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

3. Optimization of heat-shock timing during the 2nd meiotic division and the 1st cleavage

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Abstract The results of a series of experiments conducted in our laboratory on the ornamental common carp (koi), aimed at optimizing heat-shock chromosome-set manipulation procedures, are described. The timing of heat–shock initiation was expressed in the relative unit of embryological age (τ_0) in order to standardize this parameter, the absolute time for heat-shock initiation being calculated from duration of one τ_0 at two different pre-treatment water temperatures. Heat shocks were applied within the periods of 0.05–0.60 τ_0 and 1.20–2.20 τ_0 which, respectively, cover the successive phases of the 2nd meiotic division and the 1st cleavage. The highest production of diploid gynogenetic offspring was observed when heat shocks were initiated at 0.15–0.25 τ_0 and at 1.5 τ_0, after insemination, corresponding to anaphase of meiosis-II, and metaphase of the 1st cleavage, respectively. Similar results were obtained irrespective of the different pre-treatment water temperatures, thus confirming the possibility of standardizing heat-shock timing by τ_0.

Key words Common carp · Chromosome-set manipulations · Heat-shock timing · Induced gynogenesis

Introduction

Heat shock, as an agent for inducing chromosome-set diploidization, has been used in several experiments to induce 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). Although its effectiveness is dependent on the initiation time after insemination, optimum timing varies between different studies, presumably because the various incubation temperatures used prior to heat shock cause post-fertilization processes to develop at different rates. We have, therefore, concluded that additional investigations in this area were desirable in order to further optimize temperature-shock methods for chromosome-set manipulation in the common carp.

In order to standardize timings we have used the unit of relative embryological age, "τ_0", which is equivalent to the duration of one mitotic cycle during synchronous cleavage. According to the theory of Dettlaff and Dettlaff (1961), the duration in absolute time of one τ_0 is temperature dependent, but a given embryological stage (within the optimal temperature range for any given species) has a constant value if expressed in τ_0 units. Consequently, it is assumed that the 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) and Rothbard (1991) in experiments on induced gynogenesis in the common carp. In a preliminary study on heat shock at the first cleavage (Cherfas et al. 1993), the absolute time for heat-shock initiation was calculated from the duration of one τ_0 at the pre-treatment temperature of 20 °C and 25 °C used by Ignatieva (1974). The similarity of the two survival curves obtained suggested that a standardization of heat-shock timing can be achieved.

The present paper describes the results of further optimization experiments conducted in 1991–1992. The experimental procedure was improved since the two pre-treatment water-temperature variants were carried out simulataneously, using eggs of the same female. Thus, the two series of each experiment differed in pre-treatment water temperature only, while all other experimental conditions were identical. In addition, a new (more precise) calculation of the duration of τ_0 was used (Ignatieva and Saat, personal communication).

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Materials and methods

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

The experimental procedures were identical to those described by Cherfas et al. (1993). Standard techniques of artificial propagation of the common carp (Rothbard 1981) were used, and eggs of a single female were employed in each experiment. Inseminated eggs (100–500) were shocked and incubated, attached to Petri dishes placed on trays, within a temperature-controlled water circulation system. Three experiments (exp. 13/91, 14/91, 15/91) studied the effects of heat shock at the 2nd meiotic division and four (exp. 2/91, 5/91, 3/91, 3/92, 4/92) the effects of heat shock at the 1st cleavage.

According to recent data (Ignatieva 1974; Saat 1991), the 2nd meiotic division in the common carp is completed at 0.48 τ_0, and the 1st mitotic division at 2.1–2.3 τ_0, after insemination. Heat shocks were initiated (at 0.05 τ_0 intervals) within the ranges 0.05–0.60 τ_0, and 1.2–2.2 τ_0 (at 0.1 τ_0 intervals) after insemination, in order to disturb meiosis-II and mitosis-1, respectively. The period from 0.6 to 1.2 τ_0 has previously been shown to be ineffective (Gomelsky et al. 1989; Cherfas et al. 1993). Two synchronous temperature series (20 °C and 24 °C) were used, in which heat shock was initiated at the same embryological age after insemination, expressed in τ_0, but at different absolute times. The absolute time for heat-shock initiation was calculated (Table 1) from the duration of one τ_0 at these temperatures, according to Ignatieva and Saat (personal communication).

The parameters of temperature and duration of heat shock were chosen on the basis of 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, presumed haploid, groups of the same gynogenetic progenies were used as controls in each experiments to estimate the quality of eggs, the fertilization rate (i.e., the number of embryos starting gynogenetic development), and the frequency of spontaneous diploid gynogenetic larvae.

The results were analyzed at two time intervals, namely as embryos surviving prior to hatching and as diploid (presumed gynogenetic) larvae of normal appearance surviving 2–3 days after hatching. The results were compared by a t-test.

Table 1 Time for heat-shock initiation after insemination

Pre-treatment water temperature (°C)	Duration of one τ_0, (min)[a]	Investigated period (min)	
		2nd meiotic division (0.05–0.6 τ_0, 0.05 τ_0 intervals)	1st mitotic division (1.2–2.2 τ_0, 0.1 τ_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)

[a] According to Ignatieva and Saat (personal communication)

Results

Heat shock at the 2nd meiotic division

The data 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 τ_0, or later than 0.30 τ_0, after insemination. Highest embryo survival (although lower than in the control) was observed when heat shock was initiated at 0.15–0.25 τ_0 after insemination.

The effective period for heat-shock initiation coincided with the period of high survival in shock-treated offspring (0.10–0.25 τ_0). The highest output of diploid gynogenetic larvae was obtained when heat shock was initiated within 0.15–0.25 τ_0 after insemination. 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 0.45–0.60 τ_0 in exp. 14/91; Table 2). The patterns of results at the two temperature series were not significantly different [$t_{(df=11)} = 1.07$ for survival and 0.15 for 2n-larvae production]. The highest production of diploid gynogenetic larvae was 36.5% of the total number of inseminated eggs, or 52.2% of the number of live embryos prior to hatching, when heat shock was initiated at 0.15 τ_0 (Exp. 15/91, Table 2).

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 1.6–1.9 τ_0 after insemination.

Although a few diploids were also obtained at other periods, a peak output of diploid larvae was obtained when heat shock was initiated in the period 1.4–1.6 τ_0

Fig. 1 The output of 2n-gynogenetic larvae relative to the 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 the mean values of three experiments

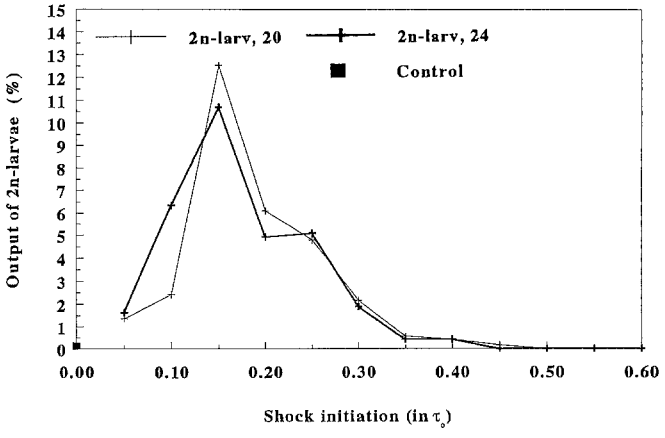

Table 2 Results of the experiments with heat shock applied at the 2nd meiotic division at 20 °C and 24 °C incubation temperature prior to heat shock: embryo survival and the production of 2n-larvae

Shock initiation	20 °C prior to heat shock				24 °C prior to heat shock			
	No. of eggs	Survival prior to hatching	2n-larvae		No. of eggs	Survival prior to hatching	2n-larvae	
(τ_0)		(%)[a]	(No.)	(%)[a]		(%)[a]	(No.)	(%)[a]
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[b]	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[b]	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[b]	284	93	1	0.4				

[a] From the total number of inseminated eggs
[b] No-shock gynogenetic control

after insemination. Based on the output of gynogenetic diploid larvae, the most effective heat-shock timing corresponded to 1.5 τ_0 after insemination (Fig. 2), which coincided with the beginning of the sharp decrease in embryo survival. Very similar production curves for 2n-larvae were obtained in the two experimental series of 20 °C and 24 °C pre-shock water temperatures (Fig. 2), though 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]. The highest output of mitotic diploid larvae was 8.4%, from the total number of inseminated eggs, or 15.3%, from the number of live embryo prior to hatching, when heat shock was initiated at 1.5 τ_0 (Exp. 2/91, Table 3). The output of mitotic diploid larvae was somewhat higher in the 24 °C pre-shock water temperature series (Fig. 2, Table 3).

Discussion

A strong resemblance between the two curves of diploid larvae output, obtained at two pre-shock water tem-

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 the production of 2n-larvae

Shock initiation	20 °C prior to heat shock				24 °C prior to heat shock			
	No. of eggs	Survival prior to hatching	2n-larvae		No. of eggs	Survival prior to hatching	2n-larvae	
(τ_0)		(%)[a]	(No.)	(%)[a]		(%)[a]	(No.)	(%)[a]
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[b]	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[b]	311	82	1	0.3				
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[b]	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[b]	526	62	0	0.0				

[a] From the total number of inseminated eggs
[b] No-shock gynogenetic control

peratures, was revealed for both investigated periods, i.e., the 2nd meiotic division and the 1st cleavage. Thus, we can conclude that each investigated τ_0 corresponded to the same phase of the cell cycle in the two series, irrespective of the difference in absolute time for shock initiation after insemination. Therefore, the results obtained have shown that heat-shock timing in experiments on chromosome-set manipulations in the

Table 4 Optimum heat-shock timing observed in different experiments on induced gynogenesis in the common carp

Temperature prior to shock (°C)	Duration of τ_0 (min)[a]	Optimum timing		2n-larvae %[b]	Reference
		min	τ_0		
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[c]	Sumantadinata et al. 1990
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	–[c]	Sumantadinata et al. 1990
		55	2.9	–[c]	Sumantadinata et al. 1990

[a] According to Ignatieva and Saat (personal communciation)
[b] In relation to: control (1), number of fertilized eggs (2), total number of inseminated eggs (3)
[c] no data

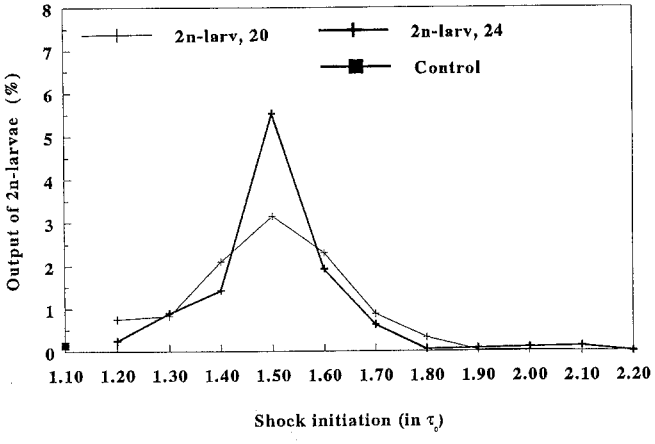

Fig. 2 The output of 2n-gynogenetic larvae relative to the total number of inseminated eggs in gynogenetic progenies heat shocked during the 1st mitotic division, after incubation at 20 °C or 24 °C. Curves are based on mean values of four experiments

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 our experiments: 0.15–0.25 τ_0 and 1.5 τ_0, after insemination. The first period corresponds to mid anaphase-II, and the second one to metaphase of the 1st cleavage (Ignatieva 1979; Saat 1991).

We have transformed the data of other investigations on induced gynogenesis in the common carp in which heat shock was applied, to enable comparison with those of the present study (Table 4). This was done by dividing the absolute reported time of heat-shock initiation 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, the optimum timing in most studies lies within the same range of embryological age, and is concentrated within the period 0.10–0.25 τ_0 (in experiments on meiotic gynogenesis) and 1.4–1.6 τ_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 an experiment on mitotic gynogenesis in the Indonesian common carp (Sumantadinata et al. 1990). We conclude that either gynogenesis resulted from suppression of the 2nd cleavage in this study (1st cleavage was not investigated) or (less likely) that the duration of one τ_0 in the Indonesian common carp is different.

Based on the results of the present investigation, an embryological age of 0.20 (0.15–0.25) τ_0 and 1.5 (1.4–1.6) τ_0 can be recommended for the induction of chromosome-set diploidization at the 2nd meiosis and the 1st cleavage, respectively. These timings 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 the common carp may be applicable also for other fish species.

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