) populations.

Cytological differentiation under rapid growth conditions occurred in 91 day-old females and at 49 mm, while females in the slower growing population required at least 119 days and a size of only about 42 mm to initiate the transition. Thus, in the two-pond series, it appears that rapid growth accelerated development compared to that observed in the slower growing population. However, the slower growing individuals appeared to pass these developmental milestones at a slightly smaller size. This suggests that reliance on either criterion (age or size) is not absolutely accurate as a sole estimator of the status of gonadal differentiation. These data appear to support the conceptual model proposed by Shelton (1990) and indicate that optimal time/size for initiating hormone-induced sex-reveral treatment cannot be defined without considering the growth history if the candidate fish (Figure 3).

Interpretation was more complicated when a wider array of growth conditions was considered. In the ten-pond series, those populations which had the most rapid growth had passed through anatomical differentiation and were in the process of cytologicial development when the first samples were taken 32-39 days post-hatching. Therefore, the gonadal age/size-relationship from the populations in these ponds is not considered to be accurate since we missed some of the sequence. In three other populations with quite different growth conditions, and for which a good series of histological samples were available, the same general relationship was observed as in the two-pond series (Table 4). In the population with the slowest growth (population density at draining = 19.6/m^2), females underwent anatomical differentiation between 70 and 112 days of age at about 43 mm; while, a population with slightly more rapid growth (7.2/m^2), passed into anatomical differentiation in 56-70 days at 50-54 mm. The other population (3.1/m^2) had initial anatomical differentiation by day 50 and in a size range of 37-43 mm. Cytological diffentiation occurred in the other two populations between 112 and 140 days and 45-50 mm. Further, within this population, larger females, which were of the same age (50-day old) were undergoing initial cytological differentiation at 57-60 mm.

The size at differentiation within the latter population is in contrast to the proposed pattern, but clearly indicates that variation occurs within a uniform-age population (single progeny group) in response to within-population growth differences. That is, the more rapidly growing individuals appear to pass through developmental stages earlier (younger age) than their slower growing siblings. This has pertinent implications for intrapopulation developmental differences between jumbers and laggards, and suggests the desirability of size grading fish before treating for hormone-induced sex reversal.

The ages/sizes of gonadal differentiation for common carp documented in this study are reasonably compatable with earlier data (Tables 3 & 4), and provide some partial explanation for the slight differences reported in the literature. Other factors are important in influencing growth and may also impact age/size relationships; both food supply and temperature are interactive with density-dependent effects (Machacek et al. 1986). Density-dependent growth cannot be isolated from effects of food competition, but temperature influence can be somewhat ameliorated if data are considered within a single year, as adjacent ponds will have similar climate-modulated thermal regimes.

Sex Determination

Another consideration for studies involving alteration of the phenotypic sex is the genetic basis for sex determination and whether progeny sex ratios of individual males and females is different from population sex ratio. This is pertinent when progeny testing is utilized to identify functionally sex-reversed individuals, and assumes that a sex ratio of 1:1 occurs both in the population and from individual crosses. Shelton et al. (1983) documented significant deviations from the expected in pair-spawning studies of untreated tilapia species.

The genetic basis for development of phenotypic sex is determined at fertilization and usually translates gonadal sex with fidelity. This is the basis for production of monosex populations by gynogenesis or breeding sex-reversed broodstock, both involving the homogametic sex. Komen and Richter (1993) found evidence for a minor female sex-determining gene among inbred lines of common carp which altered the usual direction of sex differentiation based on the presumed sex-chromosome induction. They indicated that in stocks which posses the minor sex-determing gene, the outcome of hormone treatment for sex reversal might be affected. In other cyprinids, some unexpected sex ratios have

been reported. Oshiro (1987) found only male progeny in gynogens of two female goldfish, while two others produced the expected all-female gynogenetic progeny. Shelton (1986b) found only females in gynogenetic grass carp and offspring of sex-reversed males (genetic females) and normal females.

During the course of this three-year study, numerous pairings were made between males and females from the common carp stock at the University of Oklahoma. Similar crosses were planned for the selected line of Dor-70, but stocks of this group did not mature soon enough. Pair crosses were made by hypophysized artificial propagation, where eggs and milt from different individuals were selectively used. Eggs from a single female were separated into two batches and each was fertilized with milt from a different male. The milt from each male was used to fertilize eggs from two females. The protocol would have permited examination of individual contribution to sex ratio, if there had been deviations from the expected.

Results: Twenty-one single-pair progeny groups were produced using 12 females and 12 males, which had survival adequate for a statistically valid sample to be sexed at the end of the test period (Table 5). Each progeny group is identified by a numerical designation representing individual females and males. The first number(s) of the cross code indicates the female broodstock, while the second number(s) is the male. Missing numbers in the chronology are crosses where insufficient progeny survived for a valid test. None of the 21 crosses produced progeny with a sex ratio different from the expected 1:1.

The carp broodstock tested in our experiments have been maintained for 12 years in our facility. They have not been exposed to intentional selection, therefore, the stock is considered to be reasonably heterogenous. In contrast, selected lines such as the Dor-70 or the line used by Komen and Richter (1993) might have individuals which would produce progeny with a disparate sex ratio.

Androgen Treatments

The primary focus of our investigations on hormone-induced sex reversal of the common carp was based on the examination of treatments described by other workers which appeared to be the most efficacious (Table 6). Preliminary efforts prior to the present funding were unsuccessful in repeating the achievements of Nagy et al (1981). Our results were more similar to those of Basavaraja and Rao

(1988) and Ali and Rao (1989) in that hormone exposure early in gonadal development produced a high proportion of sterile individuals rather than functionally sex-reversed ones. The studies of Komen et al. (1989) and Gomelsky (1986) provided additional insight into a more appropriate time regime for treatment. Thus, we speculated that treatment too early in the gonadal differentiation process and with excessive concentrations of hormone, might singularly or in combination be counterproductive to the goal of functional sex reversal (Shelton 1990). In our initial investigations we developed trials which emphasized the most successful treatments of Komen et al. (1989). Later, we considered the results of our growth/gonadal differentiation studies and modified the protocol further.

A primary focus of the current study was the examination of immersion treatment as an alternative to oral administration of androgen. We based the initial trials on the experience with salmonids (Baker et al. 1988; Piferrer and Donaldson 1989). The indirect effects of hormone exposure as reported by Gomelsky (1986) were not included as he is continuing this work independent of the current study (B.I. Gomelsky, personal communications, 1992). Finally, we have arrived at a somewhat more thorough understanding of the inter-related dynamics of growth and gonadal differentiation and a modified perspective on a hormone-treatment protocol which may be more broadly applicable.

Androgen Treatment - Immersion

The initial efforts in immersion therapy were directed toward examination of the effects of age at treatment. Fry of various ages were exposed to a concentration of 400 µg/l methyltestosterone for 2 hours. A stock solution of androgen was made by dissolving in ethyl alcohol so that the alcohol carrier would be at a concentration of less than 0.1% in the final immersion liquid. Age at treatment ranged from 1-day pre-hatching to 6 weeks of age. A variable number of carp were treated depending on the age. Several thousand individuals were used in pre-hatching and larval stages, while later age groups included 200 individuals. Following treatment, fish were nursed in earthen ponds for the rest of the growing season until they could be sexed.

In the second series of immersion trials, methyltestosterone concentration was increased to 1 mg/l and two exposure durations were used over three age groups. Again, ethyl alcohol was the solvent for the stock solution. Treatment durations

were 2 and 12 hours for 10-, 11-, and 12-week-old fry. Two-hundred fish were used in each treatment. Treated fish were grown in ponds until they could be sexed at the end of the growing season.

A final immersion study examined the effect of various carriers (solvents) of methyltestosterone on the efficacy of sex reversal. Approximately 5,000 four-day-old fry were used in each treatment. Three solvents were used to make stock solutions of the androgens. Ethyl alcohol was use for reference to prior tests and was less than 0.1% in the treatment solution which contained 1 mg/l of androgen. The other carriers were acetone and propylene glycol, each were at less than 0.05% in the final immersion bath containing 1 mg/l of methyltestosterone. The latter two solvents have been reported to increase the efficacy of androgens in immersion treatment (Hunsinger and Howell 1991; S. Watts, Department of Biology, University of Alabama, Birmingham, personal communications, August 1992, respectively). Treated fish were nursed in ponds until they could be sexed.

Results: The immersion treatments at 400 µg/l were ineffective in altering the sex ratio of any treatment group (Table 7). No sex ratio in the treatment groups differed from the expected 1:1. Each control group which was companion to the treatments was also composed of around 50% males. The single treatment (pre-hatching embryos) that appeared to have an androgen effect, was re-examined after additional pond growth and the sex ratio was not different from 1:1 (51% male; N=53). The treatment of 1-week-old fry had low survival in the pond and appeared to have a skewed sex ratio, but toward females; a second sample could not be examined. The female gonads in the treated groups were not reduced compared to the controls, so it appears that there was no androgenic effect.

The second series of tests involved older fish, a higher concentration of androgen, and included a longer exposure time. None of the 2- or 12-hour treatments for the 10-,11- or 12-week-old fish had sex ratios different from the expected. Similarly, the companion control groups all had sex ratios of nearly 50% males. Again, the ovaries of fish in the treated groups were not different in development from the control groups.

In the final experiment involving immmersion, several gaps in the evaluation were caused by total mortality of particular groups. Among the treatment groups with sufficient surviving fish to evaluate, all appeared to be unaltered. The two treatments using propylene glycol as the methyltestosterone solvent had sex ratios skewed toward males, which might indicate increased efficacy. However, I feel

this will need to be re-tested before a convincing argument can be made for recommending its use. All groups had sample sizes which were below or marginal (> 30) considering the required level for detecting a statistically significant departure from 1:1. The low survival may be a result of a delayed treatment effect. Post-treatment mortality was insignificant, but because the exposure and observation times were brief, only immediate toxicity would have been known.

Androgen Treatment - Oral

The oral delivery of androgen provides the most flexible treatment protocol with the greatest potential for controlled exposure to the target organ. Since growth rate was considered to be important in establishing the most appropriate size/age fish for the initiation of androgen treatment, growth during hormone therapy may also influence sex-reversal efficacy. Therefore, the initial trials addressed the question with fish which were pond nursed to a presumed appropriate size and of various ages.

Swim-up fry of various progeny groups were differentially stocked in 400 m^2 ponds and nursed for five weeks. Pond growth was monitored and fish for hormone treatment were selected from the pond which had the desired size (mean = 22.8 mm) at five-weeks of age. Two hormone concentrations were used in the feed (25 or 50 mg/kg) and all groups were fed daily at 15% of their body weight. Growth rates were monitered and feeding was adjusted daily. Fish in each treatment were confined to a net (hapa) of O.1 m^2 size at four stocking densities which included 100, 200, 400, and 600 per hapa. The four hapas of each age and hormone-treatment level were suspended in a separate outdoor circular tank of 600 liters capacity, individually supplied with water inlet, outlet and aeration. Treatment was started at 35-, 42-, and 49-days of age for each stocking density. Each age group had a companion control group. Hormone treatment was continued for 35 days from each starting date. In addition, two groups were established for treatment inside the laboratory in aquaria (100 l) and two in hapas in tanks (1000 l); each had a companion control. All were stocked with 200 fish per group and treatment was started at age 42-d. The two laboratory treatment groups consisted of fish from the same population as the 24-group study, but were size selected (<31 mm and >31 mm). Thus, the experimental design included 24 treatments and three controls in the outside facilities and four

treatments and four controls inside the laboratory. At the end of treatment, groups were stocked into individual 400 m^2 ponds for the remainder of the growing season until they were sexed.

Results: Growth Rate -- The mean size at the start of treatment with 35-day-old fish was 22.8 mm (range = 20-28 mm)(0.18 g); size at 49 days of age ranged from 29.5 to 34.5 mm (0.8-1.1 g). Mean growth in all similarly stocked hapas (six per group) was inversely related to stocking density (Figure 6), but only the lowest density grew significantly faster. Size (TL) at 12 weeks of age was 39.6- (SE = 1.2), 40.5- (SE = 1.1), 43.3- (SE = 1.0), and 47.8-mm (SE = 0.8) in the high to low stocking densities, respectively. Overall growth rate was relatively slow and size was smaller than desired at the end of hormone treatment. Fish were stocked in ponds and sexed later in the year, but the size for efficient sexing was a little small, so for the treatments with sufficient residual live fish, additional time permitted growth to a size larger than 15 cm.

Sex Ratio -- Effectivness of hormone treatment must be considered within the bounds of size as well as age during treatment. Fish in the treatment which started at 35 days of age had an average size of 22.8 mm, and size after 35 days of androgen feeding ranged from a mean size of 41.6 mm at the high density (two hapas each) to 48.6 mm in the low-density hapa. The composition of phenotypic sex was significantly altered from the expected (Control = 49% males); between 72 and 79% of MT-25 treated fish were males (Table 8). The number of females was insignificant, but about one-fourth of the fish could not be sexed and were presumed to have been sterile. Between 71 and 98% of the MT-50 treated fish were males and the remainder were presumed to be sterile. Some of the fish were small when sexed and a portion of these groups were restocked for later re-evaluation. In one of the two treatments which had been evaluated as having 98% males, the subsequent sexing of 50 additional fish gave a sex ratio of 43% male, 25% female and 32% sterile. It must be assumed that among the other groups some of the individuals identified as males might have been classified as sterile, if they had been sexed at a slightly larger size. Thus, from the standpoint of functional sex reversal, the efficacy of these treatments must be questioned.

In the treatments started at 42 days of age, density dependent growth was evident. Mean size at the end of the 35-day period ranged from 41.3 mm (high density) to 47.7 mm (low density), which was slightly lower, though not

signficantly, than the mean sizes of the previous treatment groups. After the pond growth period a representative sample of fish was sexed. The results were similar to those of the 35-day-old treated groups. The proportion of males was about the same in the MT-25 groups, and similarly higher in the MT-50 groups. Of interest is the relatively low number of fish judged to be sterile among the MT-50 groups. However, upon later reDexamination (N = 50) three groups with 96-100% males, the sex ratios were 43-49% male, 17-22% female and 32-40% sterile. Thus, in this series of treatments, the conservative interpretation indicates that only about one-fourth of the treated fish in the populations were affected by the androgen treatment and these were sterilized, rather than being sex reversed.

Growth among the treatment groups started at 49 days of age followed similar trajectories as in the previous groups. Mean size at the initiation of treatment (49-d) ranged from 29.5-34.5 mm and was 39.6-47.8 mm at the end of treatment 35 days later. The sizes were inversely related to hapa density (Figure 6). Hormone treatment efficacy was evaluated at the end of the season. The number of fish identified as males was generally higher in these groups than in the previously discussed ones. However, again when the groups with apparent altered sex ratios were re-evaluated (N = 45-52), about one-fourth of the fish examined were classified as sterile.

Growth rate was somewhat better among the populations treated in the laboratory compared to the outside populations, at least they had reached a slightly larger mean size at the end of treatment (46.8-51.7 mm). The water quality conditions may have been less variable because of the lack of phytoplankton populations inside. Number of males in the treatments ranged from 66-96%, and with similar levels of females and sterile fish among each treatment. The treatment that started with the smaller fish (<31 mm) had the highest level of males in the aquaria but not in the hapas. Thus, no conclusions as to the significance of this size dichotomy in treatment effect can be derived. The most interesting aspect of this series of treatments was that the male sex ratio of the controls in the hapas was somewhat high, suggesting that within the closed system shared with the treated fish, diffusion of metabolites may have influenced the sex ratio, as similarly reported by Gomelsky (1986).

The primary question which was tested in the follow-up studies involved the most-effective hormone dose within the postulated appropriate time-frame. Progeny for treatment were produced through artificial propagation as in previous years. Four females and three males provided six genetic groups which were

stocked in separate 400 m^2 ponds for nursing. The presumed appropriate size for initiating hormone treatments was estimated to be about 30 mm (0.8 g). Hormone therapy started with slightly older and larger fish since the series in the previous year suggested that early treatment was not effective for functional sex reversal. At the end of the nursing period, the ponds were sampled and the presumably most-appropriate population was selected to supply the desired-size fish. The mean size of fish from the selected population was 27.9 mm. Jumpers within the population (>35 mm) were culled. The size ranged from 23 to 35 mm.

The treatment facilities were the same as described for the previous study. Hapas were used to confine fish within the tanks; four hapas with 200 fish per net were sequentially placed in each tank when hormone treatment started. Fish for later treatment initiation were retained in hapas in separate tanks until the start of treatment. This removed potential for pretreatment exposure to hormones or metabolites and all fish were exposed to equal total stocking level within any one tank. Treatment of the first group was started at 41 days of age, the second on day 47 and the third on day 54. Three levels of hormone-laden feed were offered at two feeding levels. Methyltestosterone concentrations in the feed were 10, 25, and 50 mg/kg and each was fed daily at either 10 or 15% of the body weight. Periodic sampling guided adjustments of daily ration. The combinations of hormone concentration and feeding levels provided a increasing gradient of androgen dose while maintaining appropriate feeding levels for efficient growth. An additional treatment group was added but with different conditions. The objective was to examine efficacy of MT introduced into the feed via different alcohol carriers but also with an older group of fish. Treatment for this group was initiated at 83 days of age and compared MT-treated feed prepared with either the usual ethyl alcohol solvent or the proposed alternative, isopropyl alcohol.

Results: The first feeding period was started at 41 days of age with fish having a mean size of 27.9 mm (0.4 g). Hormone treatment lasted for 35 days. Mean size at the end of hormone treatment at 76 days of age was 53.8 mm (2.5 g). Fish were transferred to growout ponds until they could be evaluated. Fish were maintained in ponds longer than the previous year so as to avoid the complication of attempting to evaluate gonadal condition at too small a size; all fish evaluated in sex reversal experiments from this series were larger than 14 cm when examined.

Low survival in some ponds resulted in sample sizes which were too low for statistical confidence. Of the six populations within the first treatment age-group, three had less than 30 surviving fish to examine (Table 9). The number of males within each treated population was not different from the expected 50% level, and there were comparable numbers of females, although in some treatments sterility was evident. Because of the poor survival, no trend can be suggested, althought the proportion of sterile fish did appear to increase with increasing dose levels. The variable hormone concentrations used in conjunction with different feeding levels, was intended to permit an examination of the effect of sequentially increasing effective-dose levels. This was expressed in terms of "Pharmachologically Effective Dose" (PED) as a daily intake of micrograms of hormone per gram of body weight (Shelton 1990).

The second age-group at the start of treatment (47-d) had a mean size of 31.1 mm (0.5 g). After the 35-d treatment period at age 82 days, the mean size was 57.8 mm (3.2 g). The mean growth profile for groups during hormone treatment is summarized in Figure 7. Mean growth rate was higher in the groups fed at 10% during the first few weeks of treatment, but after about week 9, the mean size of the fish fed at 15% was higher. Survival among these treatment groups was again low in two populations (Table 9). However, it appears that no trend in hormone efficacy was related to increasing PED and that a similar percentage of the populations were females, not sterilized individuals.

Hormone treatment was started in a third age group on day 54 at a mean size of 36.2 mm (0.9 g). Treatment stopped 35 days later at 89 days of age with a mean size of 62.3 mm (4.1 g). Survival was good among the various treatments in this age group; all had adequate sample size at gonadal evaluation to be confident of the results. The proportion of males in the various treatment groups was generally not outside the expected ratio of 1:1 (Table 9). One exception was the treatment with the lowest hormone dose, which had significantly more males that would be expected. All control groups, which were each derived from same population but examined at different ages, had sex ratios well within the expected range. Two of the treatment groups had substantial numbers of fish with sterile gonads, but in comparison to the other groups in the treatment hierarchy, there is no obvious explanation for their occurrence.

In the final sex reversal experiment of this series, two treatments were conducted which had the objective of testing the option of using isopropyl alcohol as a steroid solvent instead of ethyl alcohol. Both are effective in solubilizing

methyltestosterone but ethyl alcohol has been more commonly used, despite the fact that isopropyl alcohol is more readily available. This experiment was developed subsequent to the primary treatment protocol, so was started with older fish, used only one hormone level (50 mg/kg) fed at 15% of the body weight, and had a single duration (37 d). This treatment resulted in the most successful of all protocols tested in this series of sex-reversal trials.

At the start of the hormone treatment with fish of 83 days of age, the mean size in the population was 56.6 mm (3.2 g); size range was from 40 to 74 mm (Table 9). At the end of the treatment the ethyl alcohol/MT-group had an mean size of 78.3 mm (51-104 mm) and a average weight of 9 g, while the isopropyl/MT-group averaged 90.0 mm (73-108) and 12.5 g. Both hormone treatments were highly effective in inducing apparent functional sex reversal. Each had over 90% males and no sterile individuals were found. The companion control group had the expected male composition. While the primary purpose of this particular experiment was to compare the two hormone solvents, the most valuable outcome was a treatment regimen which was the most successful of any tested during the study.

It is noteworthy that the age/size relationship in this test is in agreement with the conditions of treatment which were successful in androgen-induced sex reversal reported by Komen et al. (1989) and Gomelsky (1986). The low success of the majority of sex reversal treatments in the present study appear to be attributal to a combination of initiating treatment too early in relation to the growth history and an effect of general slow growth during androgen exposure. The size at the end of the treatments was generally smaller than expected, considering the duration of the experimental period.

Discussion and Conclusions

One of the most significant outcomes of the present research effort was the demonstration of the importance of the relationship between growth history and the development of functional gonads. Growth dynamics affects the age/size of critical events in the ontogeny of the reproductive system. Efforts to manipulate the phenotypic expression of these genetically orchestrated milestones must recognize and incorporate these considerations in protocols which have the objective of altering the pathway.

The variability in growth rate of fishes under varying conditions may have profound effects on the time chronology of developmental events. In the present study the focus was placed on select milestone stanzas in gonadal differentiation, specifically the transition from the histologically recognizable phases of anatomical and cytological differentiation. Size alone is not sufficient to judge the chronological developmental status, but the growth history to that point in time also must be considered. In general, stages were reached and passed at a younger age among more rapidly growing individuals but the size of this transition appeared to occur at a somewhat larger size relative slower growing individuals. Thus, in a population with relatively slow growth, as in one with a high density, differentiation appears to require longer, but occurs at a smaller absolute size.

The growth-rate modulated gonadal-differention relationship was found be of major importance with reference to androgen-induced sex reversal. It was pertinent in the selection of size or age of fish to be treated and also had a major influence on efficacy of treatment in relation to growth to a particular, critical size within the period of hormone exposure. The success of many androgen treatments reported in the literature, were not duplicated in the present study despite starting with similar age and size of fish. This non-repeatability was apparently a result of low growth rate in our studies.

Shelton (1990) attempted to amalgamate information from successful sex-reversal treatments for common carp and gonadal differentiation reported under different environmental conditions. But, the effects of growth dynamics on the process of gonadal differentiation were not known and could not be considered. However, considering the present knowledge, I propose a modified conceptual-ization relating successful sex reversal and probable gonadal conditions (Figure 8). Based on the sex reversal success of Komen et al. (1989), Gomelsky (1986), and in the present study, both the success and lack of success, I suggest the following set of considerations which most likely would allow achieving functional sex reversal of common carp under various conditions.

Figure 8 depicts the growth trajectories based on Komen et al. (1989) and from the present study, with superimposition of the general age/size relations under which sex reversal was achieved. Annotated information on gonadal differentiation is based on Parmentier and Timmermans (1985) and the present studies involving density dependent factors. These recommendations assume the use of an efficacious androgen at an appropriate level (e.g. methyltestosterone at 50 mg/kg of feed given at 10-15% body weight;PED 4-7.5). Functional sex

reversal of common carp should be accomplished by starting treatment with fish of 55-70 days of age within the size range of 35-50 mm (TL) or weight range of 0.9-3.5 g. Treatment should encompass about 35 days (age range of 80-120 d) or until fish reach a size of 75-85 mm (> 9-12 g). Treatment which is started earlier, at a smaller size, and/or where growth is slow during treatment can be expected to have a significant proportion of sterile fish. Successful treatment appears to be related to starting at an age/size at which gonadal differentiation is nearing the end of the anatomical phase, but continuing treatment well into the cytological differentiation condition. As to the treatment conditions which are too latent, it is probable that lower success will result if treatment starts at a size larger than 75-85 mm (>9-12 g) and older than about 75-85 days.

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Table 2. Density- dependent growth for common carp during first-season pond nursing.

Final Density (no./m²)	Survival (%)	Final mean size TL (mm)	S.Dev.	Wt. (g)	Duration (days)
1990					
39.0	18.0	43.5	10.7	1.8	160
8.4	9.7	52.4	14.3	2.4	160
1991					
19.6	15.7	48.7	9.7	0.7	157
7.2	5.8	64.2	4.6	3.9	157
3.2	7.5	92.2	5.8	11.2	154
3.2	25.0	92.9	6.5	11.5	157
3.1	24.8	106.5	15.8	18.0	155
3.1	12.4	105.3	12.4	17.5	152
2.4	9.6	104.8	7.4	16.7	159
1.9	3.8	118.9	11.9	29.9	147
0.7	1.9	168.2	10.9	76.0	133
0.2*	0.2	254.9	23.5	300.0	168
1992					
24.5	7.0	27.9	-	0.6	66
23.6	11.4	41.1	-	0.9	66
19.9	13.3	32.5	-	0.8	66
11.7	12.7	42.6	-	1.2	67
0.4	0.5	129.3	-	51.3	65
0.3	0.1	135.9	-	43.0	65

*) Water quality caused mortality at day 50.

Table 3. Gonadal differentiation of female common carp based on previous investigations.

Anatomical		Cytological		Source
age (d)	size (mm)*	age (d)	size (mm)	
68-110	40-59	117-138	65-102	Parmentier and Timmermans (1985)
60-85	30-39	90-123	-	Davies and Takashima (1980)
> 40	>18-22	-	-	Ryanzantseva and Sakun (1971)
-	-	-	95-156	Bieniarz (1986)
-	-	-	147-210	Pospisil and Smisek (1971)
60-78	~65	98	~75	Gomelsky (1986)

*) Total length; conversion from standard length (TL = 1.25 SL).

Table 4. Age/Size relationship of gonadal differentiation for female common carp under different growth conditions based on population density.

Population density at harvest (no./m^2)	Anatomical differ.		Cytological differ.	
	age (d)	size (mm)	age (d)	size (mm)
Year 1				
8	70	37	91	49
39	84	33	119	42
Year 2				
3.1	50	37-43	50*	57-60
7.2	56-70	50-54	112-140	57-59
19.6	70-112	43	112-140	45-46

*) Same-age fish from the same population, but faster growing individuals at more advanced developmental stage.

Table 5. Progeny sex ratio from single-pairings of common carp broodstock maintained at the University of Oklahoma.

Parental Cross*	Progeny number sexed**	Proportion of males (%)
1 - 1	50	50
1 - 2	129	42
2 - 1	248	42
2 - 2	97	57
3 - 3	100	50
3 - 4	216	45
4 - 3	136	50
4 - 4	122	52
7 - 5	156	44
8 - 6	88	49
8 - 7	95	47
9 - 6	100	51
11 - 6	61	44
11 - 7	63	38
12 - 8	37	51
12 - 9	100	63
13 - 8	55	44
13 - 9	84	41
14 - 10	55	60
14 - 11	50	56
15 - 12	50	50

*) First number = female broodstock; second = male.

**) Sexed by gonadal examination.

***) No progeny group had a sex ratio significantly different from the expected 1:1 (Chi-square, 95%).

Table 6. Summary of methyltestosterone-induced sex reversal treatments for common carp.

Treatment (days)		Dosage (µg/g)	Efficacy (%)**		Source
Start	Duration	(PED)*	Male	Sterile	
2	30	40-80	2	98	1
2	30	40-80	39	61	2
2	50	40-80	16	88	2
2	90	3-6	100[g]	-	3
14	42	20	44	56	4
21	35	20	45	55	4
21	35	15-20	7	93	5
42	35	4-8.5	93	5	5
62	36	10-20	83[g]	-	6
78	36	10-20	51[g]	-	6
8-62	36	10-20	71-89[g]	-	7

*) PED = androgen concentration in feed X Daily feeding rate

**) All fish sexed by gonadal examination.

g) Treatment of gynogenetic fish.

1. Ali and Rao (1989), 2. Basavaraja and Rao (1988), 3. Wu et al. (1986), 4. Shelton (unpublished data), 5. Komen et al. (1989), 6. Gomelsky (1986), 7. Nagy et al. (1981).

Table 7a. Methyltestosterone immersion treatments of common carp; 400 µg/l with ethyl alcohol carrier (< 0.1%).

Treatment		Efficacy (%)				Control (%M)
Age (d)	Duration (h)	Male	Female	Sterile	N	
-1 (egg)	2	76	21	3	32	49
1	2	50	44	6	16	49
7	2	33	67	0	3	45
14	2	-	-	-	0	51
21	2	51	44	5	57	50
		51	49	0	50	(re-examination)
28	2	49	41	10	76	50
		48	52	0	51	(re-examination)
35	2	51	49	0	77	50
42	2	47	53	0	98	59

Table 7b. Immersion at 1 mg/l MT.

70	2	54	46	0	50	60
	12	62	38	0	50	
77	2	43	57	0	51	58
	12	60	40	0	50	
84	2	50	50	0	50	63
	12	48	50	0	50	

Table 7C. Immersion at 1 mg/l MT with carriers* < 0.05%.

EOH*						
4	2	44	56	0	18	51
	12	-	-	-	0	
Acetone						
4	2	-	-	-	0	
	12	-	-	-	0	
Propylene glycol						
4	2	68	32	0	22	
	12	59	41	0	32	

*) Ethyl alcohol = usual solvent for methyltesterone; acetone based on Hunsinger & Howell (1991); propylene glycol based on personal communications, S. Watts, Dept. Biol., Univer. Alabama, Birmingham, AL, USA.

Table 8. Methlytestosterone 35-day oral treatment of common carp in 1991.

Treatment					MT-25				MT-50			
Start age (day)	Start size (mm)	End	Rate (#/hapa)	Surv. (%)	M	F (%)	St.	N	M	F (%)	St.	N
35	23	48.6	100	80/89	76	0	24	80	74	0	26	65
		44.2	200	98/25	72	0	28	57	71	0	29	45
		41.5	400	94/90	78	0	22	51	98	0	2	50
									43	25	32	50*
		41.6	600	91/91	79	2	19	49	98	0	2	51
									45	20	35	48*
42	29.2	47.7	100	41/-	[100	0	0	17]	-	-	-	0
	32.5	42.8	200	19/74	78	0	22	32	96	0	4	52
									46	22	32	51*
	30.5	41.4	400	61/81	70	0	30	57	100	0	0	50
									49	18	33	49*
	28.2	41.3	600	25/79	74	1	25	65	100	0	0	50
									43	17	40	52*
49	34.5	47.8	100	63/30	78	0	22	55	[96	0	4	23]
	33.5	43.3	200	83/45	88	0	12	56	98	0	2	50
									52	32	16	55
	32.0	40.5	400	82/81	96	0	4	52	100	0	0	50
					68	27	5	48	71	22	7	45
	29.5	39.6	600	55/63	94	0	6	49	78	0	22	50
									43	40	16	56*
42-Aq	29.3	51.7	200	93					96	4	0	52
	32.5	51.3	200	60					66	21	13	52
42-hapa**												
	29.3	46.8	200	99					75	17	8	48
	32.5	50.9	200	99					86	11	3	54

Controls - age group (d):	35	42	49	42-Aq	42-hapa
% Male:	49	45	50	60/58	75/88***

*) Fish were of marginal size for sexing in the initial treatment evaluation; surviving fish in select treatments were restocked and a second sample was later examined for sex ratio.

**) Fish used in laboratory aquarium/hapa treatments were initially size-graded.

[ ]) Indicates sample size was too small for statistical confidence in discriminating sex ratio departure from 1:1.

***) Controls were exposed to MT leaching from feed.

Table 9. Methyltestosterone 35-day oral treatment for common carp in 1992.

Treatment						Efficacy			
Start		MT	Feed	PED*	End	M	F	Ster.	N
Age (d)	size (mm/g)	(mg/kg)	level (%BW)		size (mm/g)	(%)			
41	27.9/0.4	10	10	1.0	53.8/2.5**	63	37	0	52
			15	1.5		55	37	7	54
		25	10	2.5		[60	40	0	20]
			15	3.7		[56	35	9	23]
		50	10	5.0		50	32	18	50
			15	7.5		[90	10	0	20]
47	31.1/0.5	10	10	1.0	57.8/3.2	72	28	0	50
			15	1.5		68	32	0	50
		25	10	2.5		[75	25	0	20]
			15	3.7		62	38	0	51
		50	10	5.0		53	67	0	52
			15	7.5		-	-	-	0
54	36.2/0.9	10	10	1.0	62.3/4.1	70	28	2	50
			15	1.5		51	35	14	51
		25	10	2.5		46	54	0	50
			15	3.7		62	36	2	50
		50	10	5.0		58	42	0	50
			15	7.5		63	8	29	49
83	56.6/3.2	50E	10	5.0	78.3/9.0	98	2	0	73
		50I	10	5.0	90.0/12.5	94	6	0	71
Controls:		end 41d				50	50	0	52
		end 47d				51	49	0	50
		end 54d				50	50	0	51
		end 83d			70.4/5.6	52	48	0	72
		pond source				63	37	0	100

*) PED - see Table 8

**) Mean size of age groups; see Figure 7 for mean size of 10 and 15% feeding levels.

E - Ethyl alcohol solvent for methyltestosterone.

I - Isopropyl alcohol solvent for methyltestosterone.

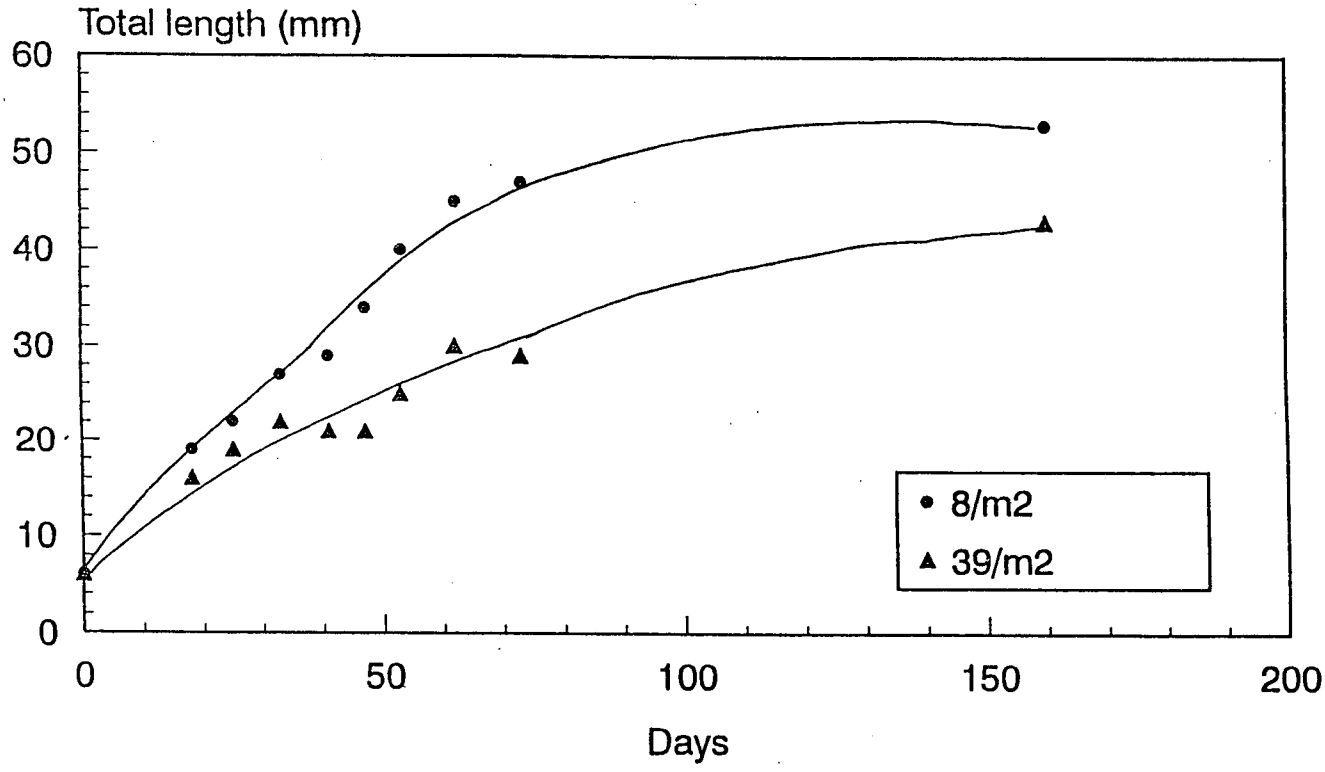

Figure 1. Growth patterns of two populations of common carp at different densities in 1990 (Fish/m^2 at draining).

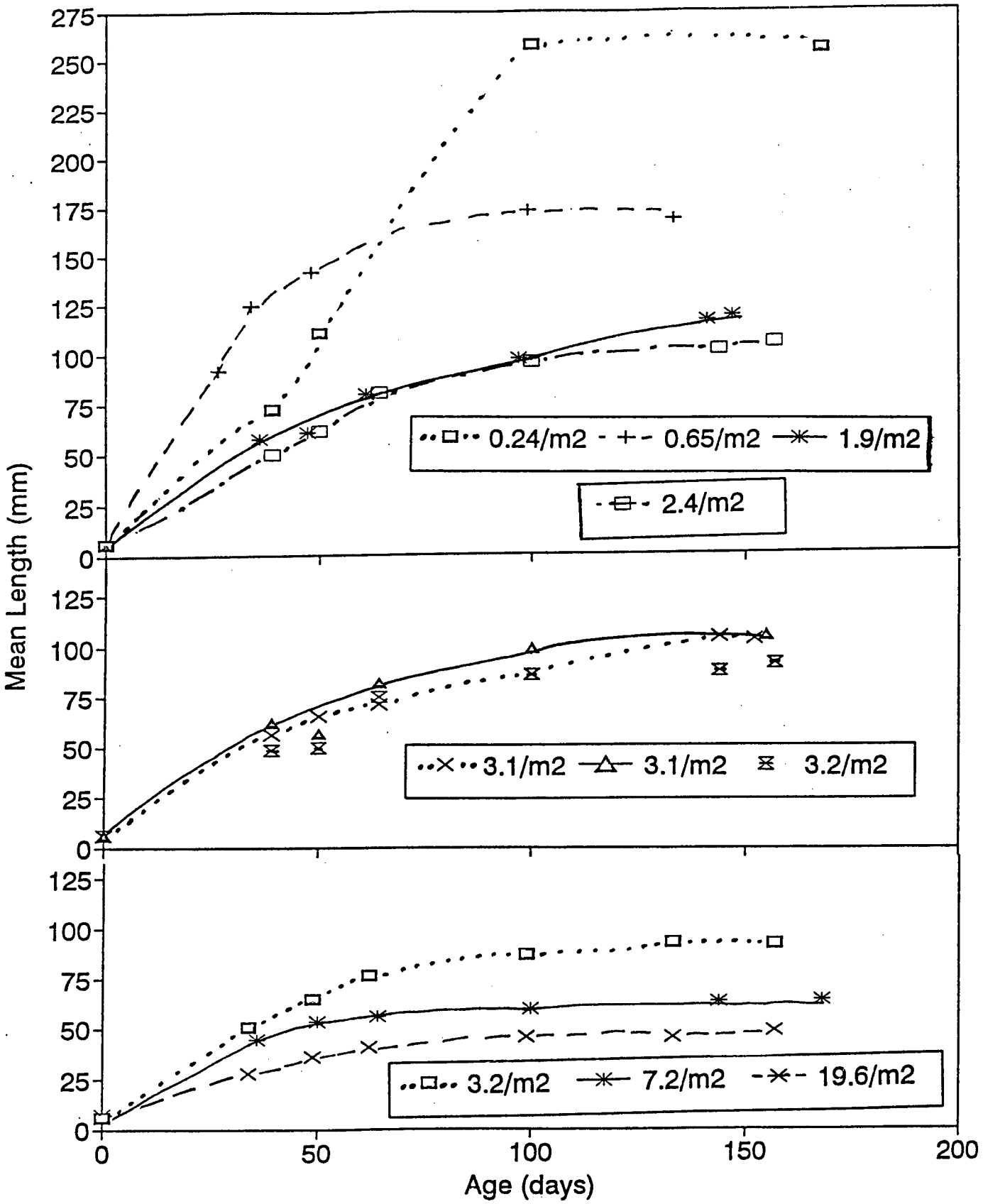

Figure 2. Growth patterns of ten populations of common carp at various densities in 1991 (Fish/m^2 at draining).

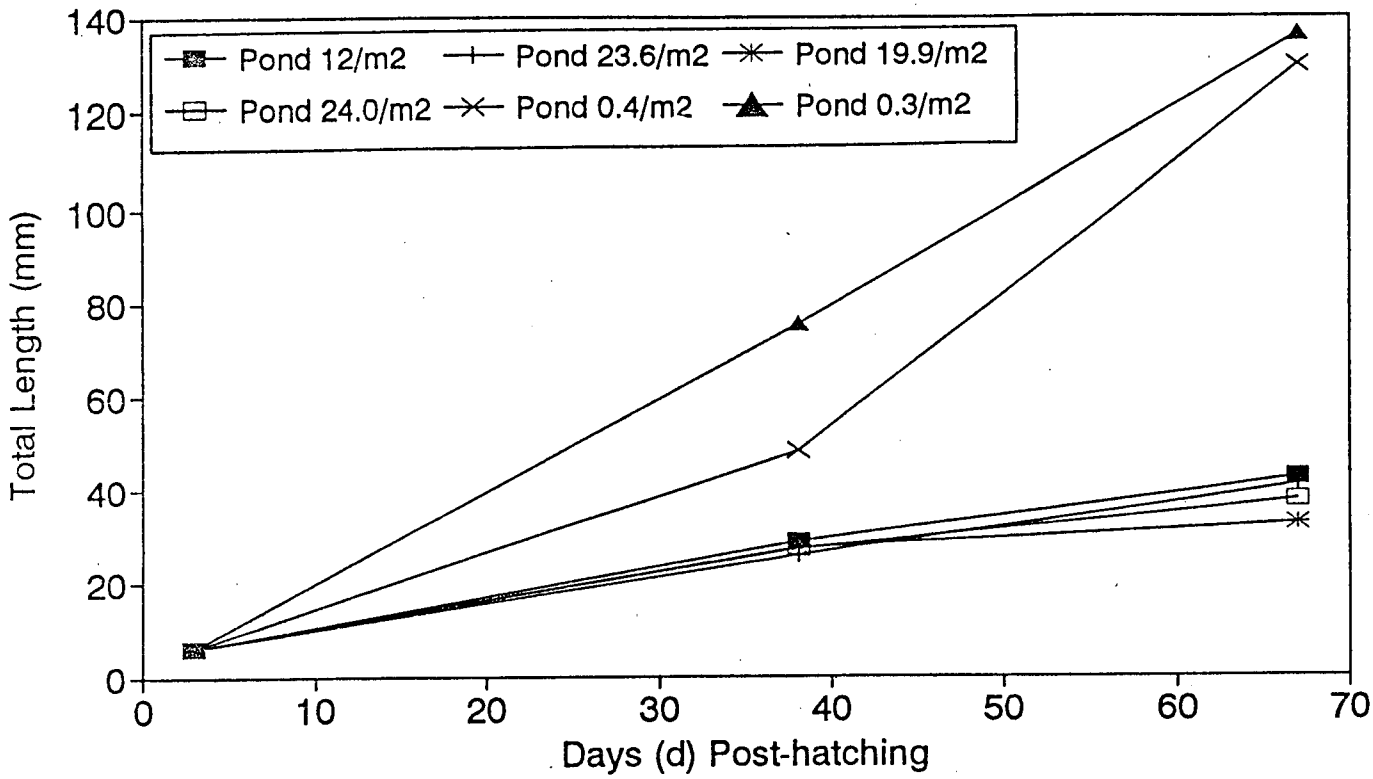

Figure 3. Growth patterns of six populations of common carp at various densities in 1992 (Fish/m^2 at draining).

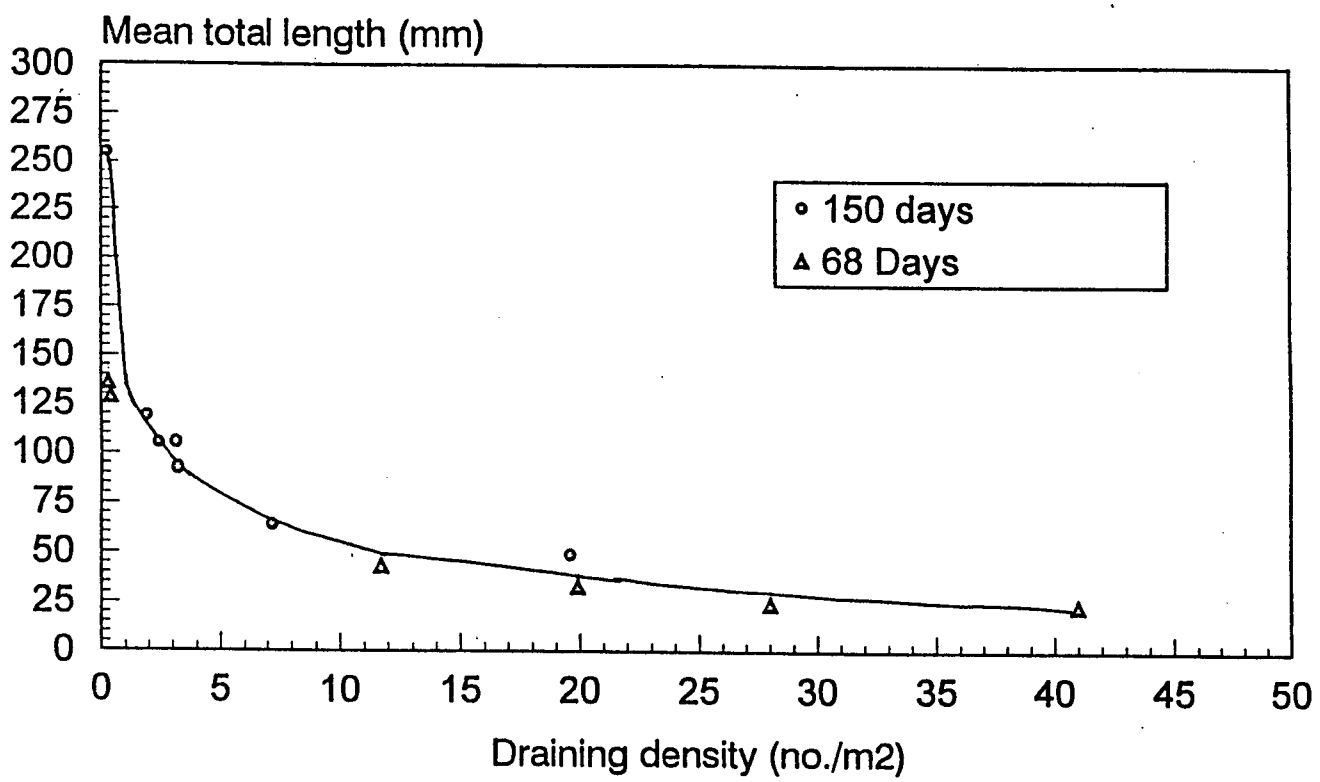

Figure 4. Size of common carp at draining after first-season nursing in ponds.

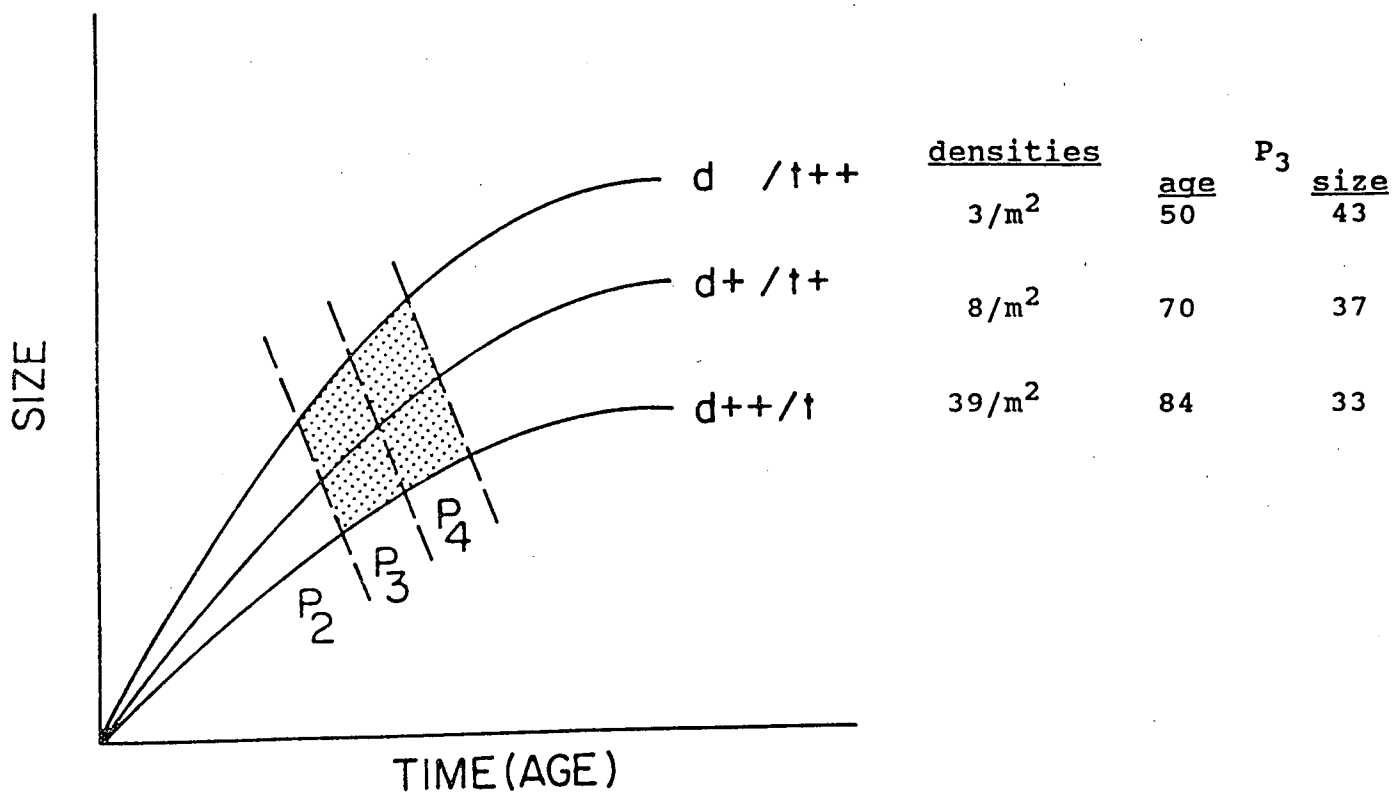

Figure 5. Conceptual Model of the effect of Size/Age relationship on gonadal differentiation; P_3 and P_4 depict anatomical and cytological differentiation periods, respectively; d/t represent different levels of density/temperature and relationship to growth rate. Three select nursery-pond densities illustrate effect of size/age relationship on initiation of anatomical differentiation (P_3).

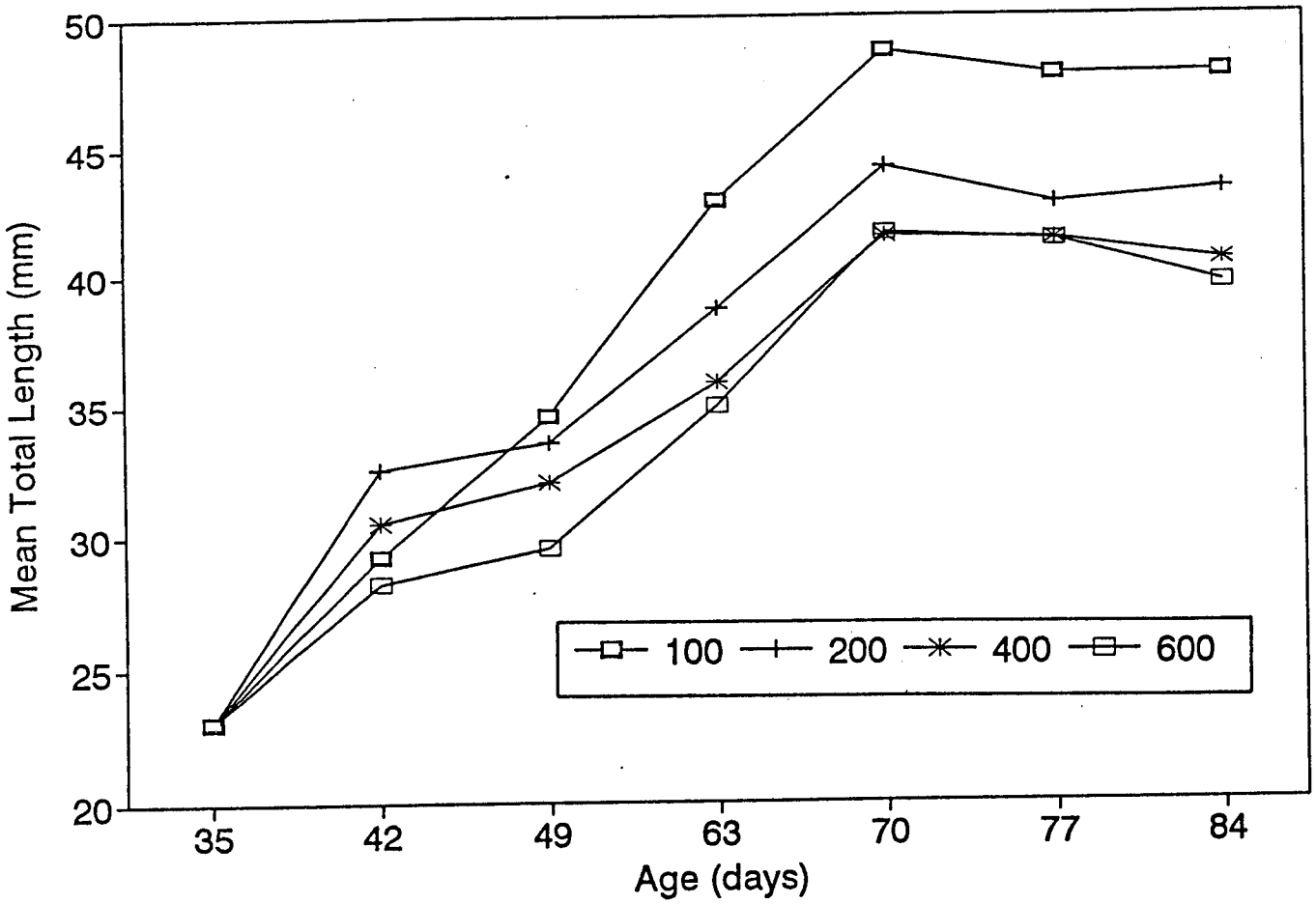

Figure 6. Growth patterns of common carp at various stocking densities in 0.1-m^2 hapas during androgen treatment.

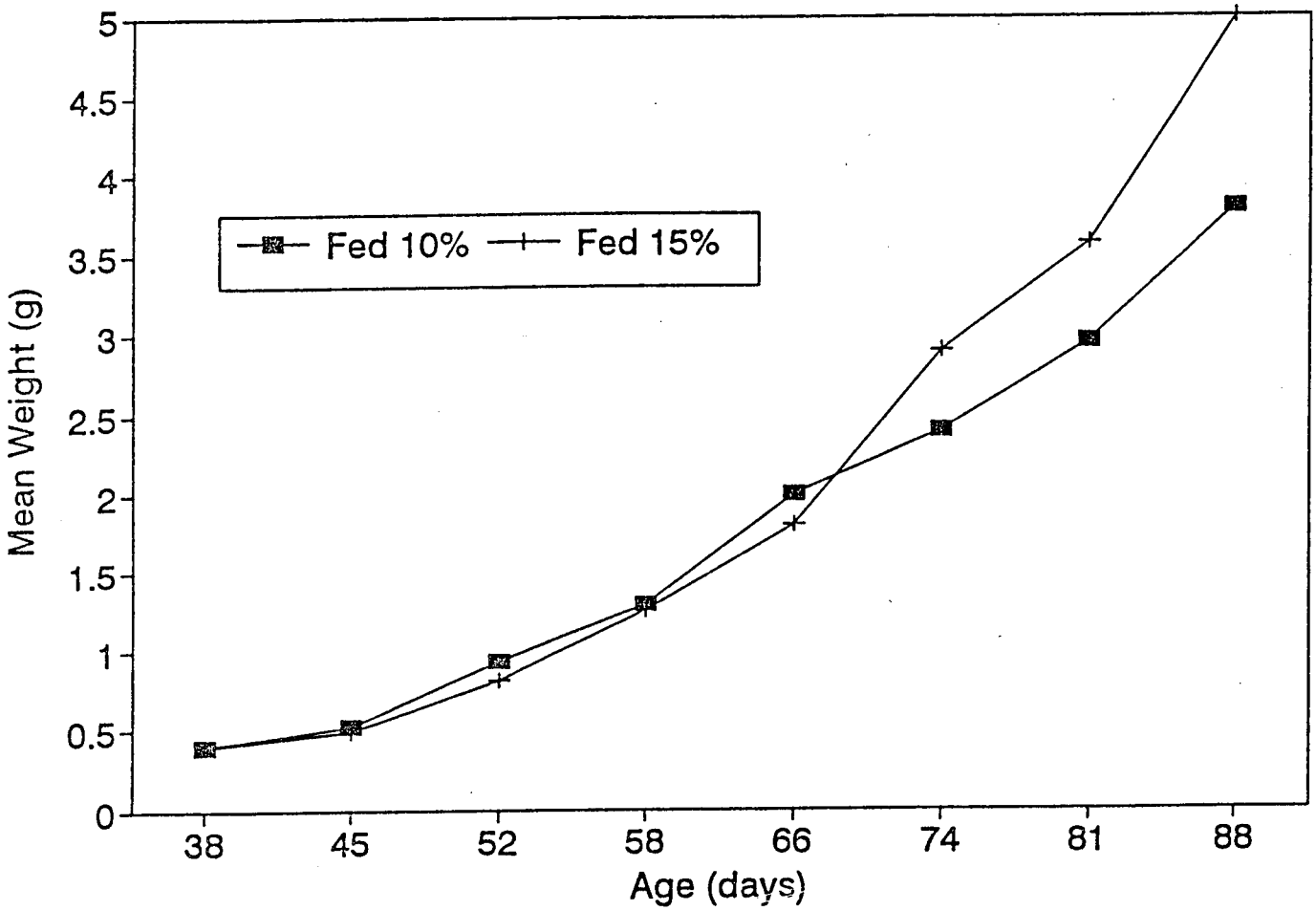

Figure 7. Growth patterns of common carp stocked at 200/0.1m^2 hapa and fed at 10 or 15% body weight during androgen treatment.

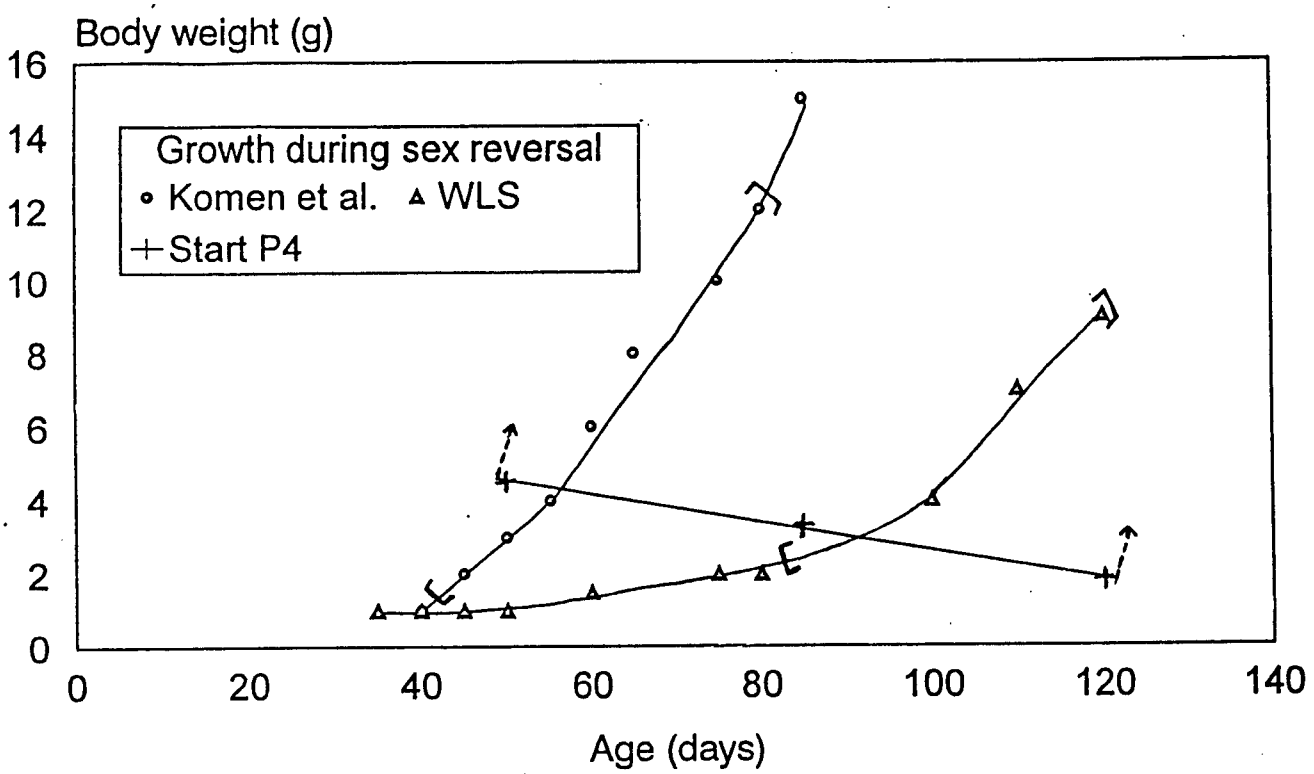

Figure 8. Growth profiles for common carp during sex reversal, with most successful treatments depicted {} and the the initiation of cytological differentiation of the gonads (P_4) based on density-dependent growth; sources = Komen et al. 1989 and present study - WLS.

5. Production and investigation of unisexual all-female progenies.

Hormonal sex inversion in the common carp (Cyprinus carpio L.)

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Abstract

Hormonal sex inversion was induced in genotypic females of the Israeli Dor-70 common carp line and ornamental common carp (koi). All-female progenies produced by both meiotic and mitotic gynogenesis were used as material for the experiments. Twenty seven to forty-day-old fingerlings (mean initial weight of 2.8-14.5 g) were fed diet containing 100 ppm methyltestosterone (MT) for 40 days. The effectiveness of androgen treatment depended on fish rearing conditions. The percentage of inverted males varied from 46.7 to 96.6% among fish reared in recirculation water systems, while among fish reared in tanks with running water only 20.0-43.8% were inverted males. Initial fish weight seems to play a stronger role in determining successful sex inversion than initial age.

Introduction

Under Israeli temperature regime common carp females are heavier by 10-15% than males at marketable size (Wohlfarth et al., 1975). Therefore, the production of all-female progenies seems a promising way for increasing pond productivity. The use of all-female populations will also prevent uncontrolled reproduction in grow-out ponds which results in over-crowding and inhibits growth of fish.

The most promising way for sex control in fish is the crossing hormonally induced sex-inverted breeders (see reviews by Hunter and Donalson, 1983; Yamazaki, 1983). The scheme for production of all-female common carp progenies (as in other species with female

homogamety) includes two consecutive steps: 1) inducing masculinization in genotypic (XX) females, and 2) crossing sex-inverted males with normal females (XX).

Several authors reported achievement of hormonal sex inversion in the common carp by oral adminisration of methyltestosterone-containing diet (Nagy et al., 1981; Wu et al., 1981; Gomelsky, 1985; Komen et al., 1989, 1993). Nevertheless, the sex-inversal technique for common carp is not regarded optimized yet (Shelton, 1990), the main gaps in knowledge being the optimal period for androgen treatment under various fish growth conditions. The onset of this period is determined by the timing of sex differentiation, which, in turn, depends on a number of factors such as age and size of the fish, water temperature, etc. Komen et al. (1993) also noted that the response to androgen treatment was highly dependent on the genetic backgroud of the carp used in experiments.

The main aim of the present investigation was to achieve hormonal sex inversion in genotypic (gynogenetic) females of Israeli common carp line Dor-70 and ornamental carp (koi). It was planned to re-evaluate the effectiveness of a procedure for androgen treatment which has successfully induced the sex inversion in Central Russian common carp (Gomelsky, 1985), and to optimize it for other rearing condinions and genetic material.

Materials and methods

The experiments were conducted at the Fish and Aquaculture Research Station, Dor, during 1992-1993. The following three all-female gynogenetic progenies were used: meiotic and mitotic gynogenetic progenies obtained from Dor-70 females (exps. 15/92 and 21a/92, respectively; Cherfas et al., 1993), and meiotic gynogenetic progeny obtained from koi females (exp. 15a/92, Cherfas et al., unpublished).

Gynogenetic larvae were stocked in earthen ponds for primary nursing soon after the onset of active feeding. Samples of fish were collected on days 27, 34 or 40 after hatching and transfered to controlled conditions for androgen treatment. Dor-70 meiotic gynogenetic progenies were sampled twice - 27 (exp.1) and 40 (exp. 2 and 3) days after hatching. The other two progenies were sampled

only once (Table 1).

Recirculation water systems or tanks with running water were used for fish rearing during and after the period of feeding androgen-containing diet. The recirculation water systems, designed according to Yarzhombek et al.(1982), consisted of two or three 120-l tanks for fish rearing and one tank serving as a biological filter to which the rearing tanks were connected. The biofilter tank was filled with synthetic fibres substratum. The water passed from the fish tanks to the filter by means of airlifts and returned through siphons. The running water tanks were 480 l in volume, and the water flow was 1.2 l/min. Water temperature in both systems was maintained at 25-26°C.

Seven experimens were performed, and their description is given in Table 1. Experiments 1, 2, 4 and 6, conducted in the recirculated water systems had the following scheme: The fish in one tank of the recirculation system were fed with 100 ppm MT-containing food, while the fish in the other tank(s) of the same system were fed androgen-free diet. The occurence of some musculizing effect on fish in the latter groups, expected according to earlier results of Gomelsky (1985), was examined.

MT-containing diet was presented for 40 days in all experiments. The actual duration of androgen treatment in the recirculation systems was longer since the water was not changed when feeding the MT-containing food was ceased.

The MT-containing food was prepared as described by Gomelsky (1985). 17α-methyltestosterone (Sigma Chemical Company) was suspended in a small quantity (2-3% of food weight) of vegetable oil and the fish food pellets were impregnated with this suspention.

In most of the experiments the effectiveness of the MT treatment was determined by inspection of 6- and 10.5-month-old fish, except for the Dor-70 mitotic gynogen progenies (exp. 4 and 5) which were inspected only at the age of 10.5 months. Gonads of dissected 6-month-old fish were visually inspected and weighed, and the gonadosomatic index (GSI) was calculated as ratio the of gonad weight to total body weight (in %). Fish were regarded as sterile when the gonads were undeveloped and the sex could not be

identified. At the age of 10.5 monthes the remaining fish in each experimental group were injected with carp pituitary extract (Yaron et al., 1984) and mature inverted males releasing milt were identified. Fish not releasing milt after hypophyzation were dissected and sexed by visual inspection of the gonads. The 10.5-month-old fish in exp. 7 were not injected and all of them were sacrificed.

Samples of untreated Dor-70 gynogenetic progenies reared in ponds were used as control groups. Six-, 8-, and 14-month-old meiotic gynogens (total of 45 fish) and 7-month-old mitotic gynogens (20 fish) were analyzed. A control group of koi was kept in a separate tank and 23 6-month-old fish were analyzed.

The data of the treated groups were compared using Chi-square test.

Results

Mortalities during experimental rearing (to age of 10.5 months) were 30-45% in most of the experiments, with most of the loses occurring, in both systems, in the initial period of rearing. Higher mortalities (about 70%), caused by ectoparasitic infection, occurred in exp. 1 (Table 1).

Not a single male was found in control groups, where as MT treatment induced sex inversion in some of the genotypic females in all experiments. The percentage of inverted males observed in the experimental groups varied from 20 to 96.6% (Table 2).

In the experiments in the recirculation water systems (exps. 1, 2, 4 and 6) inverted males were observed both among the fish that consumed MT-containing diet and among fish from the other tank(s) of the same systems that recieved no androgen in their food. The frequencies of inverted males in the latter groups tended to be higher (although the differences were not significant, $P>0.05$), than those in the respective groups receiving MT-containing diet in all experiments.

When fingerlings of the same progenies, with equal initial age and weight were treated under the two different culture systems (exp. 2 and 3, 4 and 5, 6 and 7), the percentages of inverted males were always higher in the recirculation water systems than in the

tanks with running water. This difference was significant in the experiments with Dor-70 mitotic gynogens and koi ($P<0.001$) and Dor-70 meiotic gynogens ($P<0.01$).

Feeding the Dor-70 meiotic gynogens with MT-containing diet in the period from 27 to 67 days after hatching (exp. 1) was more effective ($P<0.01$) than that in the period from 40 to 80 days after hatching (exp. 2).

The lowest percentages of inverted males were obtained in experiments with Dor-70 mitotic gynogenes (Table 2). The MT treatment of 40-day-old Dor-70 meiotic gynogens (exp. 2 and 3) and gynogenetic koi (exp. 6 and 7) with similar initial weights gave similar results. The total frequency of inverted males in the treated and non-treated groups in the recirculation systems was higher in koi than in Dor-70 (83.9% vs. 67.9%), but lower in the running water tanks (25.6% vs. 43.8%), though these differences were not significant ($P>0.05$).

The testes of 6-month-old inverted males had, according to visual inspection, a normal structure which is typical for common carp males, and were at stages 2 and 3 (maturing testes) according to Gupta (1975). The mean GSIs of 6-month-old fish are shown in table 2. In exp. 1, 2 and 6, the mean GSIs of MT-treated inverted males were somewhat lower (not significant) than those of males not fed MT-containing food (Table 2).

Most of 10.5-month-old inverted males, in all experimental groups checked, released milt after hypophyzation (some of them without injection). The testes of inverted males not releasing milt after injection were somewhat retarted in development, but had normal structure and colour.

Some inverted males produced in these experiments were used as breeders in the summer 1993 spawning season.

Discussion

The data obtained have revealed the effectiveness of oral administration of MT for inducing of sex inversion in genotypic females of common carp in both recirculation and running water systems.

The results of the present investigation coincided with previous

observation (Gomelsky, 1985) that MT administered orally affect also fish sharing the same water recirculation system but not receiving MT in their food. Yarzhombek and Gomelsky (1992) suggested that the MT consumed by the fish is tranformed in the liver into soluble active metabolite(s), and that these is/are excreted with the bile to the water of recirculated system and causes the sex inversion in fish not recieving androgene with food. This assumption was partially confirmed in experiments with the guppy (Poecilia reticulata) (Yarzhombek and Gomelsky, 1992). When the bile from common carp that received high quantity of MT was added to the water of aquarium with guppy fry secondary male sex characteristics developed in all fish, including gynotypic females.

Sex inversion was more successful in the recirculated water systems than in the tanks with running water. The soluble metabolite(s) tranformed from MT may be more potent as masculinizing factor than MT itself. It is also possible that the longer exposure to androgen treatment in the recirculated systems played a role in increasing the sex-inversion effect.

The initial age of fish subjected to MT treatment varied from 27 to 40 days after hatching, and the initial mean weight - from 2.8 to 14.5 g. The highest percentage of inverted males was achieved by treating the youngest fish with lowest mean weight (exp. 1). The least successful results were obtained in experiments with Dor-70 mitotic gynogens which had intermediate initial age but the highest initial mean weight. The initial mean weight of fingerlings at the beginning of androgen treatment was similar in the most succesfull treatments of the present investigation (3-9g) and an earlier one on Central Russian common carp (6-9g) (Gomelsky, 1985, unpublished). The age of fish at the onset of androgen treatment was quiet different in the two investigations since under Russian climate conditions the carp fingerlings reached that weight only after about 2 months of primary nursing in pond. Although different progenies may differ in their response to androgen treatment, the data obtained suggest that the initial weight of the carp is a more significant factor than initial age in determining sex inversion success. We suggest that initial weight can be used as a practical guide for determinining the appropriate period of androgen treatment

for sex inversion in the common carp. Histological studies on grass carp (Jensen et al., 1983) and common carp (Gomelsky, 1985) genotypic females indicated that the critical androgen-labile development stage in cyprinids seems to occur after the anatomical gonad differentiation but prior to cytological sex diffentiation.

Acknowledgements

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Table 1. Detailes of MT treatment procedure.

Exp.	Conditions of fish rearing	Period of feeding with MT-diet*	Dose of MT in food (ppm)	No of fish Initial	Analyzed at age 6 mo.	Analyzed at age 10.5 mo.	Mean weight (g) Initial	At end of feeding with MT diet	At age 6 mo.
				Dor-70, meiotic gynogenesis					
1	Recirculation system with 3 tanks for fish rearing	27-67	100	50	10	7	2.8	15.5	44.8
		-	0**	100	10	19	2.8	14.1	40.0
2	Recirculation system with 3 tanks	40-80	100	30	8	10	9.2	29.3	74.1
		-	0**	60	17	18	9.2	27.6	96.9
3	Tank with running water	40-80	100	70	19	29	9.2	35.9	111.1
				Dor-70, mitotic gynogenesis					
4	Recirculation system with 2 tanks	34-74	100	25	0	15	14.5	28.6	-
		-	0	25	0	13	14.5	33.1	-
5	Tank with running water	34-74	100	50	0	35	14.5	43.1	-
				Koi, meiotic gynogenesis					
6	Recirculation system with 3 tanks	40-80	100	30	9	14	8.7	-***	40.6
		-	0	60	10	32	8.7	-***	37.1
7	Tank with running water	40-80	100	70	19	20	8.7	29.8	53.8

*) days after hatching;
**) sum of two tanks;
***) not registered;

Table 2. Sex disribution in experinental groups and characteristic of gonad developnent in inverted nales.

Exp.	Conditions of fish rearing	Dose of HT in food, ppn	Total no. of fish exanined	Sexual types (%) nales	fenales	sterile	GSI of inverted nales*	No. nales releasing nilt/total no. nales in sanple**
		Dor-70, neiotic gynogenesis						
1	Recirculation systen	100	17	82.4	14.6	0	1.26±0.26	5/6
		0***	29	96.6	3.4	0	1.61±0.36	14/18
2	Recirculation systen	100	18	66.7	33.3	0	1.50±0.48	6/7
		0***	35	68.6	28.6	2.8	2.01±0.35	8/11
3	Tank with running water	100	48	43.8	52.1	4.1	1.33±0.38	13/13
Control	-	-	46	0	97.8	2.2	-	-
		Dor-70, nitotic gynogenesis						
4	Recirculation systen	100	15	46.7	53.3	0	-	5/7
		0	13	53.8	38,5	7.7	-	5/7
5	Tank with running water	100	35	20.0	77.1	2.9	-	4/7
Control	-	-	20	0	95.0	5.0	-	-
		Koi, neiotic gynogenesis						
6	Recirculation systen	100	20	75.0	10.0	15.0	1.05±0.54	4/8
		0***	42	88.1	2.4	9.5	1.24±0.34	19/30
7	Tank with running water	100	39	25.6	53.8	20.5	1.12±0.33	-
Control	-	-	23	0	91.3	8.7	-	-

*) at fish age 6 nonths;
**) at fish age 10.5 nonths;
***) total data for two tanks;

Assessement of all-female progenies for culture during 1st summer (preliminary results)

Although the possible benefit from rearing unisexual all-female common carp progenies instead of regular bisexual ones was discussed in a number of papers, specific evaluation of such progeny was never carried out. In 1993 we were able, for the first, time to produce a diploid all-female unisexual population of the Israeli common carp line Dor-70. Preliminary results of their evaluation as fingelrings, are presented below.

Unisexual all-female progenies were obtained in April 1993 from crossing Dor-70 females with sex-inverted Dor-70 males ,obtained in the experiments on hormonal sex inversion in common carp (see above, Gomelsky et al.). Eggs from 5-6 females (mixed) and mixed sperm from 8-10 inverted (XX) males were used for obtaining unisexual progenies; a sample of the same eggs was fertilized with sperm obtained from 8-10 regular (XY) Dor-70 males. Fifty g eggs (about 35,000 eggs) were fertilized for obtaining the unisexual and the control progenies. Standard techniques of artificial common carp reproduction were used. The number of larvae obtained was calculated at 4-5 days after hatching. The output of larvae was similar in "unisexual" and control progenies, i.e., ca. 12,000 in both cases.

The larvae were initially stocked into two different ponds (10,000 larvae in pond of 0.04 ha) for preliminary nursing during 40 days (May 5 to June 15). The results after this preliminary rearing were:

	Unisexual	Control
No. of fry	3,570	3,474
Survival (%)	35.7	34.7
Mean weight (g)	4.5	5.0

The fry were then transferred four similar (0.04 ha) experimental ponds, two of which were stocked with "unisexual" fish and other two with control fish, at 400 fish per pond (1 fish/ m^2). Mean weights (g, estimated by sampling the ponds) by mid September 1993 were:

Pond	Unisexual	Control
1	293	-
2	294	-
3	-	334
4	-	251

DESCRIPTION OF COOPERATION

The research team met during the initial year of funding (at a Symposium on Carp Genetics, held in Szarvas, Hungary in September 1990) when the plans for the coming spawning season were discussed. The U.S. principle investigator spent the period January-June 1992 in Israel on a sabbatical leave from his home institution. Collaboration with the Israeli team was valueable. During this period, different questions related to the project experiments were discussed, and the techniques associated with ploidy manipulation were practiced. Time was shared between the Dor Research Station and the Fish Breeding Center at Kibbutz Gan Shmuel. Appreciation is sincerely expressed to the personnel of both facilities, and to the University of Oklahoma for making this valuable experience possible.

In an effort to establish a common fish stock, the Dor-70 carp line was spawned in Israel in 1991 and some progeny were sent to the U.S. counterpart later that year. These have been nursed during the interim but did not mature soon enough to be used during the funding period.

EVALUATION OF THE RESEARCH ACHIEVEMENTS

The investigations have been carried out according to the project program, and the research objectives of the project were attained. The most important achievements are as follows:

1. In the investigations on induced gynogenesis and polyploidy, precise data concerning the efficiency of temperature shock at different phases of the 2nd meiotic division (heat and cold shocks) and the 1st cleavage (heat shock) in common carp were obtained; based on these data the optimum timing for suppression of the 2nd meiotic division and the 1st cleavage in common carp was established. The possibility for standardizing temperature shock timing (independently of pre-shock water temperature) using the relative unit of embryological age τ_0 was confirmed. Protocols for obtaining diploid meiotic and mitotic gynogens, tri- and tetraploid progenies in a large scale were specified.

2. Specific investigations on the nature of spontaneous diploidization in koi were carried out for the first time; the data obtained indicated that this phenomenon

has, at least in some cases a genetic determination, and that this property is heritable. Transformation, occurring at early meiosis stages, may be responsible for production of unreduced eggs.

3. In the investigations on hormone-induced sex reversal in common carp, the importance of growth history to the developmental relationship of the functional gonad was demonstrated. These factors need to be considered for sex reversal therapy. It was shown that growth dynamics affects the age/size relationship of critical events in ontogeny of the reproductive system. Efforts to manipulate the phenotypic expression of these genetically controlled events, must recognize and incorporate growth factors into protocols which propose to alter the usual phenotypic pathway.

Additional data concerning higher effectiveness of recirculated water systems than those with running water for sex inversion were obtained. Earlier observations showing the possible involvement of some soluble metabolite(s) of high activity (transformed from the treatment hormone) have been confirmed.

The importance of the methods of chromosome-set and sex manipulations for aquaculture has been noted above. The project yielded new data for optimizing the induced gynogenesis and sex inversal techniques in the common carp. The results obtained were already implemented in the studies on the Israeli Dor-70 common carp line. Two type of Dor-70 gynogens have been obtained in 1992-1993. Some of these (all-female) fish were transformed into phenotypic males by the androgen treatment. As a result, stocks of meiotic gynogenetic females and males, and mitotic gynogenetic females and males of Israeli common carp have been created for the first time. These, in turn, have provided the opportunity for a number of interesting further studies. Thus, meiotic gynogenetic inverted males (XX) have been crossed with regulat Dor-70 females. Unisexual all-female progenies Dor-70 were obtained from this crossings on a large scale and are cuurently under testing. Mitotic gynogenetic Dor-70 females are planned to be used (after reaching sexual maturity) for obtaining genetically homogeneous progenies, i.e., clones. Mitotic gynogenetic inverted males (of expected inbreeding coefficient = 1) can be also used for producing topcrosses with regular females to benefit from the expected heterosis effect.

The results obtained were presented at the following forums: The 4th International Symposium on Genetics in Aquaculture (April-May 1991, Wuhan, China); International Satellite Symposium "Frontiers of Biotechnology in Agriculture 1991" (August 1991, Sea of Galilee, Israel); Japanese-Israeli Symposium on Aquaculture - JISA (November 1992, Haifa, Israel); Annual Meeting of the World Aquaculture Society (July 1992, Florida, USA); International Symposium "The Carp" (September 1993, Budapest, Hungary). Some results will also be presented at the 5th International Symposium on Genetics in Aquaculture (June 1994, Halifax, Canada).

LIST OF PUBLICATIONS

1. N.B. Cherfas, G. Hulata & O. Kozinsky, 1993. Induced diploid gynogenesis and polyploidy in ornamental (koi) carp, *Cyprinus carpio* L. 2. Timing of heat shock during the first cleavage. *Aquaculture*, 111: 281-290.
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