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Artifical gynogenesis and mapping of gene-centromere distances in the paradise fish, *Macropodus opercularis*

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Summary. An efficient method has been developed for the production of gynogenetic paradise fish in order to obtain genetically homogeneous strains in a relatively short period. Diploidy of the gynogenetic embryos was restored by inhibition of the second meiotic division, and consecutive generations of normal, fully viable offspring were reared. Crossing-over frequencies and the relative positions of four marker loci were determined by analysing electrophoretic patterns of serum transferrin and esterases in the gynogenetic progeny of heterozygous females.

Key words: Gynogenesis – Paradise fish – Inbreeding – Gene mapping

Introduction

Homozygosity is often a precondition of genetic analysis, however it is not easily attained in higher eukaryotes. Long established inbred strains of certain specific species, such as the mouse or the rat and chicken, do exist however. Inbreeding by conventional techniques of sibmating or backcrossing progresses rather slowly.

In some lower vertebrates the eggs fertilised with X-ray irradiated (Hertwig 1911; Fankhauser 1937; Parmenter 1933; Subtelny 1958) or UV-treated (Nace et al. 1970) sperm are activated and develop into inviable haploid embryos. Occasionally – in about 1% of the eggs – normal embryos appear as a result of non-disjunction. To generate normal viable offspring various treatments of the activated eggs are known: hydrostatic pressure (Dasgùpta 1962; Romashov and Belyaeva 1965; Jaylet and Ferrier 1978; Streisinger et al. 1981) or heat shock treatment (Nace 1968; Golovinskaya 1968; Purdom 1969; Stanley and Sneed 1974; Cherfas 1975; Nagy et al. 1978; Streisinger et al. 1981).

These treatments result in the inhibition of either the first mitotic or the second meiotic division depending on the timing of application. The consequence of such propagation is increased homozygosity of the offspring which carries an exclusively maternal genome. Accordingly, such gynogenetic individuals are completely homozygous (Streisinger et al. 1981) or contain partly heterozygous sister chromatids due to crossing-over resulting in recombination during first meiosis. In the latter case, as Nagy and Csányi (1982) showed, the progress of inbreeding strongly depends on the distribution of recombination probabilities of the genes, i.e. those of high recombination frequencies tend to remain heterozygous longer during successive generations of gynogenesis. The level of uniformity of the gynogenetic offspring is not so strongly dependent on the distribution of crossing-over probabilities, so isogenic strains can be produced in a relatively short period.

In this study we describe a method of obtaining gynogenetic offspring of the paradise fish, *Macropodus opercularis* and a subspecies *Macropodus opercularis concolor*. We have chosen this fish because of its several desirable characteristics. The generation time in 3–4 months, mature females lay a large number of eggs weekly (up to 1,500), which develop rapidly and hatch within 24 h. The fish are hardy and easy to care for. Since our method of gynogenesis has been developed to allow for the analysis of behaviour by using inbred and recombinant inbred strains, the elaborated behaviour patterns of the paradise fish were one of the main reasons for choosing this species.

Materials and methods

Animals

Female paradise fish *Macropodus opercularis* of a random bred population and of strains maintained by brother-sister inbreeding for 10–14 generations were used. We also worked with females of inbred *Macropodus opercularis concolor* and hybrids of the two subspecies. Sperm was collected from either carp (*Cyprinus carpio*) or goldfish (*Carrassius auratus auratus*)

males. The fish were kept in 80 l aquaria at 26–28 °C temperatures under a 14–10 h light/dark cycle.

Artificial gynogenesis

Female *Macropodus* fish had received intramuscular injection of chorion gonadotropin (Pregnyl/Organon; 2,000 I.U./ml saline), in 10 I.U./g body weight dose 24 h before intended propagation. Next day the eggs were collected by gentle stripping in dry plastic Petri dishes. The number of eggs obtained from a single female varied between 800 and 1,500 depending primarily on size and age. The eggs of individual females were separately fertilized by X-ray irradiated sperm obtained from distant fish species.

Irradiation was carried out immediately after collecting the milt from the gonadotropin-injected (2 I.U./g) carp or goldfish males. The milt was placed into hermetically sealed vials surrounded with ice, put into the irradiation chamber and exposed to 100 krads. Following the X-ray treatment the sperm was diluted with 2× Hanks BSS solution in 1 : 5 ratio and motility was checked microscopically. Forty µl of the sperm suspension was added to approximately 1,000 eggs and following gentle mixing, 50 µl filtered tap water was added. One minute later a few more drops of water were added. Fertilization was followed by various cold shock treatments aimed at increasing the yield of diploid offspring. During treatment, portions of fertilized eggs were immersed in pre-cooled tap water, and by the end of treatment they were transferred back to 26 °C. The independent variables of the treatments were the onset, duration and temperature of cold shock. The latter was regulated with ±0.5 °C accuracy.

Several hours after treatment the eggs were examined under a low-power microscope and any which appeared infertile were removed. The developing eggs were transferred to plastic dishes lined with nylon mesh and placed into an 80 l tank at 28 °C provided with intensive filtration.

Determination of ploidy

Freshly hatched fish larvae obtained from three experimental groups were transferred to a 0.01 M colchicine solution for 120 min and *after photographing* squash preparations of entire larvae were made.

In *Group 1* normal paradise fish fry was produced by artificial fertilization of eggs with *Macropodus* sperm suspension. In *Group 2* and *Group 3* the *Macropodus* eggs had been fertilized with X-ray irradiated goldfish sperm, but while eggs in *Group 2* were not exposed to any more treatment, fertilized eggs in *Group 3* were cold shocked at 15 °C for 20 min starting three minutes following fertilization.

As expected, freshly hatched larvae in *Group 2* showed such major morphological abnormalities as microcephaly, curved tail, distorted abdomen, etc. These abnormalities are generally known as the haploid syndrome (Beatty 1967).

Among developing embryos in cold treated *Group 3* two morphologically distinguishable subgroups were found. There were regularly developing, morphologically normal gynogenetic diploid embryos, hatching into normal fry – *subgroup b* – and a considerable proportion of the developing embryos showed some abnormalities, sometimes even to the degree of the haploid syndrome. These latter have been included in *subgroup a* at the evaluation of DNA content of *Group 3*.

Absorbance of Feulgen stained nuclei was measured by Vickers M85 type microdensitometer.

Polyacrylamide gel electrophoresis of the blood plasma

Plasma proteins were separated according to Davis (1964) modified for slab gels. The pH of the sample buffer and the stacking gel were modified to 7.4 when separating ^{59}Fe-labelled transferrins, because of the Fe-binding properties of transferrin (Princiotto and Zapolski 1975). Transferrin phenotypes were visualised by autoradiography after separation of 2 µl isotope-labelled plasma samples on 7% polyacrylamide gels.

Plasma esterases were stained by l-naphtylacetate-Fast Blue BB reaction after separation of 2 µl plasma aliquote on 9% gel slabs (Harris and Hopkinson 1976).

Results

Diploid gynogenesis: optimal treatment

We have found that among gynogenetic *Macropodus* about 1% diploids spontaneously occur. In order to increase the ratio of viable diploid offspring various treatments have been attempted as a means of restoring diploidy. Pressure or heat shock treatment used suc-

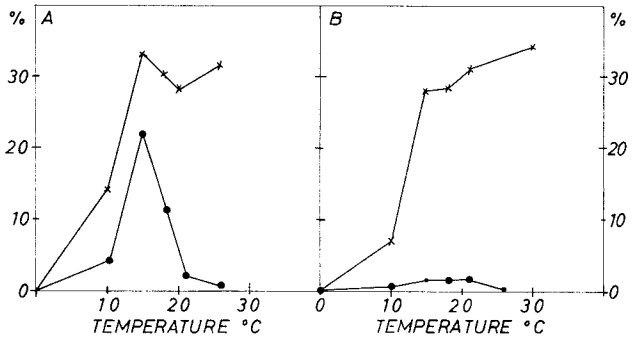

Fig. 1 A, B. Establishment of optimal conditions for gynogenetic propagation. Effect of the temperature of cold-shock on the survival of gynogenetic embryos (–×–×–) and the proportion of viable diploid fry (–●–●–). Cold shock was applied at 3 min (**A**) and 25 min (**B**) after fertilization and lasted for 20 min. Values are given in percentage of total fertilized eggs

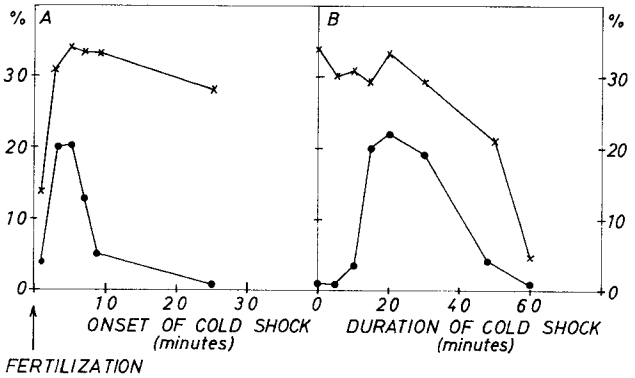

Fig. 2 A, B. Establishment of optimal conditions for gynogenetic propagation. Effect of the onset (**A**) and duration (**B**) of the cold treatment on the survival of gynogenetic embryos (–×–×–) and the proportion of viable fry (–●–●–). Cold shock temperature was 15 °C and in (**A**) lasted for 20 min, in (**B**) commenced 3 min after fertilization. Values are given in percentage of total fertilized eggs

Table 1. Survival rates (Mean ± SD) of gynogenetic offspring up to feeding larvae stage in percentage of number of fertilized eggs

Group	No. of females	Survival rates (%)		
		24 h	Hatching	Feeding stage
Diploid control[a]	18	62.8 ± 14.9	55.2 ± 14.7	45.8 ± 17.8
Haploid control	42	32.8 ± 12.9	14.4 ± 11.7	1.0 ± 0.7
Diploid gynogenetic				
of inbred parentage	14	40.2 ± 17.3	32.6 ± 13.7	17.8 ± 6.3
of non-inbred parentage	17	37.9 ± 16.2	30.8 ± 10.2	8.3 ± 5.1

[a] Eggs were fertilized with *Macropodus* sperm

cessfully by others in order to inhibit first mitosis apparently do not work in *Macropodus*. We succeeded in preventing the second meiotic division by a mild cold treatment of eggs following fertilization and obtained 20–25% diploid offspring. The establishment of an optimal cold treatment is summarized in Figs. 1 and 2.

These experiments were repeated four times with eggs obtained from different females. The shape of the curves and the location of the peaks were the same throughout, the actual values varied depending on the individual providing the eggs. We found the proportion of viable diploids to be higher when using the eggs of females of inbred origin. A summary of gynogenetic propagation experiments is shown in Table 1.

The ploidy of three groups of gynogenetic larvae was determined and compared to the control group in Table 2.

Cells of normally developing cold-treated gynogenetic fry (Group 3 – subgroup b) contain the same amount of DNA as the control (Group 1) and twice the amount of the untreated – presumably haploid larvae (Group 2). It is quite notable that the cells of cold-treated gynogenetic individuals showing morphological abnormalities (Group 3 – subgroup a) contain an intermediate amount of DNA, suggesting aneuploidy. Note the relatively large variance in this group. A similar phenomenon was found in carp gynogenesis (Gervai et al. 1980).

Gene-centromere distances

Recombination frequencies of four independently segregating marker *loci* were determined by PAG electrophoresis of blood plasma in gynogenetic progenies of heterozygous F_1 hybrid females obtained by crossing homozygous individuals of various strains. Genotypes of the parental females were $Tf^{B/C}$ $Est\text{-}1^{b/c}$ $Est\text{-}2^{e/f}$ $Est\text{-}4^{g/0}$ and $Tf^{B/B}$ $Est\text{-}1^{b/b}$ $Est\text{-}2^{e/e}$ $Est\text{-}4^{h/g}$. Detailed descriptions of these markers – *Est-1, Est-2, Est-4* and *Tf* – have been published elsewhere (Monostory et al.

Table 2. DNA content of cells of normal and gynogenetic fish larvae

Treatment	Absorbance in A.U. Mean ± SD
Group 1	
diploid control[a]	15.9 ± 1.2
Group 2	
gynogenetic untreated with morphological abnormalities	7.4 ± 2.3
Group 3	
gynogenetic cold-shocked	
Subgroup a with morphological abnormalities	11.3 ± 4.9
Subgroup b normal development	16.4 ± 2.3

[a] Eggs were fertilized with *Macropodus* sperm

Table 3. Initial observation of esterase genotypes in families of gynogenetic origin[a]

Genotype	Incidence
Parental[b]	
$Est\text{-}1^{b/c}$ $Est\text{-}2^{e/f}$	14
$Est\text{-}1^{b/b}$ $Est\text{-}2^{e/e}$	10
$Est\text{-}1^{c/c}$ $Est\text{-}2^{f/f}$	17
Recombinant	
$Est\text{-}1^{b/b}$ $Est\text{-}2^{e/f}$	8
$Est\text{-}1^{c/c}$ $Est\text{-}2^{e/f}$	9
$Est\text{-}1^{b/c}$ $Est\text{-}2^{e/e}$	7
$Est\text{-}1^{c/c}$ $Est\text{-}2^{e/e}$	12
$Est\text{-}1^{b/c}$ $Est\text{-}2^{f/f}$	–
$Est\text{-}1^{b/b}$ $Est\text{-}2^{f/f}$	–

[a] Data were obtained from three families. Genotype frequencies of individual families did not differ significantly, so they were summarized in Tables 3 and 4

[b] Genotype of F_1 hybrid female parent was $Est\text{-}1^{b/c}$ $Est\text{-}2^{e/f}$

Table 4. Genotype frequencies in families of gynogenetic origin

Marker locus	Parental alleles[a]		Incidence					
			Observed			Corrected[b]		
	P_1	P_2	P_1P_1	P_1P_2	P_2P_2	P_1P_1	P_1P_2	P_2P_2
Tf	C	B	11	40	10			
Est-1	c	b	38	21	18	38	28	30
Est-2	f	e	17	31	29	36	31	29
Est-4	0	g	29	51		29	26	29
	h	g	1	20	23	23	20	23

[a] Female parents were F_1 hybrids carrying P_1 and P_2 alleles
[b] See the basis of correction in the text

1984). From these earlier investigations concerning the inheritance of serum esterases we know that there are certain lethal allelic combinations in *Est-1* and *Est-2* loci which have been consistently absent in all segregating populations studied. Therefore, in order to estimate recombination frequencies of these genes, population size and genotype frequencies had to be corrected. Initial observations on *Est-1, Est-2* genotypes in gynogenetic offspring are summarized in Table 3.

Correction of genotype frequencies shown in Table 4 was based on the assumption that *Est-1*$^{b/b}$ *Est-2*$^{f/f}$ should have been as frequent as *Est-1*$^{c/c}$ *Est-2*$^{e/e}$ and also *Est-1*$^{b/c}$ *Est-2*$^{f/f}$ should have occurred as frequently as *Est-1*$^{b/c}$ *Est-2*$^{e/e}$. Accordingly, for calculating recombination frequencies, the population has been supplemented with 12 and 7 individuals of the respective genotypes. Correction in *Est-4* was based on the assumption that frequencies of P_1P_1 and P_2P_2 homozygotes are theoretically equal.

Diploid gynogenetic progeny has a set chromosomes derived from sister chromatids of half of a meiotic tetrad. Heterozygous progeny will develop from a zygote containing sister chromatids with an odd number of exchanges between the centromere and the particular locus. Thus, the frequency of heterozygous progeny (r) is a direct measure of the recombination frequency – in this case the gene-centromere distance.

The frequencies of heterozygous offspring range from 0.27 to 0.66 (Table 5) for the various loci examined. Distances between marker loci and centromeres were calculated according to the mapping functions derived for tetrad analysis (Barratt 1954), on the basis of coincidence values zero, 0.2 and 1.0.

It has been pointed out by Nagy and Csányi (1982), that the probability for a gene of recombination frequency r to be heterozygous in the i-th gynogenetic generation is r^i, hence the coefficient of inbreeding $F_{(i)} = 1 - r^i$. The degree of uniformity or as he defines the degree of genotypic identity – probability that two individuals selected from the i-th gynogenetic generation are of the same genotype – either homozygous or heterozygous – with respect to the gene in question is:

$$I_{(i)} = 1 - 1/2\, r^{i-1} - r^i + 3/2\, r^{i+1}.$$

It might be of interest just how the level of homozygosity and the level of uniformity changes during successive gynogenetic propagation. Therefore, respective estimations of $F_{(i)}$ and $I_{(i)}$ for the genes investigated are also given in Table 5. The calculations were based on data from Table 4.

Table 5. Estimates of recombination frequencies (r), gene-centromere (G-C) distances, and coefficients of inbreeding (F) and genotypic identity (I) in the 3rd gynogenetic generation (i = 3)

Locus	Recombination frequency	G-C map distance (cM)[a]			$F_{(3)}$	$I_{(3)}$
		k = 0	k = 0.2	k = 1.0		
Tf	0.66	33	40	∞	0.71	0.78
Est-1	0.27	13.5	15	18	0.98	0.95
Est-2	0.30	15	16	20	0.97	0.94
Est-4	0.314[b]	16	17	21	0.97	0.93

[a] k = coefficient of coincidence. k = 0.2 and 1.0 represent estimations of chromosome interference (1-k) values found to be reasonably predictive (Nace et al. 1970)
[b] average value of 0.303 and 0.325

Discussion

Gynogenetic reproduction of *Macropodus opercularis* and *Macropodus opercularis concolor* was developed for the purpose of rapid inbreeding. X-ray irradiated sperm of distant species – goldfish, carp – ensured the exclusively female origin of the gynogenetic genome since hybrids of the above species with paradise fish do not develop beyond blastomeres.

Diploidy of zygotes was restored by inhibiting the second meiotic division with cold shock as indicated by the occurrence of 27–66% heterozygotes among the off-

spring of heterozygous females. The yield of viable diploid gynogenetic offspring varies, it reaches 25% under optimal conditions and on the average inbred females produce a higher proportion of viable diploids. As Table 2 shows, aneuploidy along with morphological abnormalities occurs in the cold-treated group, as was also shown in the gynogenesis of carp earlier (Gervai et al. 1980). Males were found among gynogenetic progeny indicating ZW/ZZ or polygenic sex determination in the paradise fish.

Recombination in four marker loci were followed in order to support the supposed mechanism of non-disjunction and also to see if only in a few characteristics the effect of gynogenesis on the rate of inbreeding. As calculated, F and I coefficients of Table 5 show homozygosity of the esterase genes in the gynogenetic line is well above 90% after three consecutive gynogenesis.

The changes of genetic parameters, namely the inbreeding coefficient, the fixation index and the degree of genotypic identity in successive gynogenetic generations have been described previously (Nace et al. 1970; Nagy and Csányi 1982). All these parameters are functions of the distribution of recombination frequencies in the genome. They also emphasized the information given by the newly defined genetic parameter – the degree of uniformity or genotypic identity – regarding rearing genetically uniform, but not necessarily homozygous stocks for either animal husbandry or the study of animal behavior.

In a recent article Nagy and Csányi (1983) suggest that following a certain optimal number of consecutive gynogenetic propagation alternating gynogenesis and sibmating results in the segregation of the highly recombinant genes and thus counteracts the tendency of conservation of heterozygosity in these loci.

In this respect the availability of gynogenetic paradise fish males is a great advantage. Breeding schedules based on the above considerations lead to practically inbred strains in eight generations even under the least favourable conditions, i.e. in species with many highly recombinant genes.

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