Arch. Pol. Fish.	Archives of Polish Fisheries	Vol. 16	Fasc. 4	437-446	2008

GENETIC INACTIVATION OF DACE, *LEUCISCUS LEUCISCUS* (L.), GAMETES USING UV IRRADIATION

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ABSTRACT. The results of genetic inactivation of dace, *Leuciscus leuciscus* (L.), gametes using UV irradiation are presented. Dace sperm was genetically inactivated with UV irradiation doses ranging from 384 to 3840 J m^{-2}. The most effective dose, expressed as a high percentage of hatched haploid larvae, was 1920 J m^{-2}. The UV treatment did not kill the spermatozoa, and following irradiation the activated spermatozoa exhibited a high percentage of motility. The percentage of hatched larvae in all the experimental groups, in which genetically inactivated dace eggs were fertilized with spermatozoa from the yellow form of ide, *Leuciscus idus* (L.), was much lower than in the control groups. All haploid larvae showed morphological abnormalities known as "haploid syndrome" that included stunted bodies and poorly formed retinas. The optimal doses of UV irradiation ranged from 3456 to 4608 J m^{-2}, as it was within this range that almost 100% haploid larvae were produced at a hatching rate of over 15%. Lower UV doses led to abnormal embryonic development.

Key words: UV TREATMENT, SPERMATOZOA, OOCYTES, GYNOGENESIS, ANDROGENESIS

INTRODUCTION

Chromosome set manipulation is used widely in aquaculture (Kucharczyk 2002). Artificial gynogenesis is a method of producing individuals from female gametes without a genetic contribution from the male gametes (Kucharczyk 1996). This genome manipulation is done in two steps: the first is the genetic inactivation of the spermatozoa, and the second is the application of an environmental shock. To produce viable gynogenetic diploids, the eggs are fertilized with genetically inactivated spermatozoa and then treated with an environmental shock shortly after egg activation to inhibit the second polar body release or suppress the first cleavage (Chourrout et al. 1980). Artifi-

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DOI 10.2478/s10086-008-0030-4

cial androgenesis is a method of producing individuals without the contribution of maternal chromosomes. In this genome manipulation, the female genome in the oocyte is inactivated using ionizing radiation (May et al. 1988, Lin and Dabrowski 1998). The duplication of the paternal chromosomes is done through an environmental shock to suppress the first cleavage in the genetically inactivated eggs (Masaoka et al. 1995, Kucharczyk et al. 1998). Androgenesis can been used to produce homozygous (inbred) lines and nucleo-cytoplasmatic hybrids between different species, in the study of sex determination, or to aid in the preservation of endangered species (Arai et al. 1992, Bongers et al. 1994).

The inactivation of the genetic material in spermatozoa and oocytes can be accomplished with ionizing radiation such as gamma or X-ray (Komen et al. 1988), but it is technically difficult, raises safety concerns (Arai et al. 1992), and leaves residual chromosome fragments in the sperm (Varadaraj 1990). UV irradiation is used routinely to inactivate the genomes of spermatozoa, which, in turn, can be used to induce gynogenesis in various fish species (Kaastrup and Hørlyck 1987, Komen et al. 1988,1991, Varadaraj 1990,1993, Malison et al. 1993, Linhart et al. 1995, Luczynski et al. 1997, Mims et al. 1997, Rothbard et al. 1997, Shelton et al. 1997). The induction of gynogenetic and androgenetic development is usually applied in the production of monosex populations or inbred lines (Mirza and Shelton 1988), in studies on genetics, molecular biology, immunology, or toxicology (Komen et al. 1991, Varadaraj 1993), as well as for gene resource conservation, especially in the restoration of endangered species, populations, and stocks (Flajshans 1998). Although dace, *Leuciscus leuciscus* (L.), is a species of only limited aquacultural and angling interest, it is very important as a biological component of environments. Since some wild populations have become extinct, different methods for restoring their genetic material had to be tested. The aim of the present study was to prepare genetically inactivated dace spermatozoa and oocytes using UV irradiation.

MATERIALS AND METHODS

BROOD STOCK AND COLLECTION OF GAMETES

Dace spawners were caught in the Marózka River (northern Poland), and samples of the yellow form of ide (golden orfe), *Leuciscus idus* (L.), were obtained from the

Oleśnica Fish Farm (southern Poland). The fish were transported to the hatchery and kept in 1000 dm^3 tanks at a controlled temperature (12-15°C) and photoperiod (14L:10D). Spawners received injections of Ovopel (Unic-Trade, Hungary) (Kucharczyk 2002). The ide spawners were induced to reproduce before their natural spawning season according to methods described by Targońska-Dietrich et al. (2004).

In each experiment, milt was collected from a few males. Sperm quality was expressed as the percentage of motile spermatozoa. Motility was estimated by microscopic (500X) observations of sperm activated with 0.5% NaCl. Samples with 70-80% (or more) motile spermatozoa were pooled and used for further treatments. For each experiment eggs were stripped from one female 30-36 hours after the last hormone injection.

GENETIC INACTIVATION OF SPERMATOZOA

Pooled milt was diluted in 0.85% NaCl at a ratio of 1:9, and then 2.5 ml of diluted sperm was spread in a 1 mm layer on Petri dishes, and placed on a shake table with a cycle of ~ 1 s for irradiation. A UV lamp (30 W, 6.4 W m^{-2}, Philips, Netherlands) was switched on for at least 30 min before the onset of irradiation. Also prior to irradiation, control samples of eggs (150-200 eggs per sample) were fertilized with a small volume (0.05 ml) of undiluted sperm (egg quality control group C), and with 0.5 ml of diluted, non-irradiated sperm (sperm irradiation control group D1). The quantity of the sperm used to fertilize the control group eggs depended on the spermatozoa concentration, so that in the control (undiluted sperm) and treated (diluted sperm) groups the same number of spermatozoa were 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 10 min, and the UV radiation dose ranged from 384 to 3840 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 group D2). The entire procedure was conducted in darkness to avoid the genetic photo-reactivation of the sperm (Kaastrup and Hørlyck 1987). The eggs were incubated in a laboratory recirculation system at 12°C, which is recognized as the optimal temperature for ide embryonic development. All control and experimental groups were in duplicate. After UV irradiation, the spermatozoa were activated by adding water and their motility was noted.

GENETIC INACTIVATION OF OOCYTES

During irradiation, the eggs were placed in Petri dishes in artificial ovarian fluid composed for common carp (Bongers et al. 1994). The dishes with eggs were placed on a shake table with a cycle of ~ 1 s, which permitted the the eggs to roll in the fluid. A UV lamp (30 W, 6.4 W m^{-2}) was switched on for at least 30 min before the onset of irradiation. Also prior to the irradiation of oocytes, control samples of eggs (150-200 eggs per sample) were fertilized with a small volume (0.05 ml) of sperm (egg quality control group C). Non-irradiated eggs treated in ovarian fluid were also fertilized using the same volume of milt (ovarian fluid quality control group D1). Experimental groups of eggs were fertilized with 0.05 ml of sperm following different exposure times to UV irradiation: 1, 3, 6, 8, 9, 10, 12, or 14 min (UV radiation dose ranged from 384 to 5376 J m^{-2}). After irradiation, the eggs from the additional control groups were fertilized (treatment in ovarian fluid control group D2). The entire procedure was conducted in darkness. The eggs were incubated in a laboratory recirculating system at 12°C. All control and experimental groups were in duplicate.

Egg survival was calculated as the percentage of hatched larvae and as the percentage of larvae that started swimming. The ploidy level of the larvae was determined by either observed haploid syndrome or by using color markers.

STATISTICAL ANALYSIS

Differences in the hatching success and survival of dace larvae were tested with analysis of variance (ANOVA) and the post hoc Duncan's multiple range test ($P < 0.05$).

RESULTS

The survival of larvae in all the experimental groups fertilized with genetically inactivated spermatozoa (Fig. 1) and oocytes (Fig. 2) was much lower than that in the control groups. These results contrasted with embryo survival to the eyed-egg stage in that there were no significant differences between treatment and control groups (ANOVA, $P > 0.05$). In the first experiment, the highest percentages of hatched haploid larvae were observed in the group in which the eggs were fertilized with sperm exposed to UV irradiation for 5 min at a dose of 1920 J m^{-2} (Fig. 1). Generally, the lowest hatching rate of haploid ide was noted when spermatozoa was irradiated for 2 (at a dose of 768 J m^{-2})

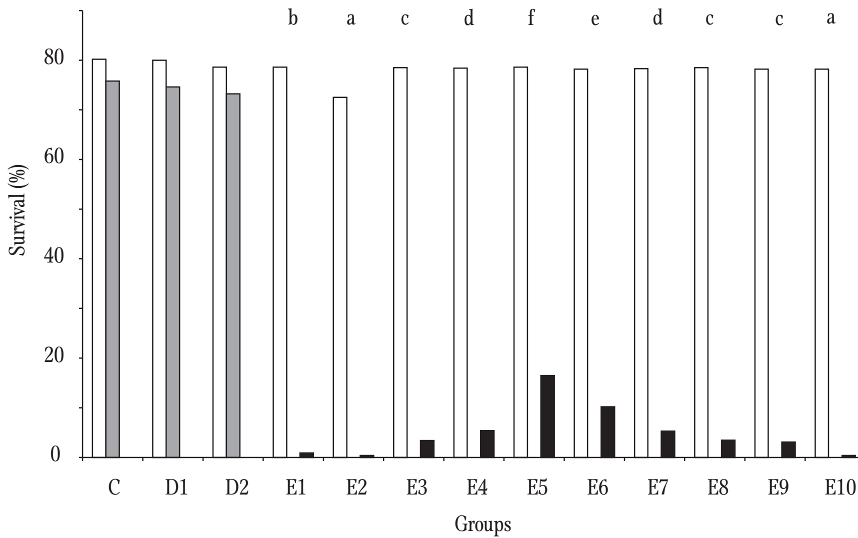

Fig. 1. Effect of UV irradiation duration on the genetic inactivation of dace, *Leuciscus leuciscus*, sperm expressed as the mean percentage of embryo survival to the eyed-egg stage (white column) and the mean percentage of hatched haploids (dark) and diploids (grey). Males had dark (wild) coloring and females had yellow coloring (orfe), respectively. C, D1 and D2 control groups and E1-E10 experimental groups. Data (hatched haploids) with the same letter index did not differ statistically.

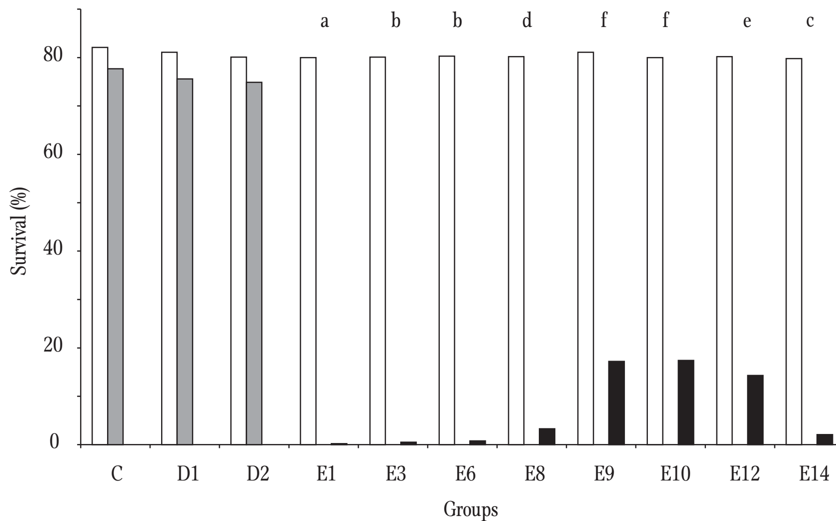

Fig. 2. Effect of UV irradiation duration on the genetic inactivation of dace, *Leuciscus leuciscus*, oocytes, expressed as the mean percentage of embryo survival to the eyed-egg stage (white column) and the mean percentage of hatched haploids (dark) and diploids (grey). Females had dark (wild) coloring and males had yellow coloring (orfe), respectively. C, D1 and D2 control groups and E1-E14 experimental groups. Data (hatched haploids) with the same letter index did not differ statistically.

and 10 (at a dose of 3840 J m^{-2}) min. All haploid larvae had morphological abnormalities that are referred to as "haploid syndrome" and include stunted bodies and poorly formed retinas. All of the hatched haploids and diploids in groups fertilized with spermatozoa irradiated from 3 (at a dose of 1152 J m^{-2}) to 10 (at a dose of 3840 J m^{-2}) min were yellow, whereas all fish from the control groups had wild (dark) coloring. The spermatozoa motility of diluted (irradiated and non-irradiated) sperm prior to, during, and after the experiments was high and oscillated between 60 and 70%.

The survival of larvae in all the experimental groups of genetically inactivated eggs fertilized with orfe sperm was much lower than in the control groups (Fig. 2). These results contrasted with embryo survival to the eyed-egg stage, in which there were no significant differences between the treated and control groups. The only exception was group 1 from the first experiment. All haploid larvae had morphological abnormalities referred to as "haploid syndrome" and included stunted bodies and poorly formed retinas. In the groups to which the lowest UV doses were applied (1, 3, and 6 min), dark diploid, aneuploid, as well as a few "blond" haploid larvae were noted. Many of the abnormal dark-colored larvae, which are morphologically similar to "typical" haploids, were probably aneuploids. In the groups of eggs exposed to UV for 9 min or more (at a dose of over 3400 J m^{-2}) all of the hatched haploids were yellow, whereas all the fish from the control groups had wild (dark) coloring.

DISCUSSION

The lower hatching rate of ide larvae from eggs fertilized with UV irradiated sperm in comparison to control groups is observed in many fish species. Data similar to that in the present work have been reported by other authors, including Stanley (1976) for grass carp, *Ctenopharyngodon idella* (Val.); Chourrout et al. (1980) for rainbow trout, *Oncorhynchus mykiss* (Walbaum); Komen et al. (1988) for common carp, *Cyprinus carpio* L.; Varadaraj (1993) for tilapia, *Oreochromis mossambicus* (Peters); Linhart et al. (1995) for tench, *Tinca tinca* (L.); Kucharczyk and Luczynski (1996) for common bream, *Abramis brama* (L.); and Luczynski et al. (1997) for northern pike, *Esox lucius* L.

In both the experiments described in the present work, the hatching rate of haploids was relatively high, especially in comparison to many other species, whose embryos usually died before or during hatching (Kaastrup and Hørlyck 1987, Komen et al. 1988,

Kucharczyk et al. 1996). Generally, the optimal doses of UV irradiation used to inactivate the genome material in dace was 1920 J m^{-2} (5 min of sperm irradiation) for spermatozoa and 3840 J m^{-2} (10 min of sperm irradiation) for oocytes. This was indicated by high embryo survival to the eyed-egg stage and the highest haploid larva hatching rate. Similar data for other cyprinids were reported by Komen et al. (1988) for carp, by Kucharczyk et al. (1996) for common bream, and by Kucharczyk (1999, 2001) for ide. The optimal UV doses for inactivating the spermatozoa of other fish species differed (i.e., Chourrout et al. 1980, Kaastrup and Hørlyck 1987), but these varied depending on fish species, type of sperm dilution, the dilution ratio, irradiation methods, etc.

The genetic inactivation of oocytes is much more difficult than that of spermatozoa. The high level of survival to the eyed-egg stage in the control (D1 and D2) and treated groups suggests that stirring is not harmful to the eggs. A similar observation was made by Bongers et al. (1994). The current results indicate that UV treatment inactivated the nuclear DNA in ide oocytes. Androgenetic origin (haploid or diploid larvae) was verified using the recessive color marker ("blond"). The optimal doses of UV irradiation were 3456-4608 J m^{-2} at which almost 100% of the haploid larvae were produced at a hatching rate of over 15%. These doses were higher than those recognized as optimum UV oocyte treatment for common carp (2500 J m^{-2}) by Bongers et al. (1994) and for northern pike (660-1320 J m^{-2}) by Lin and Dabrowski (1998), and were similar to those reported by Kucharczyk et al. (1998) for common bream (2700-3500 J m^{-2}). Viable UV doses for different species might result from differences in chorion structure, egg size and shape, as well as the position of the female pronucleus (Myers et al., 1995). Lower doses of UV irradiation (384 -1152 J m^{-2}) resulted in the development of abnormal embryos and of a few dark-colored diploids due to the incomplete inactivation of the maternal nuclear genome. Ionizing irradiation might generate chromosome fragments of maternal genome (Arai et al. 1992, Lin and Dabrowski, 1998) in androgenetic offspring, which might cause abnormal embryo development. This probably occurred when ide oocytes received too low an irradiation dose. UV treatment at doses of 2304 J m^{-2} and lower resulted in abnormal ("black") and androgenetic haploid ("blond") larval development. The current results indicate that it is possible to apply UV to inactivate dace gametes.

ACKNOWLEDGMENTS

This study was financed under the auspices of the project "Optimizing the production of stocking material of rheophilic cyprinid species under controlled conditions" of the Sector Operational Program "Fisheries and Fish Processing 2004-2006" (00040-61535-OR1400009/07).

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Received – 12 July 2008

Accepted – 26 September 2008

STRESZCZENIE

GENETYCZNA INAKTYWACJA GAMET JELCA *LEUCISCUS LEUCISCUS* (L.) PRZY UŻYCIU PROMIENIOWANIA UV

Przedstawiono wyniki inaktywacji materiału genetycznego przy zastosowaniu promieniowania UV gamet jelca *Leuciscus leuciscus* (L.). Inaktywację genomu plemników, po uprzednim rozcieńczeniu w 0,85% roztworze NaCl w stosunku 1:9, uzyskano po poddaniu nasienia dawce promieniowania w zakresie od 384 do 3840 J m^{-2}. Najbardziej efektywną dawką promieniowania, wyrażoną jako wysoki procent wyklutych haploidów, okazała się dawka 1920 J m^{-2}. Podanie nasienia promieniowaniu UV nie uszko-

dziło plemników: po naświetleniu nasienia aktywowane plemniki wykazały wysoki procent ruchliwości. Oocyty jelca również były poddane działaniu promieniowania UV w celu zniszczenia jądrowego materiału genetycznego. Odsetek wyklutych larw we wszystkich grupach doświadczalnych, gdzie użyto inaktywowanych jaj jelca, które następnie zapłodniono nasieniem żółtej formy jazia, *Leuciscus idus* (L.) był znacznie niższy niż w grupach kontrolnych. Wszystkie haploidalne embriony wykazywały cechy wad rozwojowych, które określono mianem „syndromu haploidalnego", np.: skarłowaciały pokrój ciała, słabo wykształcone gałki oczne. Za optymalną dawkę promieniowania UV uznano przedział wartości od 3456 do 4608 J m^{-2}, gdzie stwierdzono prawie 100% haploidalnych embrionów oraz ponad 15% wyklutych larw. Niższe wartości dawek promieniowania wpłynęły na nieprawidłowy rozwój embrionów.