Szent István University

CRYOPRESERVATION OF WELS CATFISH (*SILURUS GLANIS*) AND PIKEPERCH (*SANDER LUCIOPERCA*) SPERM ACCORDING TO PRACTICAL ASPECTS

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A társ-témavezető jóváhagyása

1. BACKGROUND AND OBJECTIVES

1.1. Background

The process of induced propagation of wels catfish (*Silurus glanis*) is based on a long standing technology. However, certain problems can suntil appear: the method of collecting male gametes is suntil 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 expertise and the danger of using an immature female with a less developed body structure is suntil present. As a result of listed problems the success of propagation becomes questionable (URBÁNYI et al. 2007).

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 so 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. Experiments concerning sperm cryopreservation of Volga pikeperch (*Sander volgensis*) mentioned briefly in my thesis can have an importance in the production of pikeperch – Volga pikeperch hybrids.

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 suntil not very widespread in aquaculture opposite to eg. the 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, on 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.

Cryopreservation group of our Department has 15 years of professional practice. This thesis, focusing on the application of the method in fish farms, is an important stage of a long-standing cryopreservation work.

1.2. Objectives

As described in the introduction part before starting my experiments I had the following aims:

- After the development of cryopreservation method for a large amount of wels catfish sperm I attempt to apply cryopreserved sperm successfully in a hatchery adapted to the process of normal wels catfish propagation in the farm.
- In case of wels catfish I attempt to prove that vitality and development of larvae originating from eggs fertilized with cryopreserved sperm is similar to those of larvae originating from eggs fertilized with fresh sperm thus supporting farm adaptation of cryopreserved sperm.
- I attempt to cryopreserve the sperm of pikeperch and Volga pikeperch then as a preliminary experiment I test the method on pikeperch species in hatchery environment.
- I attempt to examine the sperm of wels catfish and pikeperch by the help of a computer assisted semen analysis system (CASA).

2. MATERIALS AND METHODS

2.1. Experiments on wels catfish (*Silurus glanis*)

2.1.1. Method for 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 $NaHCO_3$ solution. From the diluted sperm treated this way 4 ml was pipetted into a 5-ml straw.

In the process of cryopreservation 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 my MSc thesis (BOKOR 2005) and former experiments of the cryopreservation group of the Department of Fish Culture on African catfish (URBÁNYI et al. 1999; HORVÁTH and 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 Bőcs 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 Bőcs
- in Százhalombatta farm of TEHAG Kft.
- in Ördöngös farm of Aranykárász Co.
- in Szeged farm of Szegedfish Kft.

In the Attala experiment in 15 May 2007 cryopreserved sperm collected ourside 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 Ördöngös 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 I joined to the propagation work of the farm with the method that I ensured the adequate amount of cryopreserved sperm 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 replcates. 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 Fish Culture. 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 Fish Culture to 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. Applied statistical methods

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 Chi^2-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 and Volga pikeperch

2.2.1. The cryopreservation of pikeperch (*Sander lucioperca*) sperm

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. Experiments on Volga pikeperch were performed in the first research place at the same time with pikeperch.

First experiment

Keeping and handling of fish

The stock of pikeperch taking part in the first experiment originated from Aranyponty 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.

Examination of the sperm

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. In each chamber the male gametes were counted and then an average was calculated. Cell number/ml (N) was calculated from the average number of cells (X) counted in the Bürker-chamber by the help of the following formula:

$$N = X \times 25 \times 10 \times 1000 \times 1000$$

Explanation: the area of one Bürker-chamber is 1/25 mm^2, its height is 1/10 mm so I have the number of cells in 1 µl. By multiplying this number with 1000 I have the number of cells/ml while the last number is the ratio of dilution.

Composition of cryopreservation media

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).

Cryopreservation of sperm

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.

Fertilization with cryopreserved 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.

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.

2.2.2. Cryopreservation of Volga pikeperch (*Sander volgensis*) sperm

Experiments on Volga pikeperch were performed in line with the first experiment on pikeperch in the same place.

Parent fish were collected by fishing at Balatoni Halászati Zrt. from Lake Balaton. Sperm of 5 Volga pikeperch was used for the experiment. Sperm collection and motility examinations were done using the same method as at pikeperch. After motility tests sperm of the 2nd and 3rd and the 4th and 5th individuals was mixed in two doses. Glucose diluent was applied for cryopreservation in this case, too. Methanol with a concentration of 10% was used as cryoprotectant. Sperm was diluted in a 1:1 ratio with a cryopreservation medium and freezing was carried out in nitrogen vapour. The same cryopreservation and thawing methods were applied as at pikeperch.

Eggs were placed into a Petri-dish with a diameter of 5 cm with about 150-400/dose. These doses were fertilized with the content of a half straw (0.25 ml). Gametes were activated with water and eggs were let to stick to the bottom of the Petri-dish where incubation happened. Hatching rate was also counted.

2.2.3. 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 ccHCl), 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.

2.2.4. 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 2.2.1). In case of research done on Volga pikeperch the effect of cryopreserved and control sperm on hatching was examined by one-way analysis of variance where Tukey-test was used as post-test.

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 ($P \leq 0.05$).

2.3. Testing of CASA system

The CASA system consisted of a trinocular optical phase contrast microscope (Nikon Eclipse E600; Nikon; Tokyo, Japan), with an attached Basler 312fc/c digital camera (Basler Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analyzed using the Integrated System for Semen Analysis (ISAS) software (Proiser; Valencia, Spain). Sampling was carried out using a 10× negative phase contrast objective (without intermediate magnification). The sperm was activated in Makler chamber placed on the stage of the microscope.

Motility and the 3 parameters of the movement (VCL, VSL, VAP) was recorded at 15, 30 and 60 seconds after activation. These times were decided previously in order to maintain repeatability between samplings (after activating the sample, the Makler chamber must be mounted with a special cover slip and positioned under the objective, and it is generally necessary to correct focus and field location). Image sequences were saved and analyzed afterwards.

CASA software settings were adjusted for analyzing fish spermatozoa. The CASA settings were: 30 frames/s for acquisition, for 1 s acquisition time; VCL≥10 μm/s to classify a spermatozoon as motile; 10–80 μm^2 for head area.

3. RESULTS

3.1. Results of the experiments on wels catfish catfish

3.1.1. Results of the experiments on cooling time and sperm-eggs ratio

The GSI (gonadosomatic index) of male individuals from Tuka was 2.00±4.00%. 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±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±0% and 51±1%) was observed. 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°C/minute. It was observed that the temperature of the straw was only -45°C after 3 minutes while after 5 minutes it was -104°C.

3.1.2. Results of the cryopreservation of wels catfish catfish sperm collected out of spawning season

The average weight of the testes of wels catfish 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 form Köröm (out of spawning season) had similar hatching rate to the sperm from Szeged.

3.1.3. Results on using cryopreserved wels catfish catfish sperm at hatcheries

Results of different hatching experiments depended on the propagation method and on the quality of sperm. Fertilization with cryopreserved sperm form Attala resulted 97±1% of fertilization rate while control fertilization rate was 93±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±2% while in the control group this rate was 94±6%. There was no statistically significant difference between the hatching rate of larvae originating from cryopreserved or fresh sperm.

The hatching rate of larvae originating from cryopreserved sperm in Köröm was 84±5%, while this rate in case of larvae originating from fresh sperm was 69±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±3%, while the result of the control group was 50±6%. There was also no statistically significant difference between the two groups. Hatching results of the experiments carried out in Ördöngös at the place of Aranykárász Kft. were about 57±22% in case of larvae originating from cryopreserved sperm and 22±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±3%, while this rate in case of the control group was 83±1%. These results also differ significantly (P=0.0249) but now in favor of the control group.

3.1.4. Results of 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. Results of the experiments on pikeperch and Volga pikeperch

3.2.1. Results of the experiments on cryopreservation of pikeperch sperm

First experiment

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

The density (spermatozoa/ml) of pikeperch sperm samples counted in a Burker chamber were the following: 1st male: 0.9375×10^{10}, 2nd male: 1.01×10^{10}, 3rd male: 0.7037×10^{10}, 4th male: 0.66875×10^{10}, average and SD: $0.83\pm0.17\times10^{10}$.

The highest fertilization rate (43±12%) was observed also in the case of using a combination of glucose diluent and DMSO as cryoprotectant. 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.

Second experiment

The volume of the sperm stripped from pikeperch males was very low (less than 1 ml/individual). The motility of fresh sperm was 45±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±22%) was observed with the use of methanol and 1:1 dilution rate, although the statistic analysis has not shown significant differences between hatching rates.

3.2.2. Results of experiments on the cryopreservation of Volga pikeperch sperm

Motility of fresh Volga pikeperch sperm was 60±20% (n=5), while motility of cryopreserved and thawed sperm was 30±10% (n=3). In this case sperm was mixed again with urine. The results of cryopreserved and fresh sperm were not significantly different in this experiment, except in the case of the 1st male (P=0.031). The highest hatching rate was observed in the case of the mixture of sperm of the 4th and 5th male. The hatching rate of the control was 60±6%.

3.2.3. Results of the preliminary experiments on the use of cryopreserves pikeperch sperm in hatcheries

Stripping sperm with silicon catheter resulted that the motility was 63±10%. Concentration of sperm was 1.8571±0.1538 × 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±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±4%, while in the case of the dose of 30 g eggs resulted 55±3% hatching rate. 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 was no replicates in the experiment.

3.3. Results of the CASA system

3.3.1. Results of the analyses of wels catfish catfish sperm with CASA system

The number of spermatozoa was between 947 and 2388 in the samples analyzed with the CASA system, which resulted in an average of 1747 spermatozoa. Motility rate was above 59% in all analyses after the 15th second of activation and the progress of time resulted in a continuous decrease in the motility and in velocity of spermatozoa.

3.3.2. Results of the analyses of pikeperch sperm with CASA system

The number of pikeperch spermatozoa were between 1230-2791, thus the average was 1747. During analyses of pikeperch sperm it was observed that the highest ratio of motility and speed were measured in the 15th second and continuous decrease was noticed with the progress of time.

3.4. New scientific results

- I have developed a method of cryopreservation of wels catfish catfish 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.
- I have examined for the first time the survival and growth parameters of wels catfish larvae originating from cryopreserved sperm. 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.
- I have successfully cryopreserved pikeperch sperm for the first time in the world, which was tested in hatchery conditions.
- I have successfully cryopreserved Volga pikeperch sperm for the first time in the world.

4. CONCLUSIONS AND SUGGESTIONS

4.1. Conclusions on the studies on wels catfish

4.1.1. Conclusions on the studies carried out on the cooling time and sperm-egg ratio

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

Large scale amount 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.

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.

4.1.2. Conclusions on using wels catfish catfish sperm collected outside of the spawning season

According to these experiments wels catfish 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 .

4.1.3. Conclusions on the large scale usage of cryopreserved wels catfish sperm

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 rate 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.

4.1.4. Conclusions on experiments on larval rearing

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. Conclusions on the experiments on pikeperch and Volga pikeperch sperm

4.2.1. Conclusions on the sperm cryopreservation of pikeperch and Volga pikeperch

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.

Concerning Volga pikeperch glucose diluent and methanol as cryoprotectant combined with 1:1 dilution rate seemed acceptable for cryopreservation of 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.

4.2.2. Conclusions on the preliminary experiments with cryopreserved pikeperch sperm in hatcheries

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.

4.3. Conclusions on the test of CASA system

High motility, fertilising and hatching rates were observed after thawing of the cryopreserved sperm using the developed cryopreservation method of wels catfish sperm. Results form computer-assisted analysis (CASA) showed also high motility rate, which proves that developed cryopreservation method can be used successfully.

Analysis of sperm of wels catfish catfish and pikeperch can successfully be accomplished by CASA system for the improvement of analysis and selection of the proper samples for cryopreservation.

4.4. Suggestions

4.4.1. Suggestions for the cryopreservation of wels catfish sperm

- I suggest the continuation of survival experiments of larvae for a longer growth period in fish ponds.
- I suggest observations regarding the better larval growth of fry originating from cryopreserved sperm using different methods.
- I suggest a comparison of motility results of CASA system with fertilization tests, furthermore incoporation of the CASA technology into the process of cryopreservation.
- I suggest the establishment of a cryopreserved gene bank combined with population genetics analyses for the proper conservation of genetic variability.

The technology of cryopreservation of wels catfish catfish sperm is in the stage of introduction into the practice for which there are some further suggestions:

- I suggest applying more intensive marketing methods and organising practical events for fish farmers.
- I suggest establishing a sperm bank for commercial purposes at the Department of Fish Culture.

4.4.2. Suggestions for the cryopreservation of pikeperch sperm

- I suggest testing the cryopreservation of pikeperch sperm in 4 and 5-ml straws both in laboratory and hatchery conditions.
- I suggest further analyses on sperm-egg ratios.
- I suggest the adaptation of pikeperch cryopreservation technique to Volga pikeperch.
- I suggest sperm analysis with CASA technology in both species before fertilisation experiments.

5. PUBLICATIONS RELATED TO THE DISSERTATION

5.1. Scientific papers

BOKOR, Z., HORVÁTH, Á., HORVÁTH, L., URBÁNYI, B. (2008): Cryopreservation of Pike Perch sperm in hatchery conditions. *The Israeli Journal of Aquaculture – Bamidgeh* 60 (3), 168-171. p.

BOKOR, Z., MÜLLER, T., BERCSÉNYI, M., HORVÁTH, L., URBÁNYI, B., HORVÁTH, Á. (2007): Cryopreservation of sperm of two European percid species, the pikeperch (*Sander lucioperca*) and the Volga perch (*S. volgensis*). *Acta Biologica Hungarica* 58 (2), 199-207. p.

5.2. Book chapters in English

URBÁNYI, B., HORVÁTH, Á., **BOKOR, Z**. (2008): Artificial fertilization in aquaculture species: from normal practice to chromosome manipulation. In: Cabrita E., Robles V., Herráez P. (eds) Methods in Reproductive Aquaculture: Marine and Freshwater Species, CRC Press, New York, 183-216. p.

5.3. Book chapters in Hungarian

BOKOR, Z. (2007): A harcsa fejezet egyes alfejezetei, In.: Horváth, L., Csorbai, B., Urbányi, B., (Szerk.): A tájidegen gyomhalak visszaszorítása őshonos ragadozó halfajokkal, Prime Rate Kft., Budapest

5.4. Proceedings

BOKOR, Z., URBÁNYI, B., HORVÁTH, L., HORVÁTH, Á. (2008): Studies on the cryopreservation of pike-perch (*Sander lucioperca*) sperm. Short Communications, Resource Management, *Aquaculture Europe 2008*, Krakkó, 100-101.

BOKOR, Z., MOSONYI, G., ITTZÉS, I., MÜLLER., T., HORVÁTH, L., HORVÁTH, Á., URBÁNYI, B. (2008): Experiments on the viability of European catfish (*Silurus glanis* L.) larvae originated from native and cryopreserved sperm in laboratory and hatchery conditions, 32nd Annual Larval Fish Conference, Kiel, 122 p.

BOKOR, Z., HORVÁTH, Á., URBÁNYI, B. (2006): A harcsa hímivartermék minőségének vizsgálata, különös tekintettel a mélyhűthetőség és gyakorlati alkalmazás eredményességére, *XXX.. Halászati Tudományos Tanácskozás*, Szarvas, 2006. május 24-25. Összefoglaló, 48 o.

BOKOR, Z., URBÁNYI, B., RADICS, F., FICSURA, A., CSERNÁK, R., OPITZER, Z., HORVÁTH, Á. (2006): Cryopreservation of sperm of some european predator fish species, *World Aquaculture 2006*, Book of Abstracts 96 p.

HORVÁTH, Á., **BOKOR Z.,** RADICS, F., CSOMA, G., HORVÁTH, L., URBÁNYI, B. (2006): Cryopreservation of wels catfish Silurus glanis sperm in 4 ml straws, *Aquaculture Amercia 2006*, Book of Abstracts 138. p.

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Education

2005-2008 PhD: **Szent István University,** Gödöllő
Faculty of Agricultural and Environmental Sciences,
PhD School of Animal Husbandry

2000-2005 MSc thesis: **Szent István University,** Gödöllő
Faculty of Agricultural and Environmental Sciences,
M.Sc. in agricultural engineering,
Specialised in Fishery and Game Management
Agricultural consultant

1992-2000 Matura: **Széchenyi István Secondary Grammar School,** Dunaújváros
Faculty of Biology and Chemistry

Scientific work, achievements

2005 **National Scientific Conference of Students**, Szarvas, Session Applied Animal Husbandry, **Special Prize**
2004 SZIU, Scientific Conference of Students**, Special Prize of the Department of Fish Culture**
2004 SZIU, **Republican Scholarship**
2003 SZIU, **Republican Scholarship**
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2002 SZIU, **Republican Scholarship**

Language skills

German state exam, basic level, type C, general
English state exam, intermediate level, type C, general
English (ICC) international exam, intermediate level

Computer skills

Windows XP, Office utilities, Adobe Photoshop, design webpages

Other skills

1999 driving licence („B" type)
2003 Fishingpond operator qualification, hunting certificate, firearms certificate
2001-2004 Membership of the Board of Rural Development of Gödöllő
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