# Gynogenesis in northern pike: UV-inactivation of spermatozoa and the heat shock inducing meiotic diploidization

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## ABSTRACT

Gynogenetic northern pike (*Esox lucius* L.) were produced using UV irradiated sperm and heat shock applied to inseminated eggs shortly after gamete activation. Milt was diluted in immobilizing solution (1:9) and UV irradiated (6.4 $W \cdot m^{-2}$) for 2-20 min, with dosage in the range of 768-7680 $J \cdot m^{-2}$. Genetic inactivation of spermatozoa was most efficient when milt was irradiated for 8 min (3072 $J \cdot m^{-2}$). Insemination of eggs with irradiated milt yielded 100% haploid larvae with hatching rate at 72.1±0.8% (mean±SD), expressed as a percentage of inseminated eggs. Haploid embryo developed and most of them hatched (showing "haploid syndrome") but all haploid larvae died within 48 hours after hatching. After insemination with irradiated sperm the eggs were exposed to a thermal shock of 34°C or 34.5°C, lasting 3 or 5 min, applied 11-16 min after gamete activation. The efficiency of heat shock and survival in experimental groups significantly depended on the source (individual female effect) and quality of eggs. The time of application and duration of the heat shock also affected the survival of embryo and percentage of gynogenetic larvae. Gynogenetic larvae were obtained in all experimental groups with hatching rate as high as 24.2±3.6%.

## INTRODUCTION

Northern pike (*Esox lucius* L.) is a key predator in lakes and is a valuable food and sport fish in Europe. Like many other fish species, northern pike exhibits sexually related dimorphic growth. Females grow faster, live longer and reach a larger size than males (Scott and Crossman 1973; Zalachowski 1965). Thus, sex control is desirable, especially for sport fisheries, and production of female monosex stock would be advantageous in creating trophy size fish.

All-female stock of fish can be obtained by the means of artificial gynogenesis, achieved by retention of the second polar body or by disruption of the first mitotic division of the eggs activated with genetically inert spermatozoa (Ihssen et al. 1990; Purdom 1983). Sex of gynogenetic fish may be reversed by using 17α-methyltestosterone or 11β-hydroxyandrostenedione in dry diet in salmonid (Donaldson and Hunter 1982; Feist et al. 1995) or cyprinid fish (Mirza and Shelton 1988). Thus, the possibility exists for producing monosex, all-female stocks of northern pike after crossing neomales with normal females, knowing that males are heterogametic (XY-type) in this species (Luczynski et al. 1997).

The aim of this study was to determine the parameters of UV irradiation for genetic inactivation of spermatozoa, and the parameters of heat shock that would enable reconstitution of diploidy in developing eggs and thus, obtain gynogenetic northern pike.

## MATERIALS AND METHODS

Three separate experiments were conducted. Eggs and milt were collected by stripping northern pike spawners

obtained from Szwaderki Hatchery (Olsztyn District, Poland) in April and May, 1995. Eggs were kept in plastic containers at 12.0-14.0°C for 1-2 hours until insemination. Milt was collected with 1 mL syringes and kept on crushed ice at 0.0-4.0°C. Sperm motility (in 120 mM NaCl activating solution) was examined using a microscope (at magnification 500x) and expressed as a percentage of motile spermatozoa (Babiak et al. 1995). Samples with less than 70% of motile spermatozoa were discarded.

### Genetic inactivation of spermatozoa (Experiment No. 1)

Eggs were obtained from one female and milt was stripped from eight males. Weight of the spawners was approximately 2-3 kg. Samples of milt (motility 80-90%) were pooled and eggs were divided into batches of approximately 900-1,000 eggs (6 mL) per treatment. Control group of eggs (K) was fertilized with 0.05 mL of intact sperm. Milt was diluted (1:9) with immobilizing solution (Tris 2.42 $g \cdot L^{-1}$, glycine 3.75 $g \cdot L^{-1}$, NaCl 5.52 $g \cdot L^{-1}$) with 2 $g \cdot L^{-1}$ KCl, preventing spermatozoa motility (Billard 1974; Billard et al. 1976). Immediately after sperm dilution the eggs of control group (D1) were fertilized with 0.50 mL of diluted milt. Samples of milt on Petri dishes (about 1 mm layer) were placed for UV irradiation on a rocking table with a cycle of about 1 s. The source of irradiation (Philips 30W germicidal UV tube, 253.7 nm, intensity of irradiation 6.4 $W \cdot m^{-2}$) was switched on 30 min before the onset of irradiation. The intensity and dose of irradiation were calculated by the method described by Goryczko et al. (1991). Separate samples of milt were UV irradiated for 2 to 20 min, at 2 min intervals. After sperm irradiation, experimental (for 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 min) and control (D2) groups of eggs were inseminated, each with 0.5 mL of diluted and irradiated (2-20) or only with diluted (D2) milt. Gametes were activated by addition of 14.0°C water. After water hardening of eggs each batch of eggs was further divided into three groups and incubated separately. From the onset of UV irradiation until the end of first mitotic division of eggs, the laboratory was illuminated only with a dim light to avoid sperm photoreactivation (Kaastrup and Horlyck 1987). The eggs were incubated at 12.5°C (±0.5°C) in small (approximately 100 mL) glass incubators with continuous water flow (Bardega and Luczynski 1990) until hatching. At the eyed egg stage the water temperature was raised and maintained at 14.0°C (±0.5°C) until hatching.

### Heat shock (Experiments No. 2 and 3)

In Experiment 2, eggs were taken from one female and milt from eight males. Samples of milt were pooled (80% motility) and eggs were divided into experimental groups of approximately 900-1,000 eggs. Control group of eggs (K) was fertilized with 0.05 mL of intact sperm, then the milt was diluted (1:9) and UV irradiated (8 min) as described above. Irradiated milt was pooled and control group of eggs (I; eggs not subjected to heat shock) and experimental groups of eggs were inseminated, each with 0.5 mL of diluted and irradiated milt. The gametes were kept in small plastic containers and were activated by the addition of 14.0°C water (time 0). Approximately 2.5 min after egg activation each group of eggs was transferred into a plastic sieve and immersed in 14.0°C water. Heat shock was accomplished by transferring sieves containing the eggs to the water baths with temperature controlled at 34.0°C or 34.5°C (±0.1°C). Eggs were subjected to high temperatures for 3 or 5 min, beginning 12, 14 or 16 min after their activation.

In Experiment 3, eggs were taken from two females (A and B) and milt was pooled from four males. Eggs from each female were divided into separate batches of approximately 900-1,000 eggs. Control groups of eggs from females A and B (K-A and K-B) were fertilized with 0.05 mL of intact sperm. Milt was diluted (1:9) and UV irradiated (8 min) as described above. Samples of irradiated milt were pooled. Control groups of eggs (I-A and I-B) (eggs not subjected to the heat shock) and experimental groups of eggs were inseminated, each with 0.5 mL of diluted and UV irradiated sperm. Gametes were activated with water at 14.0°C (time 0). Experimental groups of eggs were heat-shocked (at 34.5°C) for 3 min, beginning at 11, 12, 13, 14 or 15 min after activation.

In both experiments after the heat shock each batch of eggs was further divided into three groups and incubated separately. From the onset of UV irradiation until the end of first mitotic division of eggs, the laboratory was illuminated only with a dim light. The eggs were incubated at 14.0°C (±0.5°C) in small (approximately 100 mL) glass incubators, as described above, until hatching.

### Data analysis

The efficiency of UV irradiation of spermatozoa was evaluated based on the percentage of normally developed diploid and abnormally developed haploid larvae. Ploidy of fish was determined on the basis of morphological observations that were earlier validated with ploidy determination using flow cytometry (Lin et al. 2001). All normally developed northern pike larvae were considered as diploids. Haploids in esocids (Lin and Dabrowski 1996) showed typical "haploid syndrome", recognizable as microcephalia, shortened and twisted body, similar as it was observed in other species of fish (Kucharczyk et al. 1996; Onozato 1984; Purdom 1969). The effect of heat shock treatment on the ploidy level and survival was examined based on hatching percentage of gynogenetic diploid and haploid larvae. Statistical differences between groups were analysed with Duncan's multiple range test and pair T-test ($P<0.05$).

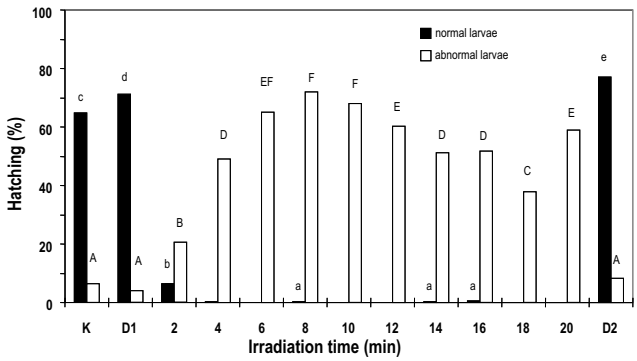


**Figure 1. The effect of the UV-irradiation time (min) on hatching percentage of eggs inseminated with irradiated spermatozoa. The columns with the same letters are not significantly different at *P*=0.05; statistics were carried out separately for normal (diploid) and abnormal (haploid) larvae – small letters mark columns showing percentage of diploid larvae hatching both in control and in experimental groups, whereas capital letters mark columns referring to haploid larvae, respectively. K – control eggs fertilized with intact sperm; D1 – control eggs fertilized with diluted sperm immediately after sperm dilution; D2 – control eggs fertilized with diluted sperm approximately 20 min after sperm dilution.**

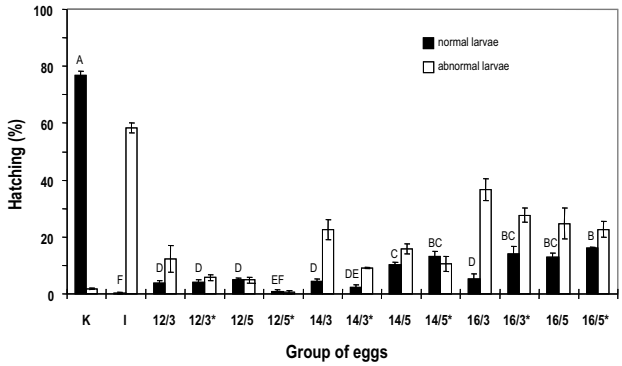


**Figure 2. The effect of a heat shock of eggs at 34.0°C and 34.5°C on the hatching percentage of normal (diploid) and abnormal (haploid) larvae. The columns with the same letters are not significantly different at *P*=0.05. Description of experimental groups of eggs: "12/3" means "12" – time of initiation of shock (min after egg activation), and "3" – duration of heat shock (min); * – eggs heat – shocked at 34.5°C.**

## RESULTS

### Genetic inactivation of spermatozoa

Hatching rate of eggs fertilized with intact sperm (control group K) was 64.8±1.0% (Figure 1). Hatching percentage was higher (72.1±4.9%) in control group fertilized immediately after sperm dilution (D1), and keeping diluted sperm for approximately 20 min (control group D2) resulted in 77.1% (±5.1%) of hatched larvae (Figure 1). Insemination of eggs with milt irradiated for 2 min (group 2) resulted in 20.5±2,0% of haploid and 6.4±3.6% of diploid larvae, whereas irradiation lasting 6 min (irradiation dose 2304 $J \cdot m^{-2}$) resulted in only abnormal embryonic development (Figure 1). The highest rate of haploid larvae (72.1±0.8%) was observed in a group inseminated with milt irradiated for 8 min (irradiation dose 3072 $J \cdot m^{-2}$).

### Heat shock

Heat shock of 34.0°C or 34.5°C (Experiment 2), applied 12, 14 or 16 min after gametes activation, lasting 3 or 5 min resulted in the retention of the second polar body and in obtaining of gynogenetic larvae in all experimental groups (Figure 2). Heat shock at 34.5°C, applied 16 min after gametes activation and lasting 5 min was most efficient and resulted in 16.2±0.3% of gynogenetic larvae (Figure 2). Heat shock at 34.5°C applied at the same time and lasting for 3 min, as well as the shock at 34.0°C lasting 5 min were also effective.

UV sperm irradiation decreased hatching percentage by approximately 20% from control group K. Also, heat shock affected egg survival in all experimental groups.

Heat shock at 34.5°C applied 11, 12, 13, 14 or 15 min after activation (Experiment 3), lasting 3 min, produced gynogenetic larvae in all experimental groups. However, for the eggs from two females the results for corresponding treatments (time) were significantly different (Figure 3). In case of the first female (A), treatment applied 12 min after activation was most efficient and resulted in 8.8±1.7% of gynogenetic larvae. Groups subjected to a heat shock at 11, 13, 14 or 15 min of development showed significantly lower percentage of gynogenetic larvae. In the second female (B), 24.2±3.6% of gynogenetic larvae were obtained after heat shock application at 11 min of development. Shock applied at 12, 13 or 14 min was also effective but heat shock applied at 15 min after egg activation was significantly less effective.

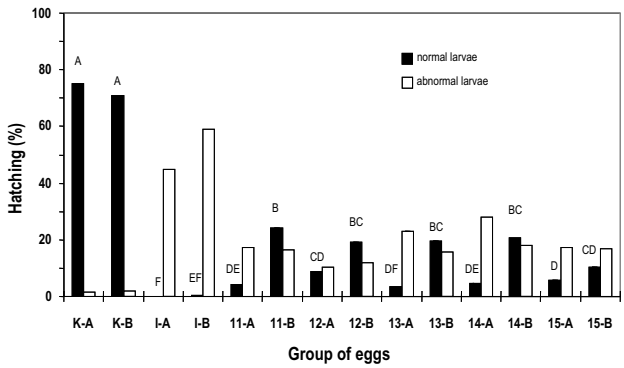


**Figure 3. The effect of thermal treatment (34.5°C) of eggs obtained from two females (A and B) at different time after gametes activation on the hatching percentage of normal and abnormal larvae. The columns with the same letters are not significantly different at *P*=0.05.**

The eggs obtained from both females were of good quality as their hatching rate in control groups K-A and K-B reached 77.0±15.0% and 72.6±3.1%, respectively. Dilution and UV irradiation of milt resulted in a decrease of hatching percentage by approximately 30% and 13% of eggs from females A and B, respectively. Also, heat shock affected survival in experimental groups, more evidently in groups of eggs collected from female B than from female A, as compared to control groups I-A and I-B (Figure 3).

## DISCUSSION

The optimum parameters of UV inactivation of sperm was irradiation lasting 8 min (irradiation dose 3072 $J \cdot m^{-2}$), which resulted in obtaining of 72.0±0.8% (n=3) of haploid larvae. At this dose genetic material of sperm was most likely damaged but spermatozoa retained their ability to activate egg development. In the Experiment No. 1 the process of spermatozoa inactivation (dilution + irradiation) did not affect survival of the embryos. However, in the Experiments No. 2 and No. 3 hatching percentage in groups inseminated with irradiated milt was lower than in control groups of eggs. The optimum dose of UV irradiation causing DNA inactivation of muskellunge (*Esox masquinongy*) spermatozoa was lower, ranging from 1440 to 2160 $J \cdot m^{-2}$ (Lin and Dabrowski 1996).

There was a clear effect of duration of UV irradiation on fertilizing ability of sperm (Figure 1), similarly to what was observed in other species of fish, such as rainbow trout (*Oncorhynchus mykiss*), masu salmon (*Oncorhynchus masou*) or bream (*Abramis brama*) (Chourrout 1982; Kucharczyk et al. 1996; Onozato and Yamaha 1983). Exposure time longer than 8 min resulted in a decreased hatching percentage in groups of eggs inseminated with milt irradiated for 10-18 min (Figure 1). However, we cannot explain the relatively high hatching percentage in a group inseminated with milt irradiated for 20 min. Location of the Petri dish under the UV lamp did not influence the hatching percentage of inseminated eggs when all batches were irradiated for 8 min (our unpublished data).

There were a few normally developed diploid larvae (0.24-0.53% of hatched embryos) present in some groups of eggs inseminated with UV inactivated milt and not subjected to heat shock (Figure 1, 2 and 3). Such a phenomenon was also recorded by other authors (Goryczko et al. 1991; Levanduski et al. 1990) and it was attributed rather to spontaneous gynogenesis than to inaccurate sperm irradiation. Dilution or storing the sperm in suspension for more than 20 min did not affect the hatching percentage in control groups, D1 and D2, respectively (Figure 1), contrary to what has been observed in muskellunge milt (Lin and Dabrowski 1996). Cytogenetic examinations confirmed that all normally developed northern pike larvae were diploids, whereas all abnormally developed specimens were haploids (Kucharczyk et al. 1999). Thus, it is possible to identify gynogenetic diploids and haploids based on their morphology, similarly as it was commonly observed in other species of fish. Most of haploid northern pike embryos hatched, but all larvae died within 48 hours after hatching. Haploid embryos could be morphologically identified starting from the eyed-embryo stage, based on their pigmentation and size and shape of the embryo. Hatched haploids showed typical "haploid syndrome", recognizable as microcephalia and shortened and twisted body. Similar haploids were identified in muskellunge by Lin and Dabrowski (1996).

Heat shock applied soon after egg insemination induced gynogenetic development in northern pike, similarly as in other fish species (Ihssen et al. 1990). Gynogenetic larvae were obtained in all experimental groups submitted to a heat shock of 34.0°C or 34.5°C applied 11-16 min after activation for 3 or 5 min. The survival in experimental groups and percentage of gynogenetic larvae in different experiments significantly depended on the source (maternal effect) and quality of eggs, similarly as it was observed in other species of fish (Diaz et al. 1993; Levanduski et al. 1990). Thermal shocks applied soon after egg activation were effective in restoring diploidy or inducing triploidy of esocid fish. Lin and Dabrowski (1996) obtained gynogenetic muskellunge larvae after application of relatively low-intensity heat shock at a broad range of parameters (temperature 28.0-30.0°C, starting 5-30 min after activation, lasting 5-30 min), with the best results obtained while using a heat shock of 30.0°C for 8-10 min, starting from 20 min post-fertilization. Relatively high-intensity heat shocks of 34.0°C for 3 or 5 min at 4-14 min after activation also resulted in high percentage of gynogenetic larvae (Luczynski et al. 1997). Moderate thermal shocks of 27.0-32.0°C, lasting for 5-25 min, beginning 10-15 min after egg activation, resulted in induction of triploidy in northern pike, producing up to 50% triploids when the shock of 28.0°C, lasting 10 min, was applied 10 min after activation (Luczynski and Woznicki 1995).

The time of application and duration of the heat shock affected the survival of embryo and percentage of gynogenetic larvae in heat-shocked groups of eggs, similarly to muskellunge (Lin and Dabrowski 1996). The survival to hatching and percentage of gynogenetic specimens in the Experiment No. 2 were significantly higher in groups heat-shocked at 14 or 16 min than at 12 min post-fertilization (Figure 2). Longer treatments, lasting 5 min, were more efficient for both tested temperatures. The optimum moment of shock application was also different for eggs from different females (Figure 3).

The temperature of heat shock affected the embryo survival and yield of gynogenetic larvae. Higher percentage of gynogenetic larvae was obtained in higher temperature when treatment was applied 14 min (for 5 min) or 16 min (for 3 or 5 min) after egg activation. However, a statistically significant difference was observed only for treatment applied at 16 min of development for 3 min (Figure 2). Generally, higher survival (haploid + diploid larvae) was observed in groups where higher percentage of gynogenetic larvae were obtained.

In the Experiment No. 2 the pair T-test was used to examine separately the effect of duration (3 min against 5 min) and temperature (34.0 against 34.5°C) of the heat shock in the group of eggs heat-shocked at 12, 14 or 16 min after gamete activation. When the eggs were submitted to a heat shock at 12 min of

development, lower percentage of gynogenetic larvae were obtained comparing to the groups of eggs heat-shocked at 14 or 16 min. In those "12 min" groups increase of shock intensity (i.e. application of longer-lasting or higher temperature treatment) resulted in lowered hatching percentage of gynogenetic specimen. It was observed when the eggs shocked for 5 min were kept in water at higher temperature (34.5 against 34.0°C), or eggs kept at 34.5°C were shocked for a longer period (5 min against 3 min) ($P<0.05$).

When eggs were submitted to a heat shock at 14 min after gamete activation a significant increase of its efficiency was observed, for both temperature tested, when the duration of shock was longer (5 min against 3 min) ($P<0.05$). Differences between groups of eggs heat-shocked for 3 min were not significant when the temperature was 34.0 or 34.5°C. The same was observed in groups of eggs shocked for 5 min. Thus, significant differences ($P<0.05$) were observed when higher differences of shock intensity appeared (2 min of longer shock duration against 0.5°C of higher shock temperature).

In the experimental groups of the eggs heat-shocked at 16 min after gamete activation the increase of shock intensity resulted in the increase of hatching percentage in all analysed cases ($P<0.05$). The most significant differences were observed between groups of eggs shocked for 3 min at 34.5°C against 34.0°C, and between groups shocked at 34.5°C for 5 min against 3 min.

Activation of eggs with UV irradiated sperm and subjecting inseminated eggs to the heat shock enabled obtaining of relatively high percentage of gynogenetic northern pike larvae. This creates possibility for hormonal reversal of sex in gynogenetic individuals resulting in obtaining homogametic XX-type males in order to produce monosex, all-female stocks.

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