

Sperm Cryopreservation of Two European Predator Fish Species, the Pikeperch (*Sander lucioperca*) and the Wels Catfish (*Silurus glanis*)

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1. Introduction

Wels catfish (*Silurus glanis*) and pikeperch (*Sander lucioperca*) are two predator fish species cultivated in the traditional Central European pond aquaculture. Their role in the pond ecosystem is the control of the populations of smaller wild fish that enter ponds during their flooding in the Spring and would represent food competition for the cultured cyprinids. In addition, both species are highly priced for their excellent boneless meat, therefore, attempts are made to improve their culture and enhance yields (Horváth et al., 2002).

The process of induced propagation of wels catfish is based on a long-standing technology. However, certain problems can still appear: the method of collecting male gametes is still based on the removal of testes. By the application of this method a particular male can only be used once for propagation. Moreover, differentiation of sexes requires a great deal of experience and the danger of using an immature female with a less developed body structure is still present. As a result of listed problems the success of propagation becomes questionable.

In recent years successful experiments have been carried out in the pikeperch in many farms and research centers for the development of induced propagation in hatcheries. Synchronization of maturation of female individuals is not perfectly developed yet, thus, successful striping requires a constant attention and control. That is why minimizing the presence of males and securing sperm in a most simple way could focus attention on females.

Application of cryopreserved male gametes for fish propagation in hatcheries can serve as a solution for all mentioned difficulties and risks. History of fish sperm cryopreservation dates back to the beginning of 1950s, since then the sperm of more than 200 fish species has been cryopreserved successfully all over the world (Rana, 1995). In spite of this the application of cryopreserved fish sperm is still not very common in aquaculture in contrast to e.g. the dairy cattle sector. Most studies done on cryopreservation of fish sperm put the emphasis on optimization of the process, on cryopreservation of a small amounts of male gametes, thus, rendering this technology to the level of laboratory experiments without any basic output for farmers.

By the help of a successful cryopreservation method applicable in fish farms not only the reduction of propagation risks would become possible but also a long-term storage of gametes of substantial breeders as already applied in case of carp. Development of a sperm bank already employed at cattle breeding would also become feasible thus increasing the role and rate of rarely used selection methods of animal breeding in fisheries.

The objective of our work was the development of working protocols for the cryopreservation of wels catfish and pikeperch sperm that can be applied to aquaculture of these species. In both species, this development required a thorough knowledge of culture conditions of the given species, studies of the cryoresistance of sperm and adaptation of cryopreservation methods to hatchery practices.

2. Materials and methods

Experiments on the cryopreservation of sperm of both species were carried out during the years 2005-2008 at various locations in Hungary. Details of experiments including their dates and locations are provided in the descriptions of experiments on each species.

2.1 Experiments on wels catfish sperm

2.1.1 Methods of collection and cryopreservation of male gametes

Gametes applied in the experiments were invariably retrieved directly from the testis removed from the abdomen after the decapitation of male wels catfish (not stripped). After removing it from the abdomen the testis was cut up and squeezed out through a dry gauze into a Petri-dish. After extraction of sperm motility of gametes was examined through a light microscope at 200× magnification.

Ten per cent methanol was used as a cryoprotectant and 6% fructose as a diluent. pH of the cryopreservation medium was adjusted to 7.73 by the help of 1 M NaHCO₃ solution. From the diluted sperm treated this way 4 ml was pipetted into a 5-ml straw.

In the process of cryopreservation liquid nitrogen was poured into a polystyrene box on the top of which a polystyrene frame was placed with a height of 3 cm and the straws were laid on it. Samples were stored in a canister storage dewar until use.

Straws were thawed in 40 °C of water for 40 seconds. After thawing the closed ends of straws were cut up and their content was poured into a test-tube or directly onto the eggs used for propagation. Motility of thawed sperm was examined according to the method already described. The method mentioned here was compiled on the basis of former experiments of the cryopreservation group of the Department of Aquaculture on African catfish (Urbányi et al., 1999; Horváth & Urbányi, 2000).

2.1.2 Experiments on cooling time and sperm-egg ratio

Male gametes applied for cryopreservation in 2005 were collected from the farm in Tuka of Szarvas-Fish Kft. and the farm in Szeged of Szegedfish Kft. by joining to their propagation processes. Males were injected with 4 mg/body weight kg carp pituitary in one dose by the assistants of the farm before the extraction of sperm. Length and weight of the body and weight of the testis were measured. Gonadosomatic indices (GSI) were determined from the

ratio of testis and body weight. After the squeezing of sperm the motility of gametes was defined as described above. After the collection of gametes the former described cryopreservation method was used with the addition that in case of samples collected in Tuka the effect of cooling time on motility and fertilizing capacity was also tested. Cooling time of samples varied between 3, 5 and 7 minutes. After the cooling period straws were placed into liquid nitrogen.

First propagation tests were completed in the Szajol farm of Fish-Coop Kft. Eggs were gained from fish by a routine propagation process. In the first experiments eggs were divided into 40, 80 and 120 g doses and each dose was propagated with 1 straw of cryopreserved sperm. Fertilized eggs were then incubated in 7 l Zug-jars. Hatching rate was counted after hatching.

The second experimental procedure was performed in the Attala fish farm of Attala Hal Kft., when eggs were divided up to two 150 g doses and one of them was fertilized with one, while the other one with two straws of sperm. In both cases freezing time was 7 minutes. At hatching the ratio of hatching and deformed larvae was determined.

In the experimental procedure the cooling rate was measured, too. A straw was filled with cryopreservation medium. The K type sensor of a Digi-Sense DualLogR digital thermometer (Eutech Instruments, Singapore) was placed into the straw which was then laid onto a 3 cm high polystyrene frame floating on the surface of liquid nitrogen. The thermometer recorded temperature data with 1 second intervals. Temperature data were collected for 6 minutes since storing capacity of the memory of the thermometer allowed the recording of this amount of data.

2.1.3 Cryopreservation and analysis of sperm collected outside of the spawning season in hatchery conditions

In 2006 wels catfish sperm was collected in January and March (aside from the spawning season) in the Köröm farm of Aqua-culture Kft. (Köröm Fish Farm, Local Government of Bócs at present) from wels catfish kept in a flow-through intensive system. They were kept in tanks in a constant water temperature of 20 °C. The method applied for cryopreservation was the same as already mentioned with the difference that sperm was frozen for 7 minutes on the polystyrene frame before placing it into liquid nitrogen. Motility of sperm was examined both in fresh and cryopreserved samples.

Propagation experiments were performed at the Szajol farm of Fish-Coop Kft. For this, cryopreserved samples originating from Szeged, 2005 and Köröm, 2006 were used. Eggs were collected from female fish by a routine propagation process. In the experimental procedure eggs were distributed into 250 and 350 g dosages and fertilized with one straw of sperm. Fertilized egg doses were then incubated in 7 l Zug-jars and hatching rates were determined after hatching.

2.1.4 Application in hatcheries

The aim of these experiments was to fertilize significant amounts of eggs (150-300 g) with large doses of cryopreserved sperm (5-ml straws) all over the country by joining to propagation work of a given farm. Reliability and repeatability of the method were also

examined. In each case one dose of eggs was fertilized with the content of one straw. In the frames of a routine propagation work fertilization with cryopreserved sperm was propagated in five different farms:

- in Attala pond farm of Attala Fish Production and Trading Kft.
- in Köröm fish farm of the Local Government of Bócs
- in Százhalombatta farm of TEHAG Kft.
- in Ördögös farm of Aranykárász Co.
- in Szeged farm of Szegedfish Kft.

In the Attala experiment in 15 May 2007 cryopreserved sperm collected outside of the spawning season was used for the fertilization of 200 g egg doses.

The experiment in Köröm was carried out in 17 May 2007 in which cryopreserved sperm was applied for fertilization also collected off season. Egg doses of 200 g were fertilized both for treated and for control groups.

Following that, egg doses of 200 g were fertilized in the Százhalombatta farm of TEHAG Kft. in 22 May 2007. This time cryopreserved sperm from Szeged was used in the experiments collected in 2006 in the spawning season.

In the fourth experiment in 21 May 2007 egg doses of 200-350 g were fertilized in the Ördögös farm of Aranykárász Co with cryopreserved sperm collected off season.

The last experiment was performed in the hatchery of Szegedfish Kft. in 23 May 2007. In the research work egg doses of 150 g were fertilized with cryopreserved sperm deriving from Szeged.

In all cases our team personally joined to the propagation work of the farm, thus, we ensured that an adequate amount of cryopreserved sperm was used for the egg doses. Process of propagation was always performed according to the practice of the certain farm. One dose of eggs was fertilized with one straw of thawed sperm. In all experiments hatching percentage of larvae was determined and in the experiment in Attala fertilization rate at 4-8 cell stage was also examined.

2.1.5 Experiments on larval survival

For the analysis of larval survival, growth and survival of feeding larvae in 2007 and non-feeding wels catfish larvae in 2008 were examined.

Analysis of feeding larval stage

When examining feeding larval stage in farm conditions, larvae were produced in Százhalombatta farm of TEHAG Kft. by applying local propagation methods. During fertilization process 1 straw of sperm was added to 200 g of eggs. After fertilization the 200 g doses of eggs were placed into 7 l Zug-jars. For fertilization of individuals in the control group native sperm from males of the farm was used. On the second day after fertilization hatched larvae were counted then after hatching the non-feeding larvae were placed into larva-tanks. On the third day after hatching when larvae started their exogenous feeding the ones devoted for the experiment were counted and placed into troughs. 1000 individuals were placed into a 100 l trough with flow-through water in 3 replicates. The stock was fed

with chopped tubifex in every 3 hours. To prevent infections 36% formalin treatment was applied in a concentration of 10 ml/trough in every 4 hours. In each trough velocity of water flow-through was 3 l/minute. Rearing in the trough lasted for 10 days according to the routine practice of the farm.

Laboratory experiments were performed in the Department of Aquaculture. Troughs of the recirculation system applied in the experiment were 40 cm long, 15 cm deep and 10 cm wide (though water depth was 10 cm due to the outlet/stub). Due to photophobia of wels catfish larvae the system was located in a dark room. In the experiment 5×100 individuals (larvae hatched after the application of control and cryopreserved sperm) took part in the treatments. In the first 4 days fish were fed once in every three hours. At the morning and evening feeding they were fed with plankton while at other feeding times experimental fish were fed with Perla Proactiv 4.0 fish diet. Following this 4-day period fish were fed with the above mentioned diet (sometimes also with plankton or Artemia) 4 times a day. Velocity of water flow-through in the troughs was 0.25 l/minute.

In addition to body length and weight condition factor, specific growth rate (S.G.R.) and survival rate (%) was measured.

Examination of non-feeding larval stage

In this developmental stage no farm study was done due to the fact that the trough system was not suitable for the accommodation of such small larvae. Moreover, the hatchery protocol for fry rearing does not use the method of rearing in troughs at this age.

Larvae examined at non-feeding life stage were produced in June 2008 in the hatchery of TEHAG Kft. The method of propagation and utilization of cryopreserved sperm was the same as described at the examination of feeding larval stage. After hatching larvae were transported to the laboratory of the Department of Aquaculture in Gödöllő where the survival of fish was tested using the rearing system built in the previous year. The experiment lasted for 4 days.

2.1.6 Experiments on larval survival

Main effects of cooling time and the quantity of eggs on hatching rate was examined in the Szajol experiment (Part 2.1.2) by the help of two-way analysis of variance ($P < 0.05$). In case of testis collected off season at two sampling times as described in Chapter 2.1.3 GSI values were compared by the help of a two-sample t-test. Hatching results gained from fertilization experiments with cryopreserved and control sperm described in Chapter 2.1.4 were analysed with a t-test, too. These statistical tests were performed using the software GraphPad Prism 4.0 for Windows. In the examinations of larval rearing results of survival were compared with Chi²-test (Kruskal Wallis test), while body length, body weight, condition factors and SGR values with t-tests (Chapter 2.1.5) using the software SPSS 13 for Windows.

2.2 Experiments on pikeperch sperm

2.2.1 Origin of fish

Experiments on pikeperch were conducted two times and in two places: the first place was the Keszthely-Tanyakereszt fish laboratory of Georgikon Faculty of Agriculture at Pannon University and the second one was the Attala hatchery of Attala Hal Kft.

2.2.2 First experiment

The stock of pikeperch taking part in the first experiment originated from Aranypony Kft. (Sáregres-Rétimajor) (8 females and 10 males: 1424-1870 g). Males were anesthetized by clove oil then sperm was manually stripped and collected with an automatic pipette. Care was taken not to contaminate the gametes with urine or feces.

Motility of fresh sperm was estimated after activating it with water. Motility was examined on slides in a 20× dilution at 200× magnification by the help of a Zeiss Laboval 4 microscope (Carl Zeiss, Jena, GDR). Density of sperm was examined by the Bürker-chamber method in a 1000× dilution.

The following diluents were prepared:

- Glucose diluent (350 mM glucose, 30 mM Tris, pH 8,0)
- KCl diluent (200 mM KCl, 30 mM Tris, pH 8,0)
- Sacharose diluent (300 mM sacharose, 30 mM Tris, pH 8,0)

Methanol and dimethyl-sulfoxide (DMSO) were used as cryoprotectants in a concentration of 10%. All chemicals used in the experiments were purchased from Reanal Zrt. (Budapest, Hungary).

Sperm (200 µl) mixed with a cryopreservation medium (200 µl cryoprotectant, 1600 µl diluent) in a ratio of 1:9 (Horváth et al., 2003; Urbányi et al., 2006) was pipetted into individually marked 0.5-ml straws after 3 minutes of equilibration time. Samples were cryopreserved in a polystyrene box filled with liquid nitrogen. A 3 cm high polystyrene frame was placed onto the top of the nitrogen then straws were laid on this frame where temperature was around -165°C. The time of cryopreservation was 3 minutes. After the freezing process straws were placed into liquid nitrogen and stored there until being used. Thawing was carried out in a 40°C water bath for 13 seconds (Horváth et al., 2003; Horváth et al., 2005). After thawing sperm motility was examined with the same method as described at fresh sperm.

Eggs were distributed to Petri-dishes with a diameter of 5 cm with 200-350 eggs/dose. Fertilization was made with thawed sperm of a half straw (250 µl) right after taking the straw out of the water. Sperm was poured on the egg doses then the gametes were activated with 1 ml of water. Next eggs were allowed to stick to the bottom of the Petri-dish by taking care of the eggs being located in one layer. Fertilization rate was counted at neurula stage.

2.2.3 Second experiment

The second experiment was performed in line with pikeperch propagation in a hatchery. In this research sperm of 4 males and eggs of 1 female were applied. In this case only glucose was used as a diluent with 10% concentration of methanol and DMSO cryoprotectants. Sperm was diluted in a 1:1 and 1:9 ratios. The process of cryopreservation and thawing was the same as the process applied in the first experiment.

Eggs were divided into 10 g doses (about 10 000 eggs according to my counts) and taken into plastic bowls. Each dose was fertilized by a thawed straw of samples (0.5 ml) then these doses were placed into 7 l Zug-jars until hatching. Finally hatched larvae were counted and hatching rate was defined.

2.2.4 Application of cryopreserved sperm to hatchery conditions (preliminary experiment)

This research was done in April 2007 in the hatchery of Attala Hal Kft. Gametes used in the experiment originated from the same farm. Male and female fish were treated by the method applied in the farm in line with local hatchery propagation. In the previous experiment it was difficult to avoid mixing of sperm with urine so in this case stripping was performed by a silicon catheter (inside diameter: 1 mm, outside diameter: 1.5 mm) which was introduced into the sperm duct. Motility estimations were made by the method described earlier. Sperm concentration was examined in a Bürker-chamber in a 1000× dilution.

Sperm originating from 3 males were used in the experiment. It was diluted in a 1:1 ratio with the following composition of cryopreservation medium: 350 mM glucose, 30 mM Tris, pH 8.0 (titrated with cHCl), methanol with a concentration of 10%. Diluted gametes were pipetted into 0.5-ml straws. Cryopreservation and thawing methods arranged to the method described in the previous chapter. Sperm was stored for 1 week in liquid nitrogen in a canister. After thawing the motility of samples was also examined.

Stripped eggs were divided into 10 and 30 g doses in 3 replicates and into a 50 g dose in one replicate. One dose of eggs was fertilized with one straw. Fresh sperm was used as a control. Each dose was poured into a 7 l Zug-jar for incubation. Hatching rate was counted after hatching.

2.2.5 Applied statistical methods

Results of experiments were evaluated with Graphpad Prism 4.0 for Windows program. Effect of cryoprotectants and diluents on motility and fertilization and the effect of diluent ratio and cryoprotectants on hatching ratio was examined by a two-way analysis of variance (ANOVA) (Chapter 2.2.2).

Results gained from the second experiment (Chapter 2.2.3), namely motility (cryopreserved and fresh sperm) and hatching results (in case of 10 and 30 g egg doses) were analysed by the help of a two-sample t-test ($P \leq 0.05$).

3. Results

3.1 Experiments on wels catfish sperm

The GSI (gonadosomatic index) of male individuals from Tuka was $2 \pm 4\%$. The motility of sperm before cryopreservation was 90%, while after thawing this rate was 0% in case of 3 minutes, 40% in case of 5 minutes and 70% in case of 7 minutes long freezing time. The motility of sperm from Szeged was 80%.

In the experiments carried out in Szajol the highest hatching rate ($51 \pm 1\%$) was observed at 7 minutes freezing time and 40 g of dose of eggs, although in the case of 5 and 7 minutes of freezing time a very similar hatching rate (between $40 \pm 0\%$ and $51 \pm 1\%$) was observed (Figure 1.). Only the cooling time had a significant main effect ($P < 0.0001$) on the results, considering that 3 minutes long cooling time gave lower hatching rate.

Hatching rate of propagated eggs was 94% in the case of fertilization with a single straw in Attala, while fertilization with two straws resulted 77% hatching rate. The control results

were 89% and 81%. It is worth to mention that the ratio of deformed larvae hatched from eggs fertilized with a single straw was only 2.4% (1.8% in control), while in the case of fertilization with two straws it was 11.2% (7.3% in control).

The cooling rate of a straw was approximately $-23^{\circ}\text{C}/\text{minute}$ (Figure 2.). It was observed that the temperature of the straw was only -45°C after 3 minutes while after 5 minutes it was -104°C .

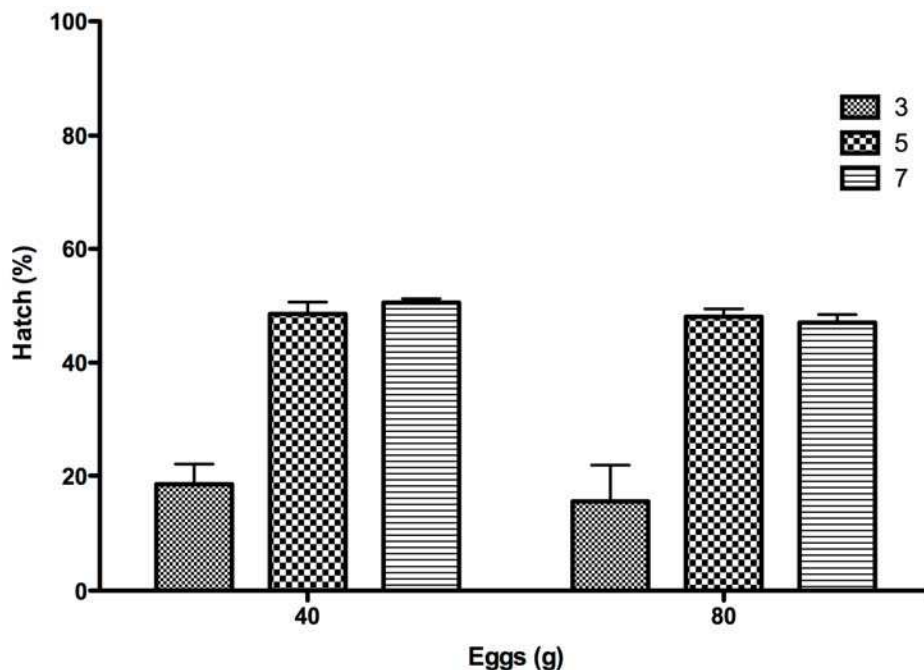


Fig. 1. Hatch rates of wels catfish eggs fertilized with cryopreserved sperm. Each batch of eggs (40 or 80 g) was fertilized with one 5-ml straw of cryopreserved sperm. Columns indicate cooling times employed during cryopreservation (3, 5 or 7 minutes). Data are presented as Mean \pm SD (N = 3).

When sperm was collected outside of the spawning season, the average weight of the testes of wels catfish from Köröm was 20.4 g and the average weight of the fish was 2.52 kg, thus GSI rate of them was lower than 1% except one male. This low GSI rate have not had adverse effects on the quality of sperm. No significant difference was observed between GSI rate in January and in March ($P = 0.4589$). The motility of fresh sperm varied between 50% and 90%. Two of the sperm samples selected for cryopreservation were excluded from further examinations because the motility of these samples was the lowest (50 - 60%). This low rate was caused likely by injuring the cells during squeezing of the testes. Some of the cryopreserved samples were thawed 5 days after freezing and their motility was about 50%.

Sperm frozen in 2005, in Szeged and 2006, in Köröm were used for the propagation experiments. Hatching rate varied between 70 - 80%, except for one sample with 20% of

hatching rate. However, according to the head of the farm the fertilization of control group was as bad as the result of the 20% hatching rate. On the basis of the results it was observed that sperm from Köröm (out of spawning season) had similar hatching rate to the sperm from Szeged.

Results of different hatching experiments depended on the propagation method and on the quality of sperm. Fertilization with cryopreserved sperm from Attala resulted $97 \pm 1\%$ of fertilization rate while control fertilization rate was $93 \pm 1\%$. There was no significant difference between the two rates ($P = 0.0084$). In the same experiment the hatching rate of the larvae was $95 \pm 2\%$ while in the control group this rate was $94 \pm 6\%$. There was no statistically significant difference between the hatching rate of larvae originating from cryopreserved or fresh sperm.

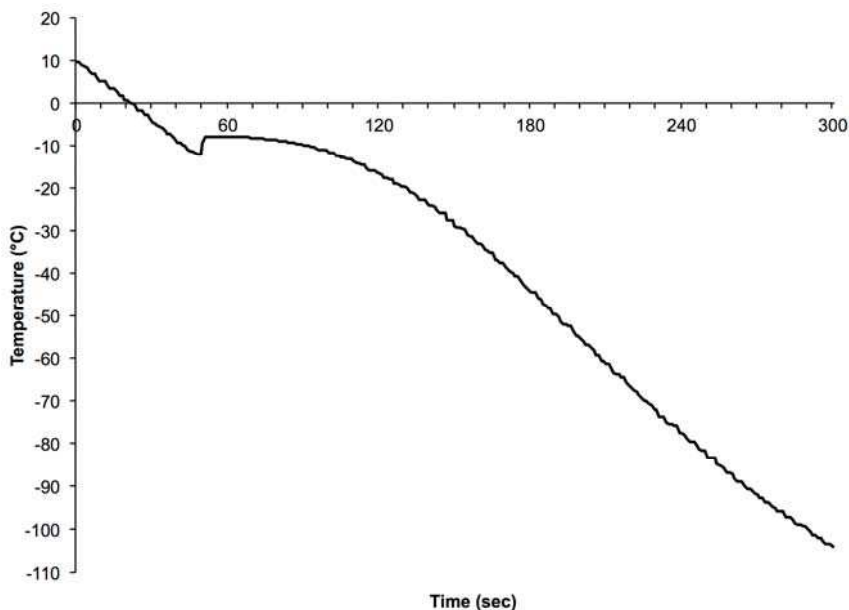


Fig. 2. The cooling profile used with 5-ml straws in the experiments on wels catfish sperm

The hatching rate of larvae originating from cryopreserved sperm in Köröm was $84 \pm 5\%$, while this rate in case of larvae originating from fresh sperm was $69 \pm 16\%$. There was no significant difference between the results. It was observed in the experiments carried out in Százhalombatta that hatching rate of larvae originating from cryopreserved sperm was $50 \pm 3\%$, while the result of the control group was $50 \pm 6\%$. There was also no statistically significant difference between the two groups. Hatching results of the experiments carried out in Ördögös at the place of Aranykárász Kft. were about $57 \pm 22\%$ in case of larvae originating from cryopreserved sperm and $22 \pm 18\%$ in case of larvae originating from fresh sperm. In this experiment a significant difference was found ($P = 0.05$) in favor of the cryopreserved group. The hatching rate of larvae originating from cryopreserved sperm in Szeged was $75 \pm 3\%$, while this rate in case of the control group was $83 \pm 1\%$. These results also differ significantly ($P = 0.0249$) but now in favor of the control group.

In the experiments on larval survival, statistically significant difference ($P = 0.034$) was observed on feeding larvae in laboratory conditions regarding 10-day body length. The results showed that larvae originating from cryopreserved sperm had a longer body. During non feeding larval period final body length ($P < 0.001$) and final weight ($P = 0.018$) differed significantly in the two groups in favor of larvae originating from cryopreserved sperm. There was no difference between the larvae originating from cryopreserved or fresh sperm in terms of larval survival.

3.2 Experiments on pikeperch sperm

In the first experiment, in spite of all efforts sperm was mixed with urine during stripping, thus the motility of pikeperch sperm was $50 \pm 17\%$. The motility of the best thawed sample was $28 \pm 21\%$, which was cryopreserved with glucose diluent and DMSO as cryoprotectant, but statistically significant difference was not be observed among the treatments.

The density (spermatozoa/ml) of pikeperch sperm samples counted in a Burker chamber were the following: 1st male: 0.9375×10^{10} , 2nd male: 1.0100×10^{10} , 3rd male: 0.7037×10^{10} , 4th male: 0.6687×10^{10} .

The highest fertilization rate ($43 \pm 12\%$) was observed also in the case of using a combination of glucose diluent and DMSO as cryoprotectant (Figure 3.). During statistical analysis of the data it was found that only the cryoprotectant had a significant effect ($P = 0.0338$) on the ratio of the fertilization.

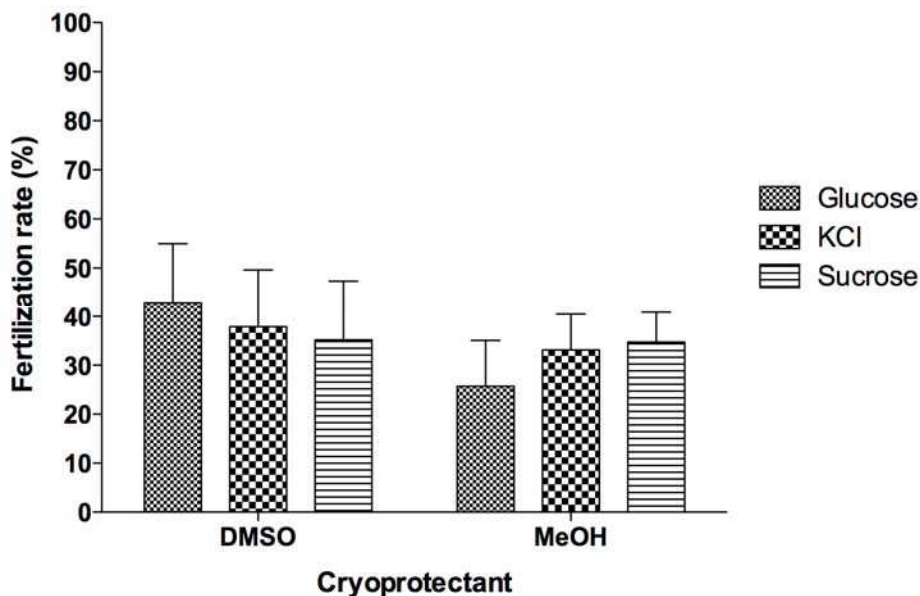


Fig. 3. Fertilization percentages of pikeperch eggs fertilized with cryopreserved sperm in the first experiment. The cryoprotectants dimethyl-sulfoxide (DMSO) and methanol (MeOH) and glucose, KCl or sucrose extenders were compared. Data are expressed as Mean \pm SD ($N = 3$).

The volume of the sperm stripped from pikeperch males in the second experiment was very low (less than 1 ml/individual). The motility of fresh sperm was $45 \pm 30\%$. Similarly to the previous experiment the sperm was mixed with urine again. Motility of thawed pikeperch sperm was very low (0 – 2%) in the samples containin the cryoprotectant DMSO, while motility of sperm frozen in presence of methanol was 40%, independently from rate of dilution. The highest hatching rate ($41 \pm 22\%$) was observed with the use of methanol and 1:1 dilution rate, although the statistical analysis has not shown significant differences between hatching rates (Figure 4.).

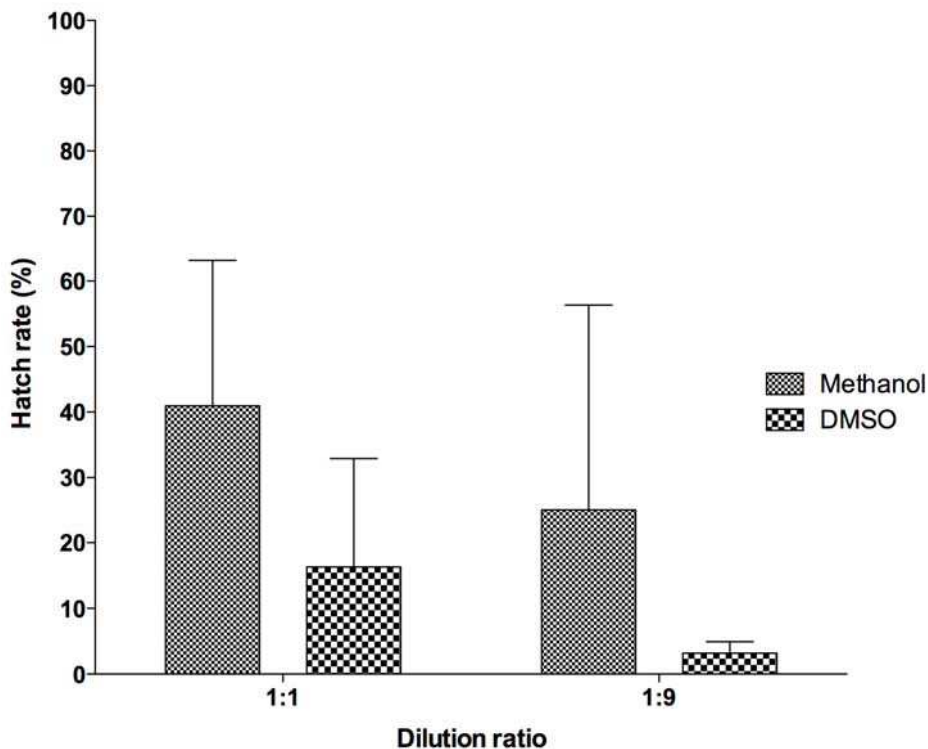


Fig. 4. Fertilization rates of pikeperch eggs fertilized with cryopreserved sperm in the second experiment. The cryoprotectants methanol and dimethyl-sulfoxide (DMSO) and dilution ratios of 1:1 and 1:9 were compared. Data are expressed as Mean \pm SD (N = 3).

In the hatchery experiment Stripping sperm with silicon catheter resulted that the motility was $63 \pm 10\%$. Concentration of sperm was $1.8571 \pm 0.1538 \times 10^{10}$, while the number of eggs/g was 1367 ± 54 , thus the number of sperm for an egg was 3.396×10^5 in the case of 10-g dose of eggs, 1.132×10^5 in the case of 30-g dose of eggs and 6.792×10^4 in the case of 50-g dose of eggs. Motility of sperm after thawing was $53 \pm 5\%$, thus there was no significant difference ($P = 0.1135$) between the motility of fresh and cryopreserved sperm.

Fertilization of the dose of 10 g of eggs with a single straw resulted $47 \pm 4\%$, while in the case of the dose of 30 g eggs resulted $55 \pm 3\%$ hatching rate (Figure 5.). There was no

statistically significant difference between the results of the different doses however the result of t-test ($P = 0.05701$) was very close to the significance level. A hatching rate of 87% was observed in the case of fertilization the dose of 50 g eggs with one thawed straw although in this case there were no replicates in the experiment. It was observed, however, that egg batches of different weight behaved differently in the hatching jars. While egg batches of 10 g stuck together in spite of the attempted elimination of egg stickiness, those in batches of 30 g or 50 g freely rolled on each other, thus improving oxygen supply of fertilized eggs and developing embryos. Thus, it is recommended to use larger batches of eggs for fertilization with cryopreserved sperm, which in turn would facilitate the acceptance of this technology in the aquaculture practice.

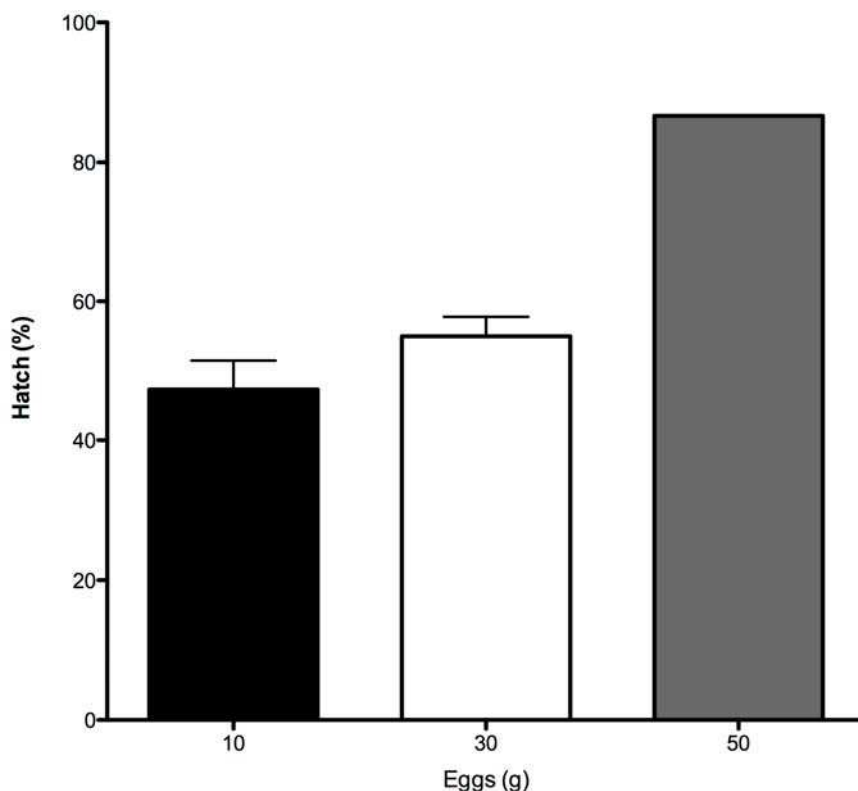


Fig. 5. Hatch rates of pikeperch eggs fertilized with cryopreserved sperm in a commercial hatchery during routine spawning work. Egg batches of 10, 30 or 50 g were used for fertilization ($N = 3$).

4. Discussion

4.1 Experiments on wels catfish sperm

Hatchery propagation of catfish species including the wels catfish faces several problems. Males of catfish species are typically oligospermic and sperm cannot be stripped but has to

be extracted from surgically removed testes (Legendre et al., 1996). As it was mentioned in the Introduction, this can lead to several problems such as shortage of sperm during induced spawning to unnecessary killing of immature females due to their erroneous identification as males.

Cryopreservation of the sperm of catfish species has been studied extensively. Several studies have been published on the cryopreservation of wels catfish sperm (Krasznai & Márián, 1985; Linhart et al., 1993; Ogier de Baulny et al., 1999; Linhart et al., 2005), however, they all reported the use of minute amounts of gametes and did not test practical utilization in the hatcheries.

It can be concluded according to the measured freezing parameters that a longer cooling time is needed for the safe cryopreservation of sperm in 5-ml straws because the temperature is not low enough (-45°C) after 3 minutes. The 7 minutes cooling time, which was used during the experiments, is suitable for these 5-ml straws.

Large amounts of eggs can be fertilised safely with a single 5-ml straw. It was observed in these experiments that the amount of the eggs, fertilised with one straw can be increased because the 2 ml sperm that can be found in a straw contained enough spermatozoa to fertilise 120 g eggs. The use of 5-ml straws has been tested on several fish species including the rainbow trout *Oncorhynchus mykiss* (Wheeler & Thorgaard, 1991; Lahnsteiner et al., 1997; Cabrita et al., 2001) or the paddlefish *Polyodon spathula* (Horváth et al., 2010), however, all previous works report a more or less reduced fertilizing capacity of sperm cryopreserved in these straws as compared to the conventional 0.5-ml French straws. The reaction of sperm to cryopreservation in different straw types seems to be species specific with the sperm of the wels catfish being especially resistant to the incurred cryodamage.

In the experiments no significant decrease was experienced in the quality of sperm after thawing. One of the reasons of this is that proper cooling time was successfully defined, which resulted the best fertilization rate. Thus, it can be said that cryopreserved sperm does not decrease the hatching rate compared to the traditional, routine method.

According to these experiments wels catfish sperm collected outside of spawning season is as suitable for cryopreservation and for fertilization at fish farms similarly to the ones collected in the spawning time.

After the successful cryopreservation and thawing of large amounts of sperm the next step is to carry out safe fertilization with this sperm on large scale. In the experiments doses of eggs between 150 - 350 g were fertilised with a single straw. According to literature data 100 - 200 g eggs can safely be incubated in one 7-l Zug-jar (Szabó, 2000). Cryopreserved sperm showed similar hatching rates to control in every experiment. These results prove that maximum sperm-egg ratio was not reached that might cause a decrease in hatching rate. According to the experiments it can be said that the improved method is suitable for wels catfish fertilization.

After improving the freezing method of sperm the next task was to examine whether the growth and survival of larvae originated from cryopreserved sperm reaches that of larvae originated from fresh sperm. The research was extended to both the feeding and non-

feeding larval periods. The results in both cases were that there is no difference in the survival of larvae fertilised with cryopreserved or fresh sperm. In the non-feeding larval period the growth of larvae from cryopreserved sperm exceeded the growth of the control, and in feeding larvae body length was higher compared to the control results.

According to these experiments the survival rate of larvae originating from cryopreserved sperm is as high as in the control and growth level of them in some cases showed better results compared to the control.

4.2 Experiments on pikeperch sperm

During the improvement of the cryopreservation technique of pikeperch sperm in laboratory cryoprotectant DMSO showed better fertilization rates than methanol but fertilization experiments in hatcheries showed opposite results. Literature data can be found on successful usage of both cryoprotectants in several fish species. The objective of this thesis is the usage of cryopreserved pikeperch sperm in hatcheries and according to the results of the experiments in the whole it was concluded that methanol and 1:1 dilution rate is suitable for freezing pikeperch sperm.

A significant variation was observed in motility after thawing and in hatching rate in the first experiments. This variability is caused by mixing of sperm with urine. This problem can successfully be eliminated when the stripping of sperm is conducted with a silicone catheter. According to this method the sperm is stripped with this silicone catheter directly from the testes preventing the mixing of sperm with urine or feces. One year later the use of catheter resulted in substantially better hatching rates.

It was observed that the increasing of the amount of eggs fertilised with a single 0.5-ml straw resulted in improved hatching rates. The reason for this can be that different amounts of eggs behaved differently in Zug-jars. The dose of 10 g of eggs were slightly stuck together, the dose of 30 g of eggs stuck in smaller batches while the dose of 50 g of eggs rolled freely. In spite of the fact that the 50 g of eggs sample had not replicates, these results suggest that fertilisation of larger amounts of eggs result in better hatching rates.

It is supposed that the eggs in the middle of the 10-g batches were more sensitive for oxygen deficiency than the more loose larger egg samples.

Another explanation for these results is that methanol in smaller eggs samples was in higher concentration, thus the toxic effects were more drastic than in larger samples. The lower sperm-egg ratio in larger egg samples had no influence on the results, suggesting that the amount of sperm was in surplus in the case of smaller egg samples.

5. Conclusion

A method has been developed for the cryopreservation of wels catfish sperm that can be used in the practice of fish farms. It is possible to fertilize 150-300 g eggs with sperm cryopreserved in large volumes (5-ml straws). The motility and hatching rate of frozen sperm correspond with the currently used routine method of fertilization with fresh sperm.

The survival and growth parameters of wels catfish larvae originating from cryopreserved sperm was investigated for the first time. According to this study it can be said that survival

rate of larvae originating from cryopreserved sperm reaches and in some cases exceeds that of control larvae. This result proves the practical usage of the cryopreservation method.

Pikeperch sperm has successfully been cryopreserved for the first time, and the developed method was tested in hatchery conditions.

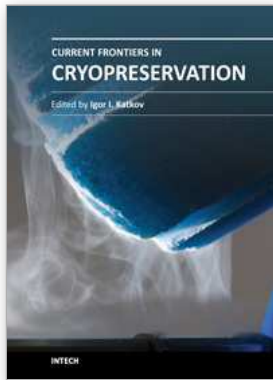
6. Acknowledgment

This work was supported by the NKFP 4/006/2004, RET 12/05 and the Bolyai János Fellowship of the Hungarian Academy of Sciences. The authors express their gratitude to the fish farms mentioned in the work for their support.

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Current Frontiers in Cryopreservation

Edited by Prof. Igor Katkov

ISBN 978-953-51-0302-8

Hard cover, 462 pages

Publisher InTech

Published online 14, March, 2012

Published in print edition March, 2012

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Zoltán Bokor, Béla Urbányi, László Horváth, Tamás Müller and Ákos Horváth (2012). Sperm Cryopreservation of Two European Predator Fish Species, the Pikeperch (*Sander lucioperca*) and the Wels Catfish (*Silurus glanis*), *Current Frontiers in Cryopreservation*, Prof. Igor Katkov (Ed.), ISBN: 978-953-51-0302-8, InTech, Available from: <http://www.intechopen.com/books/current-frontiers-in-cryopreservation/sperm-cryopreservation-of-two-european-predator-fish-species-the-pikeperch-sander-lucioperca-and-the>

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