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ARTIFICIAL GYNOGENESIS IN COMMON BREAM (*ABRAMIS BRAMA* (L.)) INDUCED BY COLD-TEMPERATURE SHOCK

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ABSTRACT. Bream, *Abramis brama* (L.), eggs fertilized with genetically inactivated sperm (UV irradiation dose of 1920 J m^{-2}) were exposed to thermal cold shock to produce meiotic gynogenotes. The shock was applied at one-minute intervals from 1 to 10 min after egg insemination. The temperature of the shock was 2.0 ± 0.1°C, and its duration was 45 min. The water temperature prior to the shock was 20.0°C. Eggs fertilized with genetically inactivated sperm (putative haploids) exhibited retarded and abnormal development. The yield of gynogenesis was relatively low, except for the group to which the shock was applied 1 min after fertilization (about 30% in comparison with the controls). Ninety fish from the control and gynogenetic groups were reared for ten months. The survival of the gynogenetic bream was twofold lower than that of the controls. The gynogenotes were highly variable in size and exhibited some morphological abnormalities. The sex ratios in the control groups were close to 1:1, whereas all the gynogenotes were female.

Key words: COMMON BREAM (*ABRAMIS BRAMA*), ARTIFICIAL GYNOGENOTES, UV-TREATMENT, SEX DETERMINATION, GENETIC MARKERS

INTRODUCTION

Artificial gynogenesis is a method of obtaining specimens from female gametes. To produce viable gynogenetic diploids, the eggs are activated with genetically inactivated spermatozoa and treated with some environmental stress in order to inhibit the second polar body extrusion or to suppress the first cleavage (Kaastrup and Horlyck 1987, Komen et al. 1988, Varadaraj 1990, Kucharczyk 1996). This method is usually applied in the production of monosex populations or inbred lines, as well as in gene resource conservation, especially in the restoration of endangered species (Flajshans

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1998). The first step in inducing gynogenetic offspring is to inactivate the genetic material in spermatozoa nuclei. The authors' earlier work showed that the concentration of spermatozoa in common bream, *Abramis brama* (L.), sperm varied in individuals from 9 to 22×10^9 spermatozoa per 1 ml (Kucharczyk 1996). Due to this, bream milt should be diluted in 0.85% NaCl to a constant concentration of 1.9×10^9 spermatozoa per ml before UV irradiation. The second step in the production of gynogenetic cyprinid fish involves exposing the activated eggs to cold-temperature shock (Komen et al. 1988, Cherfas et al. 1990). The results obtained in common bream gene resource conservation might be used further in other similar species such as vimba bream, *Vimba vimba* (L.). The situation of this last species in Poland has changed dramatically recently, and many populations are becoming or already are extinct.

Chromosome set manipulation such as gynogenesis might also be used to determine sex chromosomes. Heteromorphic sex chromosomes were identified for a limited number of wild European cyprinid species (Rudek 1974, Vujosevic et al. 1983), but these data are problematic since no such system has been found in other research.

Identifying cyprinid chromosomes well is rather difficult because they are relatively small at 1.5-3.0 μm (Rab and Collares-Pereira 1995). In the most recent studies of the common bream karyotype, which applied different methods of chromosome staining such as Giemsa, silver (Ag-NOR), C or replication (DAPI, BrdU) banding (Jankun et al. 1997, 1998), no differences were discerned between specimens of the two sexes. Distinguishing sex in gynogenotes (specimens with only maternal genome material) usually showed what kind of sex determination – homogenic or heterogenic, the females have. This information is very useful for further work on a sex determination system in fish.

This paper presents results of a study on the induction of artificial gynogenesis in common bream in which the eggs were activated with sperm (also heterologous) irradiated with UV followed by a cold temperature shock in order to prevent the extrusion of the second polar body. The second aim of this work was to determine the sex of common bream gynogenotes.

MATERIAL AND METHODS

BROODSTOCK AND THE COLLECTION OF GAMETES

Bream spawners were caught in Lake Kortowskie (Olsztyn, Poland) in May 1994. The fish were transported to the hatchery and kept in 1000 l tanks under controlled

temperature (19-20°C) and light (photoperiod L:D – 18:6) conditions. Spawners received three injections of human chorionic gonadotropin (hCG) at total doses of 1100 and 2200 IU kg^{-1} for males and females, respectively, and acetone-dried common carp pituitary extract (CPE) at total doses of 2.0 and 4.0 mg kg^{-1} for males and females, respectively (Kucharczyk et al. 1997b). Some bream breeders were marked using an isozyme genetic marker (GPI-1). Koi carp, *Cyprinus carpio* L., males were treated with acetone-dried common carp pituitary extract (CPE) at a total dose of 2.0 mg kg^{-1}.

In each experiment milt was collected from two to six (bream) or two to three (koi carp) males. The quality of sperm was expressed as the percentage of motile spermatozoa. Motility was estimated by microscopic (500x) observation of sperm activated with 0.5% NaCl. Samples of sperm with 80% (or more) motile spermatozoa were pooled and used for further treatments. Sperm concentration was estimated using the haemocytometer (Bürker chamber) method (Kucharczyk et al. 1996a). For each experiment eggs were stripped from one bream female 14 hours after the resolving hormone injection.

STANDARDIZATION OF THE GENETIC INACTIVATION OF SPERMATOZOA

The pooled milt was diluted in 0.85% NaCl to a concentration of 1.9×10^9 spermatozoa per ml. Volumes of 2.5 ml of the diluted sperm were spread in a 1 mm layer on Petri dishes and placed on a rocking table with a cycle of ~ 1 s for irradiation. The UV lamp (30 W, 6.4 W m^{-2}) was switched on for at least 30 min before the onset of irradiation. Before the sperm was irradiated, control samples of eggs (250-300 eggs in each sample) were fertilized with a small volume of undiluted sperm (egg quality control: groups C1 and C2), and with 0.5 ml of diluted, non-irradiated sperm (sperm irradiation control: groups DB1 and DB2). The quantity of sperm used for fertilizing control eggs depended on the spermatozoa concentration, so that in controls (undiluted sperm) and in treated groups (diluted sperm) the same number of spermatozoa was used for egg insemination. Experimental groups of eggs were fertilized with 0.5 ml of diluted and irradiated sperm (irradiation time ranged from 1 to 9 min, UV irradiation dose ranged from 384 to 3456 J m^{-2}). After sperm irradiation, additional control groups of eggs were fertilized with 0.5 ml of diluted, non-irradiated sperm (diluted sperm viability control: groups DE1 and DE2). The whole procedure was carried out in darkness to avoid genetic photo-reactivation of the sperm (Kaastrup and Horlyck 1987). The eggs were incubated in a laboratory recirculating system at 20°C, which had previously been proven to be the optimal temperature for bream embryonic development (Kucharczyk et al. 1997c). All the experimental groups were in duplicate.

GYNOGENESIS

The genetic inactivation procedure of the bream sperm was the same as described above, with the addition of one control group (in duplicate) to evaluate the effect of sperm irradiation (I1 and I2). In these groups, the eggs were mixed with 0.5 ml of diluted sperm that had been irradiated for 5 min at a UV irradiation dose of 1920 J m^{-2} (Kucharczyk 1996). Portions of 250-300 eggs were placed in Petri dishes and mixed with sperm. The moment of water addition was considered to be the activation time (t_o). Cold shocks were applied by transferring Petri dishes containing activated eggs at 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 min after egg activation to a tank with cool (2.0 ± 0.1°C) water. The duration of the shock was 45 min. The parameters of the cold shock (temperature, duration) were chosen based on preliminary work (Kucharczyk et al. 1996b). The eggs were incubated in a laboratory recirculating system at 20°C before and after the cold shock. All groups were in duplicate.

GYNOGENESIS USING HETEROLOGOUS SPERM

This experiment was conducted to provide evidence that the gynogenesis process is successful. All manipulations were conducted as described above with the following small differences: the bream breeders were chosen after isozyme analysis (GPI-1 homozygous); the sperm was collected from koi carp; the time of applied cold shock was 2, 4, 6, and 8 min after egg activation.

REARING LARVAE AND JUVENILES

Ninety gynogenetic (group G) and "normal" (control group C, groups K1 and K2) larvae were reared in 60 l aquaria that were part of a recirculating system. The fish density in group K2 was regulated artificially along with the natural mortality in group G. The fish were fed mixed food (alive and dry). After 10 months of rearing the fish were measured (total length – TL and body length – BL), weighed (wet weight – WW), and their sex was determined based on the microscopic examination (300x) of fresh gonads.

STATISTICAL ANALYSIS

Egg survival was calculated as the percentages of hatched embryos and those that started swimming. The ploidy was determined based on the nucleoli number with the method described by Kucharczyk et al. (1997a). Successful gynogenesis, that using heterologous sperm, was evidenced using genetic markers (GPI-1). Differences

in hatching success and survival and growth between groups of bream larvae and juveniles were tested with Duncan's multiple range test ($P \leq 0.05$).

RESULTS

STANDARDIZATION OF GENETIC INACTIVATION OF SPERMATOZOA

No diploid embryos hatched from the eggs inseminated with UV-irradiated sperm. The highest percentage of eyed-eggs was observed in groups activated with sperm irradiated for 5 min (1920 J m^{-2}). The hatching rate was low and did not exceed < 3%. (Fig. 1). The haploid embryos were very weak and only some of them hatched. The hatching interval of bream haploids was long, and sometimes the embryos hatched so late in time that the fish from control groups had already started exogenous feeding. All of the hatched haploid embryos showed morphological abnormalities and died soon after hatching. No abnormal embryos were noted in the control groups (C, DB, and DE).

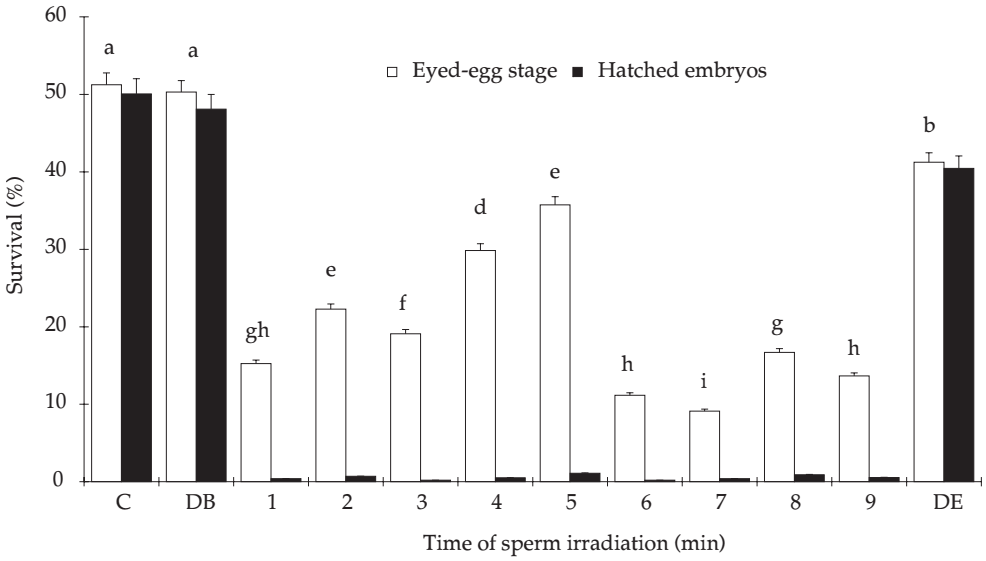

Fig. 1. Effect of the genetic inactivation of bream spermatozoa by UV irradiation on survival to the eyed-egg stage and on hatching success. Vertical bars show SE. Data of survival to the eyed-egg stage marked with the same letter did not differ significantly (Duncan's test, $P > 0.05$). Groups C, DB, and DE are described in Material and Methods.

The cells of haploid bream individuals usually had one nucleolus, or, sporadically, two active nucleoli. Depending on the specimen, the mean number of active nucleoli per nucleus ranged from 1.00 to 1.01; the cells of diploid (control) fish had one (24-30%), two (69-75%), or, sporadically, three nucleoli (mean ranged from 1.71 to 1.77).

GYNOGENESIS

Survival to the eyed-egg stage and the hatching percentage in groups exposed to the cold shock were much lower than in the controls. The lowest hatching rate was observed in the egg groups shocked at 4, 5, 6, 7, and 9 min after egg activation. Higher survival rates were noted in groups shocked 1 and 10 min after egg activation; these same groups also showed the highest percentage of gynogenotes (Fig. 2).

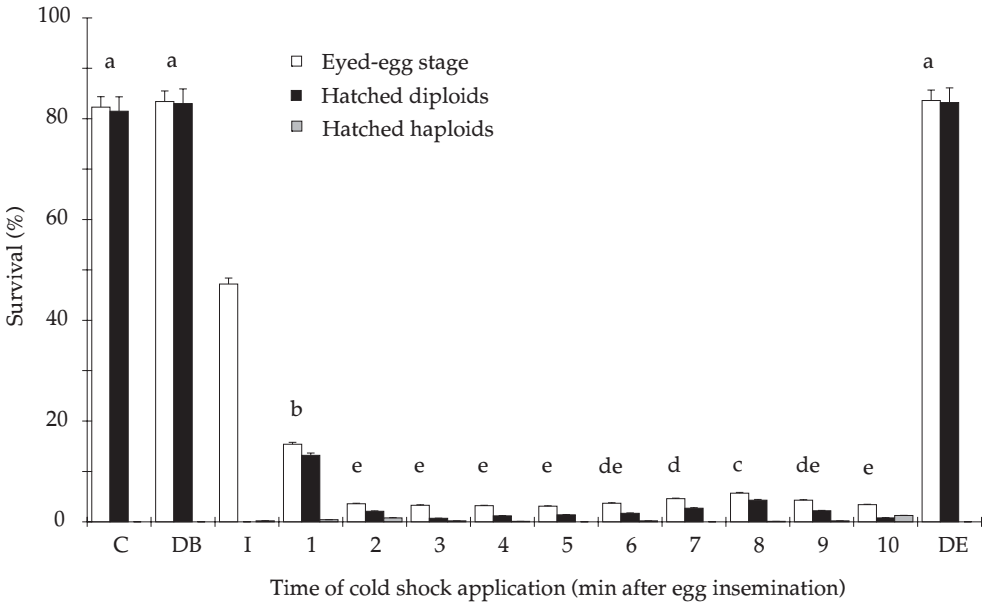

Fig. 2. Effect of the time of cold shock (2°C ± 0.1) application on bream eggs inseminated with UV irradiated sperm. Vertical bars show SE. Data of hatching of diploids rate marked with the same letter did not differ significantly (Duncan's test, $P > 0.05$). Groups C, DB, I, and DE are described in Material and Methods.

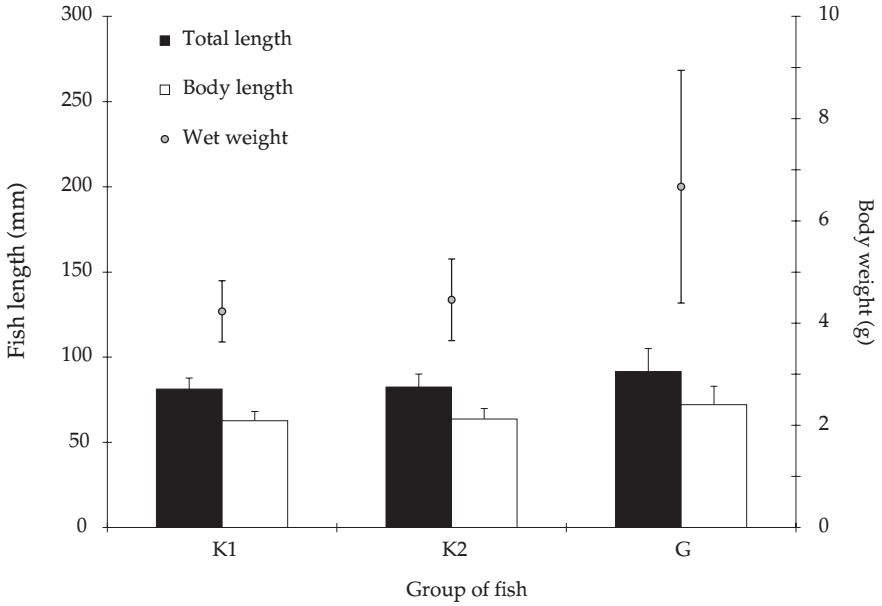

Fig. 3. Effect of the time of cold shock (2°C ± 0.1) application on bream eggs inseminated with UV irradiated koi carp sperm. Vertical bars show SE. Data of diploid hatching rate marked with the same letter did not differ significantly (Duncan's test, $P > 0.05$). Groups K1, K2, and G are described in Material and Methods.

In the experiment with koi carp sperm, the survival in the control groups (bream × koi carp) was zero. The highest survival rate in the treated groups was noted when the eggs where exposed to the shock 8 min after activation (Fig. 2). The highest gynogenesis yield, in comparison to the survival to the eyed-egg stage, was observed in groups exposed to the shock at 1 and 8 min or, in the experiment with carp sperm, at 8 min. The hatching time, morphology, and number of nucleoli per nucleus in the haploid and diploid fish were similar to those obtained in a trial for the "...standardization of the genetic inactivation of spermatozoa...". The mean number of active nucleoli per nucleus ranged from 1.00 to 1.02 in haploids and from 1.70 to 1.76 in diploids (gynogenotes and controls). All gynogenotes that were verified using isozyme analysis were homozygous.

REARING OF LARVAE AND JUVENILES

Fish size (length and weight) is shown in Fig. 3. The largest fish were observed in group G, although the differences between groups (K1, K2, and G) were not statistically significant (Table 1).

TABLE 1

Mean size (± SE) of gynogenetic (G) and control (K1, K2) bream, reared for 10-months. Groups K1, K2, and G are described in Material and Methods. There were no statistical differences between groups

Group of fish	Survival	Total length	Body length	Wet weight
	(%)	(mm)	(mm)	(g)
Control – K1	93.3	81.2 ± 6.5	62.7 ± 5.4	4.23 ± 0.60
Control – K2	-	82.4 ± 7.7	63.7 ± 6.1	4.46 ± 0.80
Gynogenotes	46.7	91.5 ± 13.6	72.1 ± 10.8	6.67 ± 2.28

Some gynogenetic fish showed development abnormalities during rearing. These fish had much slower growth and they died successively. The highest cumulative mortality was noted in group G (53.3%), whereas in the control group (K1) it was much lower (6.7%). The sex ratio of fish in both of the control groups was approximately 1:1, while all 42 gynogenetic specimens were females. Most of the gynogenetic females had synchronously developed oocytes; this observation was similar to that in the control females (Photo 1). Only two of the smallest gynogenotes had fewer and smaller oocytes.

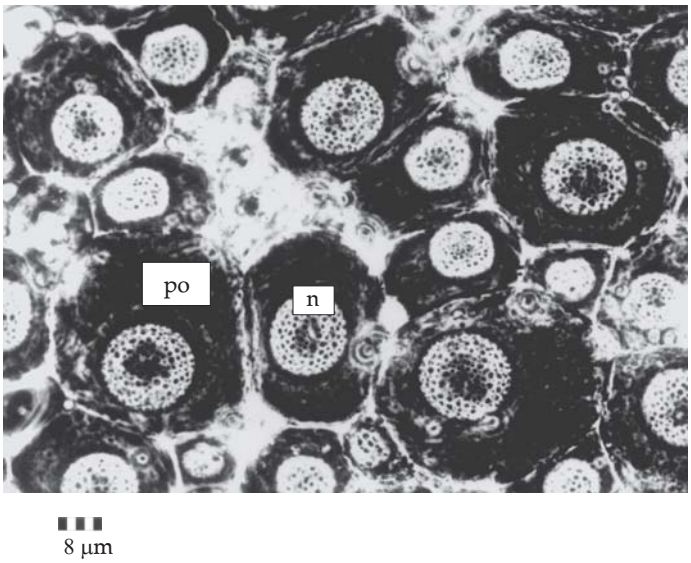

Photo 1. Photographs (500 X) of gonads of bream gynogenetic females. Descriptions: po – previtellogenic oocytes; n – nucleus.

DISCUSSION

The low hatching rate of the eggs inseminated with genetically inactivated sperm was similar to that reported in other artificial fish gynogenesis experiments (Stanley 1976, Quillet et al. 1988, Varadaraj 1993). In many fish species including rainbow trout, *Oncorhynchus mykiss* (Wal.) (Kaastrup and Horlyck 1987), common carp, *Cyprinus carpio* L. (Komen et al. 1988), grass carp, *Ctenopharyngodon idella* (Val.) (Stanley 1976), or tilapia, *Oreochromis mossambicus* (Peters) (Varadaraj 1993), haploid embryos usually die before, during or shortly after hatching. The highest survival rate to the eyed-egg stage was observed when diluted sperm was irradiated for 5 min (irradiation dose of 1920 J m^{-2}). Similar results were obtained when bream sperm was diluted to a final concentration that ranged from 1.65 to 1.90×10^9 spermatozoa per 1 ml (Kucharczyk et al. 1996a). The number of active nucleoli per nucleus observed in haploids was very similar to the data obtained for haploid bream embryos in the authors' earlier works (Kucharczyk 1996, Kucharczyk et al. 1996a, Kucharczyk et al. 1997a).

In warm – water fish, a cold shock applied to produce heterozygous gynogenetic or triploid fish by the retention of the second polar body can be very effective (Ojima and Makino 1978, Ueno and Arimoto 1982, Komen et al. 1988, Kucharczyk et al. 1997d). However, the survival in experimental egg groups is much lower than in the controls (Stanley et al. 1975, Chourrout 1984, Quillet and Gaignon 1990, Kim et al. 1994). In this study the highest survival was observed in eggs which were cold shocked 1 min after insemination, and also at 8 and 10 min after egg activation. Similar observations were reported for common carp by Komen et al. (1988) and by Cherfas et al. (1990). This bimodal response to cold shock seems to occur in warm water cyprinid eggs, and might be related to two consecutive phases occurring during meiosis. Environmental shock dissociates the microtubules of the karyokinetic spindle or, when applied at a later stage, cause the second polar body formation to be absorbed by the ovoplasma (Komen et al. 1988).

In comparison with control samples, the yield of gynogenesis might differ in different species. The highest gynogenesis yield ranges for several species were as follows: common carp 9 - 95% (Nagy et al. 1978, Linhart et al. 1986); Atlantic salmon, *Salmo salar* L., 43 - 56% (Quillet and Gaignon 1990); channel catfish, *Ictalurus punctatus* (Raf.), approximately 3% (Goudie et al. 1995); tilapia 2% (Varadaraj 1990); bream (current study) 30%. This result for bream was much higher than that obtained from an experiment with a heat shock (Kucharczyk et al. 1997d).

The gynogenetic bream reared for 10 months exhibited extensive size differentiation, some developmental abnormalities, and comparatively high mortality. This is typical for heterozygous gynogenetic fish, and is most probably due to their inbreeding. The gonads of some smaller gynogenotes (~ 5% of all the gynogenetic fish) were underdeveloped, as was observed in gynogenetic coho salmon, *Oncorhynchus kisutch* (Wal.), by Pifferer et al. (1994). The analysis of the gonads of fish from control and treated groups showed that common bream females are homogenic with regard to the sex chromosome determination system

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STRESZCZENIE

KONTROLOWANA GYNOGENEZA U LESZCZA (*ABRAMIS BRAMA* (L.)) WYWOŁANA ZASTOSOWANIEM SZOKU TERMICZNEGO ZIMNEGO

Oocyty leszcza (*Abramis brama* (L.)) zostały zaplemnione genetycznie inaktywowanym nasieniem (promieniowanie UV; dawka 1920 J m^{-2}) (rys. 1) i poddane działaniu szoku termicznego zimnego, w celu otrzymania mejotycznych gynogenotów. Szok był stosowany od 1 do 10 min (w odstępach wynoszących 1 min) od momentu aktywacji ikry. Temperatura i czas szoku wynosiły odpowiednio 2,0 ± 0,1°C i 45 min. Temperatura inkubacji ikry przed poddaniem jej działaniu szoku wynosiła 20,0°C. Ikra zaplemniona inaktywowanym genetycznie nasieniem wykazywała rozwój charakterystyczny dla osobników haploidalnych, w tym charakterystyczną deformację ciała (syndrom haploidalny). Wydajność procesu gynogenezy była relatywnie niska, z wyjątkiem grup poddanych działaniu szoku w 1 min od aktywacji (około 30% w porównaniu z kontrolą) (rys. 2). 90 ryb gynogenetycznych i kontrolnych było podchowywanych przez trzy miesiące. W tym okresie przeżywalność gynogenotów była dwukrotnie niższa niż ryb kontrolnych (tab. 1). Ryby gynogenetyczne wykazywały znacznie większe zróżnicowanie w rozmiarach ciała (rys. 3) niż ryby kontrolne. Stosunek płci u ryb kontrolnych wynosił w przybliżeniu 1:1, podczas gdy wszystkie gynogenetyczne ryby okazały się samicami (rys. 4).