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APPLICATION OF THE UV-IRRADIATED HOMOLOGOUS AND HETEROLOGOUS SPERM FOR ACTIVATION OF THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EGGS AND PRODUCTION OF THE GYNOGENETIC STOCKS*

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Abstract

Meiotic gynogenetic development of the rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) was induced in the course of egg activation performed by the UV-irradiated homologous and heterologous (European grayling *Thymallus thymallus* Linnaeus, 1758) spermatozoa. To recover diploidy in the gynogenetic zygotes, activated eggs were subjected to the high pressure shock in order to inhibit extrusion of the second polar body. Gynogenetic rainbow trout progeny hatched from the eggs activated by the irradiated rainbow trout and grayling milt with similar hatching rates of 28.19% and 29.22%, respectively. However, gynogenetic rainbow trout produced with grayling semen had shown lower survival than gynogenotes provided with the homologous spermatozoa during two years of rearing. Viable hybrids are not produced between rainbow trout and grayling which ensured that fish obtained in this experiment were true gynogenetic progenies. A Robertsonian polymorphism characteristic for the rainbow trout from the studied strain was also observed among the gynogenetic specimens that exhibited diploid chromosome number ranging from 58 to 62 and stable chromosome arm number (FN= 104). No radiation-induced fragments of the paternal chromosomes were observed in the gynogenetic individuals. Fish produced in both experimental variants were genotypic (XX) and phenotypic (gonads) females. The results confirmed that the gynogenetic protocol used in the present research is an efficient means of producing all-female gynogenetic rainbow trout stocks.

Key words: all female, gynogenesis, sex chromosomes, Salmonids, UV light

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Sexual maturation in rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) can reduce growth rate, increase mortality and cause deterioration in fillets as the gonadal growth phase causes mobilization of the nutrients mostly from the muscles. Thus, it is reasonable to harvest fish before their sexual maturation. In the rainbow trout, males usually mature during the first year of life and before reaching a market size of about 450 g. In turn, rainbow trout females reach a commercial size before maturation, making them preferable for aquaculture. As cultivation of only female individuals is economically more profitable than raising mixed stocks, several technologies to generate "all female" rainbow trout stocks have been proposed (Donaldson, 1996). Rainbow trout are XX/XY for sex determination. All-female offspring (XX) can be produced through gynogenesis (I) (Thorgaard, 1992) or indirectly by mating normal females with "neo-males" (XX) which have been produced through androgen-induced masculinization of females (II) (Feist et al., 1995). Sometimes both approaches are combined and masculinized gynogenetic rainbow trout are used as spermatozoa donors to provide "all-female" progenies (Feist et al., 1995).

Gynogenesis occurs naturally in several fish species, however, it also may be induced under control conditions (Pandian and Koteeswaran, 1998). Artificial gynogenesis is a three-step process including radiation-induced inactivation of the spermatozoa DNA (I), activation of the eggs with the irradiated spermatozoa (II) and exposing activated eggs to a temperature or pressure shock, which inhibits release of the second polar body (meiotic gynogenesis, heterozygous offspring) or by suppressing the first mitotic cleavage (mitotic gynogenesis, homozygous offspring) (Thorgaard, 1986). Yields of meiotic gynogenetic fish at hatching rarely exceed 60% and hatching rate of the mitotic gynogenotes is even lower (Chourrout and Quillet, 1982; Dabrowski et al., 2000; Paschos et al., 2001; Komen and Thorgaard, 2007). Several factors affect survival of the gynogenetic fish including the stressful effects of the manipulations performed on the activated eggs, expression of the lethal alleles (mitotic gynogenesis) and presence of the paternal chromosome fragments (Chourrout and Quillet, 1982; Chourrout, 1984; Krisfalusi et al., 2000). Fragments of paternal chromosomes may appear in the course of incomplete inactivation of the DNA from the spermatozoa. Radiation-induced chromosome fragments are considered to interrupt cell cleavage, provoke subsequent chromosome rearrangements and increase mortality among the genome manipulated fish (Ocalewicz et al., 2009, 2010). It has been also observed that chromosome fragments affect gonadal development in the gynogenetic rainbow trout females (Krisfalusi et al., 2000). To avoid such remnants of the irradiated paternal genome, application of inactivated sperm from a species that will not fertilize the egg donor species has been proposed for gynogenetic induction (Chourrout, 1986; Dabrowski et al., 2000). To verify this approach we used UV-irradiated homologous and heterologous sperm provided from the European grayling (*Thymallus thymallus* Linnaeus, 1758) to activate rainbow trout eggs. Diploidization through retention of the second polar body was induced with high pressure shocking. Survival of the gynogenetic rainbow trout was monitored during two years of rearing. Moreover, two-year-old gynogenetic rainbow trout were cytogenetically studied to confirm occurrence of only maternal chromosomes based on the presence of only XX-sex chromosomes.

Material and methods

Gamete collection and induction of gynogenetic development

Gametes were collected from rainbow trout and European grayling broodstocks kept at the Department of Salmonid Research, Inland Fisheries Institute in Olsztyn, Rutki, Poland. Rainbow trout specimens from the Rutki strain used in this experiment had a diploid chromosome number varying from 59 to 62 and stable chromosome arm number (Fundamental Number, FN) equaled 104 (Robertsonian polymorphism) (Ocalewicz, 2002). Moreover, sex related chromosomal polymorphism has been described in the fish from Rutki strain and cytogenetic markers of the X chromosome provided. The X chromosome is a subtelocentric with an accumulation of AT-rich chromatin (DAPI-positive) in the pericentromeric region. Y chromosome shows significantly reduced p-arm without AT-rich chromatin at the pericentromeric location (Ocalewicz, 2002). The European grayling had 100 chromosomes and FN= 167–170 (Jankun et al., 2003).

Eggs derived from four-year-old rainbow trout females (n=6) were pooled and placed in a plastic bowl. Then, the pool of eggs was divided into several batches allocated for the gynogenetic treatment (c. 6500 eggs) and as control groups (c. 1000 eggs). Semen from four-year-old rainbow trout males (n=3) and from three-year-old grayling (n=3) were pooled within the species.

For inactivation of the nuclear DNA in the rainbow trout and grayling spermatozoa, 0.375 ml of the semen from each species was diluted 40 × in the rainbow trout seminal fluid and subjected to a 2075 μW/cm^2 of UV-radiation lasting 12 minutes (Goryczko et al., 1991). The rest of the milt was kept intact in the refrigerator (+2°C) to be used for the control variants of the experiment. Batches of about 6500 eggs were inseminated with the aliquots of UV-inactivated semen from the rainbow trout and the European grayling (c. 150 000 spermatozoa per egg) to provide gynogenetic experimental variants G_1 and G_2, respectively. Batches of the eggs designated for the control groups were inseminated with the intact semen (0.1 ml, c. 300 000 spermatozoa per one egg) from each species, to provide rainbow trout progeny (C_1) and rainbow trout × European grayling hybrid offspring (C_2). Immediately after the addition of the semen, Billard fertilization diluent (154 mM NaCl and mM Ca^{2+}, buffered to pH 9.0 with 20 mM Tris + 30 mM glycine) (Billard, 1992) was poured over the batches of eggs. Batches were then swirled and left for 3 minutes. Inseminated eggs from the gynogenetic and control groups were then rinsed with the hatchery water and incubated at 8°C for the further treatments.

The gynogensis was induced according to a previously published procedure (Chourrout and Quillet, 1982) with some modifications. Thirty-five minutes after insemination, eggs from G_1 and G_2 batches were subjected to the high pressure shock (7500 psi) for four minutes. Fertilized eggs from the control groups were not subjected to any treatment after insemination. Eggs from the gynogenetic and control variants of the experiments were incubated separately at 8–10°C under routine conditions used at the Department of Salmonid Research, Rutki.

Survival of the gynogenetic embryos and hatchlings

The survivability of the embryos from the gynogenetic and control groups was measured at the eyed stage, 25 days post fertilization (dpf). Survival rates of the larvae were checked just after hatching (38 dpf) and at the swim-up stage when the yolk sacs were fully absorbed (50 dpf). The survival of the gynogenetic progenies after the swim-up stage was monitored during two years of rearing.

Cytogenetic analysis

After two years of rearing, four gynogenetic rainbow trout provided in the course of egg activation performed by the UV-irradiated rainbow trout sperm and five gynogenetic progeny obtained by the egg activation performed by UV-inactivated heterologous spermatozoa were sacrificed for the cytogenetic analysis. The phenotypic sex of the cytogenetically studied specimens was determined by the eye examination of gonads after fish dissection and in the course of the microscope analysis of the gonad tissue squashed with a cover slip.

Metaphase spreads were prepared from the cephalic kidney cells using the method described by Ocalewicz et al. (2012). DAPI (4',6-diamidino-2-phenylindole) staining technique was applied to identify rainbow trout X chromosomes and to evaluate genetic sex of the gynogenetic specimens. For chromosomal localization of the telomeric sequences, PNA-FISH (peptide nucleic acid – fluorescence in situ hybridization) with a telomeric probe, was used. Fluorescence *in situ* hybridization (FISH) procedure with a telomere PNA probe conjugated with FITC (DAKO, Denmark) was performed according to the manufacturer's protocol. Chromosomal DNA was denatured at 84°C for 5 minutes under the coverslip in the presence of the PNA probe. Hybridization took place in the darkness at room temperature for 60 minutes. Chromosomes after PNA – FISH were counterstained with DAPI solution.

At least ten high quality metaphase spreads from each specimen were analyzed. Metaphase cells were examined under a Zeiss Axio Imager A1 microscope equipped with a Zeiss EC Plan-Neofluar 100x/1.3 oil objective, a fluorescent lamp and a digital camera (Applied Spectral Imaging, Galilee, Israel). Images were captured and the electronic processing of the images was performed using the Band View/FISH View software (Applied Spectral Imaging).

Results

The survival rates of the gynogenetic rainbow trout and their siblings from the control groups during embryogenesis and just after hatching were summarized in Table 1. Gynogenetic embryos in the eggs activated by the homologous (G_1) and heterologous (G_2) spermatozoa were developing equally and their survival rates at the eyed stage were above 50%. In spite of very high survival rates of the normal rainbow trout (C_1) during the entire experiment, mass mortality was observed among rainbow trout × grayling embryos from the other control group (C_2) and none of these hybrids survived until hatching (Table 1). Gynogenetic progeny developing

in the eggs activated by the irradiated rainbow trout sperm (G_1) and grayling milt (G_2) hatched with similar survivability. From hatching to the swim-up stage mortality of the gynogenetic rainbow trout increased and it was higher among specimens that hatched from the eggs activated by the irradiated grayling sperm (G_2) (Table 1). After the first year of rearing 437 gynogenetic individuals from G_1 group and 175 gynogenetic specimens from G_2 group survived and after two years of rearing, 280 specimens from G_1 and 115 specimens from G_2 remained alive.

Table 1. Survival rates (%) of the gynogenetic rainbow trout provided in the course of the eggs activation performed with the UV-irradiated homologous (G_1) and heterologous sperm (European grayling) (G_2), and their control siblings; normal rainbow trout (C_1) and rainbow trout × European grayling hybrids (C_2), relative to amount of fertilized eggs

Experimental group	Eyed stage embryos	Hatching	Swim-up stage	After one year of rearing	After two years of rearing
G_1	55.5	28.19	16.48	6.82	4.37
G_2	54.31	29.22	7.03	2.77	1.82
C_1	90.54	85.97	85.37	65.0	55.0
C_2	1.55	-	-	-	-

Phenotypic sex was determined in all cytogenetically studied gynogenetic specimens which all appeared to be females. Diploid chromosome numbers among gynogenetic rainbow trout hatched from the eggs inseminated with the irradiated rainbow trout semen were 58 (n=1), 59 (n=2) and 60 (n=1). Gynogenetic rainbow trout from the G_2 group exhibited 60 chromosomes (n=3) and 62 chromosomes (n=2). Chromosome arm numbers were found to be 104 in all gynogenetic rainbow trout studied. Moreover, all analyzed gynogenetic individuals had two X chromosomes (XX) in their karyotypes. Radiation-induced chromosome fragments were not found in any of the metaphase plates analyzed.

Fluorescent signals after PNA-FISH with telomeric probe were observed only at the ends of all chromosomes.

Discussion

Gynogenetic induction using heterologous sperm has been performed in several freshwater and marine fish species. In the present research gynogenetic development of the rainbow trout was induced using genetically inactivated homologous and heterologous sperm. High pressure shock was applied to induce retention of the second polar body and reconstitute diploidy in the gynogenetic zygotes. In other studies, yields of hatched gynogenetic larvae from salmonid fish eggs activated with irradiated homologous spermatozoa and subjected to temperature shock varied from 10% (rainbow trout) to 50% (Atlantic salmon *Salmo salar*) (Pandian and Koteeswaran, 1998). Application of the high pressure shock to recover diploidy in the gynogenetic zygotes usually results in the lower mortality (Pandian and Koteswaaran, 1998; Reading et al., 2015). In this study, gynogenetic rainbow trout progenies hatched from the

eggs activated by the irradiated rainbow trout and grayling milt had survivability that was comparable to rates reported in other studies (Pandian and Koteswaaran, 1998). Side effects of the high pressure shock applied to the inseminated eggs probably affected some part of the mortality among the gynogenetic embryos. High pressure shock interrupts segregation of chromosomes by destabilizing the spindle microtubules. But fish egg microtubules play also some role in the transportation of the cytoplasmic factors involved in the early stages of embryogenesis (Webb et al., 1995). Therefore, changes in the microtubule structure generated by the hydrostatic pressure may affect early development of the gynogenetic individuals (Yamaha et al., 2002). Moreover, thermal and high pressure shocks may be harmful to the maternal transcripts from the egg cytoplasm that play crucial role at the beginning of the embryonic development before midblastula transition (MTB) (Pelegri, 2003).

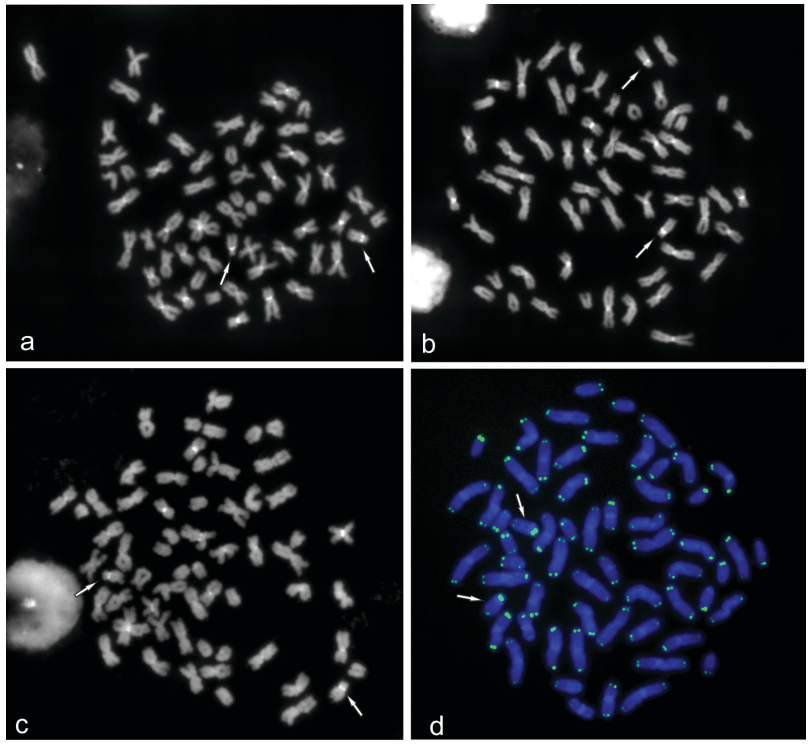

Figure 1. Metaphase spreads of the gynogenetic rainbow trout (*Oncorhynchus mykiss*) with 58 (a), 59 (b), 60 (d) and 62 (c) chromosomes after DAPI staining (a, b, c) and PNA-FISH with telomeric probe (d). Arrows show X chromosomes

Application of the heterologous sperm has been used to activate eggs from several species and such approach does not seem to affect the survival of the gynogenetic embryos and newly hatched larvae, which is in agreement with results presented here (Pandian and Koteswaaran, 1998). However, survival rates of the gynogenetic fish from G_1 and G_2 groups started to differentiate after hatching. Rainbow trout and the

European grayling are genetically distant and the mismatch between their karyotypes may result in the failure of the interspecies hybridization. Furthermore, mortality of these hybrids might be also caused by the incompatibility between the rainbow trout egg cytoplasm and the grayling nuclear genome. Such a nucleo-cytoplasmic conflict that resulted in the exclusion of the paternal chromosomes during embryogenesis was observed in the inviable masu salmon (*Oncorhynchus masou masou* Brevoort 1856) × rainbow trout hybrids (Fujiwara et al., 1997).

This kind of conflict does not seem to happen in the interspecies gynogenetic progenies as theoretically UV-irradiated spermatozoa should not transfer any parts of the paternal chromosomes to the gynogenetic zygote. Nevertheless, none of the agents employed for the genetic inactivation of the spermatozoa (UV-, ionizing radiation or chemical mutagens like dimethylsulphate) has been found to destroy paternal chromosomes completely. Residues of the partially inactivated chromosomes from the spermatozoa are sometimes observed as small chromosome fragments in the cells of the gynogenetic embryos and hatched fish (Chourrout and Quillet, 1982; Chourrout, 1984, 1986; Krisfalusi et al., 2000). Remnants of the irradiated chromosomes may incorporate into the intact chromosomes. In the androgenetic adult rainbow trout, gamma-radiation-induced terminal fragments of the maternal chromosomes have been found to be incorporated into the paternal chromosomes and detected by the FISH procedure with the telomeric probe (Ocalewicz et al., 2004). However, neither chromosome fragments nor pieces of the chromatin incorporated into the maternal chromosomes were found in the studied gynogenetic specimens (Figure 1). This might have indicated two things: the dose of the UV radiation was proper to inactivate totally nuclear DNA in the spermatozoa from both species studied here (1) or nuclear genome was not fully inactivated in some of the spermatozoa but within two years of rearing most of the gynogenetic individuals with fragments of the paternal chromosomes were preferentially lost from the cohort (2). This could partially explain lower survival of the gynogenetic trout hatched from the eggs activated by grayling UV-treated spermatozoa.

Cytogenetic survey exhibited varied chromosome numbers among the gynogenetic rainbow trout progeny. Although Rutki strain of the rainbow trout is polymorphic for the chromosome number (Robertsonian polymorphism) (Ocalewicz, 2002), we did not observe any significant differences in the survival rates between offspring of the parents showing different chromosome numbers (Ocalewicz et al., 2010).

Rainbow trout females are the homogametic sex (XX) and the induced gynogenesis results in only female progenies (Arai, 2001). This was also observed in the present experiment where all examined gynogenetic rainbow trout exhibited XX sex genotypes and female phenotypes.

To conclude, two stocks of the meiotic gynogenetic rainbow trout females were produced in the course of the rainbow trout eggs activation performed by the UV-irradiated homologous and heterologous spermatozoa. After two years of rearing randomly selected gynogenotes did not possess residues of the paternal nuclear genome in the form of chromosome fragments. The protocol used for induction of the gynogenetic development in this study may be applied for the production of "all female" rainbow trout stocks.

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