MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

INHERITED CRYORESISTANCE OF FISH SPERM

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1 Background and objectives

Polge et al. successfully cryopreserved sperm in 1949 using dry ice and glycerol. This was the first step to recognise the importance of cryoprotectants. Cryopreservation is used for preserving cells and tissues at low temperature in liquid nitrogen (-196 °C). With the preservation of gametes the males and females can be separated in time and space. This method can be economically beneficial also.

In the case of bull the sperm cryopreservation became a business. In fish more than 200 sperm cryopreservation methods were developed until the 2000s, however, nowadays it is not used in fish breeding systems just in the northern countries. The causes of this are plenty. First of all, the cryopreservation takes time and competence is also needed. Furthermore the used tools are expensive. It is also important to consider the value of cryoprotectants, temperature and the equilibration time.

Some research are giving attention to the freezing impact on the gametes. In the case of fish the only way for preserving gametes is to cryopreserve the sperm. Freezing embryos and eggs is not possible yet because of the high water content. In sperm cryopreservation studies the focus is usually on the motility of sperm, the shape and damages to spermatozoa, organelles and the flagellum, and the integrity of DNA. Furthermore adjusting concentration prior to cryopreservation, using different cryoprotectants and the freezing temperature are tested to find the right protocol. Nowadays more focus is on the gamete freezing impact on next generations' geno- and phenotype.

Babiak et al. (2002) found that in the rainbow trout (*Onorhynchus mykiss)* the semen of males originated from cryopreserved sperm is more suitable for cryopreservation than that of males produced from fertilization with fresh non-frozen sperm. They thought it can be the effect of some epigenetic changes.

1.1 Objectives

In this recearch the objectives are to find out in zebrafish *(Danio rerio)* and also in carp *(Cyprinus carpio)* analyzing more than 1 generation if the thawed sperm of males hatched from fertilization with cryopreserved sperm has

different motility parameters and fertilizing capacity than the sperm of those hatched from eggs fertilized with fresh sperm. Furthermore my research focused on the morphological parameters in the subsequent generation of carps. It was also important to discover a method with which the concentration of sperm can be measured easily and fast and if the concentration prior cryopreservation can cause any difference in motility parameters or fertilizing capacity.

2 Materials and methods

2.1 Broodstock handling and gamete collection

2.1.1 Carp

Common carp *(Cyprinus carpio)* individuals were kept in a recirculating fish housing and breeding system (Sentimento Kft., Érd, Hungary) of the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences (Gödöllő, Hungary). Scaly and mirror carps (P: $N = 66$, age: 4+, body weight: 817-3000 g, F1: N=46 hatched from fertilization with cryopreserved sperm, $N = 63$ hatched from fertilization with fresh sperm, age: 2+, body weight: 100-520 g; F2: $N = 63$ hatched from fertilization with cryopreserved sperm, $N = 68$ hatched from fertilization with fresh sperm, age: 1+, weight: 28-640 g) were used for the experiments.

Fish were raised in 10 liter plastic tanks until the age of 3-4 months and moved and kept in a 3-m^3 plastic tank in cages, with constant water quality parameters (23 ± 2 °C, pH 7.0 ± 0.2, redox potential; 230 ± 2 mV, dissolved O_2-level; 4 ± 1 mgL^{-1}). Carps were fed once a day with 10 g per kg body weight of Aqua Uni (Aqua Garant, Pöchlarn, Austria) pelleted feed.

The fingerligs were fed in the first month with artemia (*Artemia salina* nauplii; INVE Aquaculture NV, Belgium) 8 times a day. In the 2nd month the fingerlings were also fed with zebrafish food (> 100 µm – 600 µm < ; ZEBRAFEED®, Sparos Lda, Portugal). From the 3rd month artemia was not involved in the feeding and from the 4th month they were fed with larger food (1,00 – 4,5 mm; Aqua start; Aqua Uni, Aqua Garant, Pöchlarn, Austria). At the age of half year the fish were tagged abdominally (Agrident GmbH, Barsinghausen, Germany) and released in the system.

2.1.2 Zebrafish

The zebrafish were kept in 3- and 8-L polycarbonate tanks at 25 ± 2 °C (pH 7.0 ± 0.2; conductivity 525 ± 50 µS; alkalinity: 0 mM OH^-, 0 mM CO_3^{2-}, 0.4 mM HCO_3^{2-}; hardness: < 0.5 °dH; DOC: > 90%; from here onwards

referred to as system water) in a recirculating zebrafish housing system (ZebTEC® (Tecniplast, Italy)) at the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences (Gödöllő, Hungary). The photoperiod was 14-h light and 10-h dark.

Fish were fed twice a day with commercial zebrafish feed (ZEBRAFEED® diet (Sparos Lda, Portugal) and with live artemia (*Artemia salina* nauplii) (TQ type; INVE Aquaculture NV, Belgium) every other day. The fingerlings were kept in incubator (25 ± 2 °C) until the age of 1.5 weeks and were fed once a day with artemia before the water changing.

2.2 Broodstock handling prior examination

Carps were induced hormonally for spermiation and ovulation. All treatments of fish were carried out in anesthesia using a solution of 0.04% 2-phenoxyethanol (Reanal, Hungary). After that male fish were placed on a wet towel and injected intraperitoneally with 1 pellet per kg body weight of Ovopel (Interfish Kft., Hungary) containing 12-20 μg per pellet of synthetic GnRH analogue (D-Ala^6, Pro^{9}Net-mGnRH) and 8-10 mg per pellet of dopamin receptor antagonist metoclopramide 48 h before the experiments. Females were aministered 10% of the total dose 24 hours before the planned ovulation and 12 hours later, the other 90%. The zebrafish were anesthetized with tricaine methane sulfonate (MS-222, Arlos Organics™, Geel, Belgium, 168 mg/l). In case of zebrafish no hormonal treatment was used.

2.3 Creating the generations

2.3.1 Creating the P generation of carps

The P generation arrived in 2016 at the age of 1 year from Dinnyési Halgazdaság Kft. and Jászkíséri Halas Kft. to the recirculation system of the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences. From the 41 males 9 were randomly chosen. Fresh and thawed motility parameters of these fish were examined for 3 months 6 times with CASA. Between each spawning the fish rested for a minimum of 1 week.

These tests were conducted in order to find the individuals with the lowest post-thaw progressive motility and the ones with more than 40% of post-thaw progressive motility. There was no significant difference found among the sperm motility parameters of the afore mentioned fish, thus 6 individulas was randomly chosen from the 9 for creating the F1 generation.

2.3.2 Creating the F1 generation of carps

From 74 females considering size and health one was randomly chosen. The female was injected with ovopel 24 hours before fertilization. For fertilization, eggs were divided into 12 batches of 10 g, each. The batches were fertilized with 50 µl of fresh sperm from the males and 500 µl of cryopreserved sperm (due to the 1:9 dilution ratio) from the same males creating 12 groups.

Eggs were incubated in 10 l polycarbonate tanks in a recirculating system at 23 ± 2 °C. Motility parameters of the sperm were measured with CASA prior to cryopreservation. Sperm and subsequently 100 µl of system water were added to the eggs and mixed for 1 minute. After 1 minute Woynárovich solution (10 L system water, 40 g NaCl, 30 g urea) was added to eliminate the adhesiveness of eggs. More Woynárovich solution was added while mixing for approximately 1.5 hours during the process of egg swelling. After that the eggs were washed three times in 5% tannic acid for 10 s and incubated at 22-24 °C in 10 l tanks in a recirculating system. The fingerlings hatched in 3-5 days. In the first 3 days methylene blue was added to the water to avoid infections. After hatching, the egg shells were removed from the tanks. At the age of 3-4 months the fish were put to 3 m^3 polycarbonate tanks.

2.3.3 Creating the F2 generation of carps

One male from 4 F1 families (F1-c and F1-f, $N = 8$) and a single female were injected with Ovopel 24 hours before fertilization. Males were administered a single dose while the female was given 2 doses (priming dose 10%, resolving dose 90%), 12 hours apart. The sperm motility parameters were measured with CASA. The sperm was cryopreserved following the protocol of Horváth et al. (2003) and were put in liquid nitrogen. The eggs were divided into 8 batches of 10 g each for fertilization. The batches were fertilized with 50 µl of fresh

sperm from F1-f males (hatched from fertilization with fresh sperm) and 500 µl of cryopreserved sperm (due to the 1:9 dilution ratio) from F1-c males (hatched from fertilization with cryopreserved sperm) creating 8 groups.

Eggs were incubated in 10 l polycarbonate tanks in a recirculating system at 23 ± 2 °C. Sperm and subsequently 100 µl of system water were added to the eggs and mixed for 1 minute. After 1 minute Woynárovich solution (10 L system water, 40 g NaCl, 30 g urea) was added to eliminate the adhesiveness of eggs. More Woynárovich solution was added while mixing for approximately 1.5 hours during the process of egg swelling. After that, eggs were washed three times in 5% tannic acid for 10 s and incubated at 22-24 °C in 10 l tanks in a recirculating system. The fingerlings hatched in 3-5 days. In the first 3 days methylene blue was added to the water to avoid infections. After hatching, egg shells were removed from the tanks. At the age of 3-4 months the fish were put to 3 m^3 polycarbonate tanks in separate cages.

2.3.4 Creating the P generation of zebrafish

The sperm of 50 zebrafish were measured with CASA every 2 weeks 3 times. 6 individuals were chosen whose sperm had progressive motility above 80%. 6 females were randomly chosen also for creating the P generation.

2.3.5 Creating the F1 generation of zebrafish

The 6 females' batches of eggs were individually separated in two and fertilized with the 6 male's thawed and fresh spem. 12 full-sib groups were created. 6 groups were hatched from fertilization with cryopreserved and 6 from fresh sperm.

2.3.6 Creating the F2 generation of zebrafish

3 males were chosen from each group for creating the F2 generation in zebrafish. The sperm of the 3 individuals from each group were pooled. 6 females were randomly chosen and the eggs were also pooled and divided in 6. Half of the batches of eggs were fertilised with cryopreserved sperm from

the ones hatched from fertilization with cryopreserved sperm and another half with the fresh sperm from males hatched from fertilization with cryopreserved sperm creating 12 groups. The pooling was necessary because the quantity of sperm and eggs was not enough for creating the next generation.

2.3.7 Creating the F3 generation of zebrafish

3 males were chosen from each group for creating the F3 generation in zebrafish. The sperm of the 3 individuals from each group were pooled. 6 females were randomly chosen for each male groups (fresh and cryopreserved full-sibs) and their eggs were also pooled. Half of the batches of eggs were fertilised with cryopreserved and another half with fresh sperm of the males hatched from fertilization with fresh and cryopreserved sperm of the males hatched from fertilization with cryopreserved sperm creating 12 groups.

2.4 Collection, analysis and cryopreservation of sperm and fertilization

2.4.1 Carp

24 hours after the hormonal injection, carps were anasthetised. Anesthetized fish were laid out on a wet towel and the urogenital papilla was dried to prevent the contamination of sperm with water. Gentle pressure was applied to the abdominal wall, and the outflowing sperm was collected individually into 15 ml Falcon tubes and were put onto crushed ice. At least 1 ml of sperm was collected from each individual, avoiding contamination with blood or urine. After stripping, fish were placed back into the recirculating system.

Fresh and thawed sperm motility parameters were measured with CASA. The sperm was cryopreserved as follows. Samples were diluted at a ratio of 1:9 in an isotonic extender (200 mM glucose, 40 mM KCl, 30 mM Tris, pH: 8.0±0.2) and methanol (10%, v/v final concentration). Diluted samples were loaded into 0.5 ml plastic straws (Minitube GmbH, Tiefenbach, Germany). Samples were cryopreserved in the vapor of liquid nitrogen 3 cm above its surface for 3 minutes. After cryopreservation, samples were plunged into liquid nitrogen.

Samples were thawed for 13 sec at 40 ˚C (Thermo Haake P5, Thermo Electron Corp, Waltham, Massachusetts, USA).

2.4.2 Zebrafish

Zebrafish were anesthetized with tricaine methane sulfonate (MS222 Arlos Organics™, Geel, Belgium, 168 mg/l). After anesthesia, sperm was collected by stripping of males as follows. Fish were placed into a dampened foam holder and sperm from was collected into 10 µl glass capillaries by abdominal massage of the individuals using a slide forceps.

Fresh and thawed sperm motility parameters were measured with CASA. The cryopreservation followed Caetano et al.'s (2019) protocol. Sperm was diluted with an extender (200 mM glucose, 40 mM KCl, 30 mM Tris, pH: 8.0±0.2) and methanol was added to reach a final concentration of 8%. Samples were loaded into 0.25 ml straws. Programable freezer was used for cryopreservation (IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria) at cooling rate 10 °C/min. Samples were stored in liquid nitrogen and before use were thawed at 40 °C for 5 sec.

2.5 Assessment of sperm concentration and adjustment of concentration before cryopreservation

For further investigations, a method was needed with which sperm concentration can be measured easily and fastly. For measuring concentration, a CASA system (Computer-assisted sperm analysis, Minitüb GmbH AndroVison) and a microplate reader (Thermo Scientific Varioskan Lux) were tested and compared with the Bürker-Türk type hemocytometric results.

2.5.1 Assesment of sperm concentration with microplate reader

Semen was collected from 9 individuals. Firstly, 10 or 5 µl of each sperm sample was diluted in 990 or 995 µl extender (100- and 200-fold dilution; 200 mM glucose, 40 mM KCl, 30 mM Tris, pH: 8.0±0.2). Subsequently, 100 µl

from the pre-diluted sample was diluted further in 900 µL extender (1000- and 2000-fold dilution) and spermatozoa were counted using a Bürker-Türk type hemocytometer (96-well plate, 200 µl/well) and absorbance was also measured at 505 nm by a spectrophotometer (Hermo Scientific Varioskan LUX multimode microplate reader, SkanIt RE v.5.0). Each (1000- and 2000-fold dilution) dilution was repeated 3 times.

2.5.2 Assesment of sperm concentration with CASA

Samples were collected from 12 individuals. Computer-assisted sperm analysis (CASA) was conducted with an AndroVision (Minitüb, Tiefenbach, Germany) sytem equipped with a Motic BA310 microscope with a 20× negative phase contrast objective. The samples were diluted in extender to reach a 100× dilution ratio. Aliquots of 3 µl of diluted sperm were pipetted into a Makler-type cell counting chamber, covered with a dedicated coverglass and placed under the microscope. CASA was run on the immotile samples to determine cell concentration. Only cell concentration data were retained at this point. For measurement with Bürker-Türk type hemocytometer the dilution rate was 1000×. Each dilution was repeated 3 times.

2.5.3 Adjustment of sperm concentration prior to cryopreservation

Sperm of 5 males was diluted in extender containing 10% methanol (v:v, final concentration) to reach a final concentration of 0.5; 1; 2; 4×10^9 spermatozoa per ml (3 straws per dilution) which resulted in dilution ratios of 25–43-fold for 0.5×10^9, 12–21-fold for 1×10^9, 6–11-fold for 2×10^9 and 3–5-fold for 4×10^9 spermatozoa per ml. The concentrations of samples were measured by the previously used CASA system. The standard, 1:9 (or else 10-fold) dilution ratio (sperm:extender) served as a positive control which is this case corresponded to a concentration of $1.2–2.1 \times 10^9$ spermatozoa per ml. The samples were loaded into 0.5 mL straws and frozen in a polystyrene box using polystyrene frames 3 cm above the surface in the vapour of liquid nitrogen for 3 minutes. Following freezing, the samples were plunged into liquid nitrogen. After storage, the straws were thawed in a 40 °C water bath for 13 s.

2.6 Morphometry measurement

Anesthetised fish were placed on a wet towel and digital images were taken with a Nikon D7200 DSLR equipped with an AF-S Nikkor 35 mm objective. The geometric morphometric analysis using morphological landmarks were measured with tpsUtil, tpsDig2 and MorphoJ software. Nine well defined landmarks were taken in every image. The morphopetric measurement and the length measurement took place in the same day, however the weight was measured in separate day.

2.7 Statistical analysis

For the statistical analysis Prism 9 (GraphPad Software, San Diego, USA), Microsoft Office Excel (Mondo 365) and JASP (0.14.3) was used. Canonical Variates Analysis (CVA) and Discriminant Function Analysis (DFA) were used for measuring morphometric changes. After a full Procrustes fit, a mutivariate linear regression was performed (independent variables: logarithm of Centroid sizes; dependent variables: Procrustes coordinates), to remove the allometric growth effects. Regression residuals was used for the statistical analysis.

A two-sample t-test was used to compare the length between the two groups. One-way ANOVA with Tukey's multipe comparisons test was used to determine the main effect of the origin of the males and the date on CASA parameters. The significance level was $p = 0.05$.

3 Results

3.1 Carp

3.1.1 Adjustment of common carp sperm concentration prior cryopreservation

Sperm concentration of 9 common carp males was $1.849 \times 10^{10} \pm 3.853 \times 10^{9}$ spermatozoa per ml. A significant positive linear relationship was detected between absorbance measured in the plate spectrophotometer and sperm concentration assessed using a hemocytometer ($p < 0.0001$, $r^2 = 0.8289$) resulting in the equation $y = 1.720 \times 10^{11}x + 3.851 \times 10^{9}$.

Sperm concentration of 12 common carp males measured with CASA was $1.853 \times 10^{10} \pm 7.854 \times 10^{9}$ spermatozoa per ml while that measured with a hemocytometer was $1.442 \times 10^{10} \pm 6.212 \times 10^{9}$ spermatozoa per ml. The concentration values measured with CASA were in a significant linear relationship ($p < 0.0001$, $r^2 = 0.8559$, $y = 0.7317x + 8.555 \times 10^{8}$) with sperm concentration counted in a hemocytometer. For further studies, CASA was used to determine the concentration of sperm.

No significant main effect of sperm concentration was found on any of the parameters measured by CASA. The only exception was LIN ($p = 0.0112$) where the post-hoc test found a significant difference ($p = 0.0056$) between linearity value for the sperm concentration of 0.5×10^{9} spermatozoa ml^{-1} (0.86 ± 0.03) and that for the dilution ratio of 1:9 (0.74 ± 0.08).

A significant main effect ($p = 0.0156$) of cell concentration on the fertilizing capacity of cryopreserved common carp sperm was found. The post-hoc test detected a significant difference ($p = 0.0121$), between the fertilization percentage of batches fertilized with sperm frozen at a cell concentration of 4×10^{9} spermatozoa ml^{-1} ($66 \pm 6\%$) and the positive control (sperm diluted at a ratio of 1:9, $49 \pm 5\%$). The control fertilization rate was 95±5% confirming satisfactory egg quality.

3.1.2 Quality of the sperm from F1 carps originated from fresh and cryopreserved sperm

No significant difference ($p = 0.86$) was found between the fertilizing capacity of cryopreserved (87 ± 5%) and fresh sperm (86 ± 13%) of F1 males used to establish the F2 generation. On the progressive motility of cryopreserved carp sperm both the sampling date ($p < 0.001$) and the origin of males (fresh or cryopreserved sperm) had a significant effect ($p = 0.024$, $N = 46$ for cryopreserved, $N = 63$ for fresh) altough the family of fish had no effects on the results. The origin of the males did not affect other motility parameters (VCL, VAP, VSL, STR or LIN).

3.1.3 Quality of the sperm from F2 carps originated from fresh and cryopreserved sperm

In the second generation of carps no significant difference ($p > 0.05$) was found between the thawed sperm fertilizing capacity of the two groups. On the progressive motility of cryopreserved carp sperm, neither the sampling date nor the origin of males (fresh or cryopreserved sperm) had a significant affect. The origin of the males did no affect other motility parameters (VCL, VAP, VSL, STR or LIN). The density of the sperm was not affected by its origin. However the concentration was higher in the group originated from cryopreserved sperm ($1 \times 10^{10} \pm 2{,}5 \times 10^{9}$ spermatozoa/ ml) than the group originated from fresh sperm ($7 \times 10^{9} \pm 3 \times 10^{9}$ spermatozoa/ ml).

3.1.4 Difference in morphology and lenght in the second generation of carps

Morphometry studies of F2 individuals revealed that fish can be classified according to their origin (fertilization with cryopreserved or fresh sperm) with 81.09 ± 3.57% accuracy accross all tested families, based on DFA. A transformation grid for visualizing a shape change was taken where the group originating from cryopreserved sperm differed from that originating from fresh sperm at 9 measured points. Generally, fish originating from fertilization with cryopreserved sperm had a smaller head, lower back and narrower caudal

peduncle that those originating from fresh sperm. However, in the length of the individuals there was no signifficant difference (p = 0.4078). The length of fish from cryopreserved sperm (N = 173; 55 ± 13 cm) and the ones hatched from fertilization with fresh sperm (N = 176; 55 ± 13 cm) and the growth pattern of the individuals was more or less similar.

3.2 Zebrafish

3.2.1 First generation of zebrafish

There was no significant difference found (p = 0,9) between the fresh motility of the sperm of the groups originated from fresh (80 ± 11%) and cryopreserved (80 ± 14%) sperm. The progressive motility of fresh and thawed sperm was significantly different (p < 0,001) as expected. The progressive motility of thawed sperm was not significantly different (p = 0,73) between the cryopreserved (25 ± 8%) and the fresh (21 ± 12%) groups as well as the VCL, VAP, VSL, STR and LIN.

The concentration measurement with Bürker-Türk type hemocytometer showed no significant difference (p = 0,56) between the males originated from cryopreserved (5 ± 4 × 10^8 spermium/ ml) and fresh sperm (4 ± 3 × 10^8 spermium/ ml). For the fertilization 5000:1 sperm and egg ratio was used. As a control fresh sperm was used. The fertilization rate showed no significant difference (p = 0,73) between the fresh (1 ± 3%) and the cryopreserved (1 ± 1%) groups, however the fertilization rate was very low.

3.2.2 Second generation of zebrafish

The second generation was tested as the first except for the fertilization rate. Measuring with CASA, fresh motility rates were not significantly different (p = 0,281) between the ones hatched from fertilization with fresh (67 ± 12%) and with cryopreserved sperm (70 ± 9%). The progressive motility of the fresh and thawed sperm was significantly different (p < 0,001) as in the first generation. In case of progressive motility of thawed sperm, there was no significant difference (p = 0,54) between the males originated from

cryopreserved (17 ± 9%) and the ones originated from fresh sperm (21 ± 3%). The origin of the males did not affect other motility parameters (VCL, VAP, VSL, STR or LIN). In the concentration of the sperm there was no significant difference (p = 0.073) between the fish hatched from fertilization with cryopreserved ($1 \pm 2 \times 10^9$ spermatozoa/ ml) and fresh sperm ($2 \pm 1 \times 10^9$ spermatozoa/ ml).

3.2.3 Third generation of zebrafish

In the F3 generation, progressive motility of fresh sperm was not significantly different ($p > 0,05$) between the two groups. Neither was there a significant difference ($p = 0,781$) in the progressive motility of the thawed sperm between the males originated from cryopreserved (30 ± 16%) and from fresh sperm (15 ± 2%). The origin of the males did not affect other motility parameters (VCL, VAP, VSL, STR or LIN).

There was no significant difference ($p = 0,44$) in sperm density, measuring the males hatched from fertilization with cryopreserved ($2 \pm 1 \times 10^9$ spermium/ ml) and from fresh sperm (2×10^9 spermium/ ml). The fertilization rate was not statistically different ($p > 0,05$) between the fish hatched from fertilization with cryopreserved (5 ± 7%) and from fresh (5 ± 9%) sperm. There was no significant difference between any of the sperm parameters in any of the generations in zebrafish.

4 Conclusions

Adjustment of sperm concentration prior to cryopreservation can have a signifficant effect on the fertilizing capacity of sperm. In the dissertation the following densities were used: $0{,}5 \times 10^9$, 1×10^9, 2×10^9, 4×10^9 cell/ml and standard 1:9 in the staws, however significant difference ($p = 0{,}0121$) was only found between the density of 4×10^9 ml^{-1} ($66 \pm 6\%$) and the control 1:9 ($1.2–2.1 \times 10^9$ ml^{-1}) thawed sperm ($49 \pm 5\%$).

Cell density can have a positive impact on some motility parameters. In this dissertation the LIN parameter was significantly different ($p = 0.0056$) in the case of 0.5×10^9 spermatozoa/mL (0.86 ± 0.03) and the 1:9 dilution ratio (0.74 ± 0.08).

Higher sperm density can cause higher fertilization rate and also lower the motility. The reason behind it can be the offset dilution ratio which is optimal in a 1:9 ratio in the case of common carp. However in fertilization this can be compensated by a higher number of spermatozoa. This disagrees with other studies that found a lineral correlation between the fertilization rate and motility parameters. This can be caused by the variations in fertilization which can not be modelled. This factor can be the ovarian fluid, which prolongs the motility time of spermatozoa or the chemical communication between the spermatozoa and eggs.

Babiak et al. (2002) found in rainbow trout that the sperm of the males originated from cryopreserved sperm had a significantly higher ($p < 0{,}05$) fertilization capacity ($89 \pm 6\%$) than that of ones hatched from fertilization with fresh sperm ($81 \pm 7\%$). On the contrary, in our research there was no significant difference ($p > 0{,}05$) between the fertilization capacity or any other sperm motility parameters in zebrafish. In carp neither was significant difference ($p = 0{,}86$) in the fertilizing capacity between the males originated from cryopreserved ($87 \pm 5\%$) and fresh ($86 \pm 13\%$) sperm. However, on the progressive motility of cryopreserved sperm both the sampling date ($p < 0.001$) and the origin of males (fresh or cryopreserved sperm) had a significant effect ($p = 0.024$) in the first generation which did not differ in the second generation. This can be explained by the fact that spermatozoa and also the sperm compound are different in fish species.

Neither in carp nor in zebrafish was there any difference between the density of sperm analysing the 2 groups. However the post-thaw motility was

significantly different from the fresh motility which corresponded to the findings of other studies.

Some studies pointed out that the origin of cryopreserved sperm had a positive effect on the growth, while others found the contrary. The cryopreservation of sperm can also cause deformities in the offspring. This agrees with the dissertation's results, where the fish originating from fertilization with cryopreserved sperm had a smaller head, lower back and narrower caudal peduncle than those originating from fresh sperm. However, in the length of the individuals there was no signifficant difference (p = 0,4078).

5 Suggestions

The microplate reader as well as the CASA are suitable for measuring sperm density. The samples must be immobilized before the measurements in order to get accurate measurements. However, the method has to be standardised for every device. The observed objective can be different in CASA systems as well as the optimal value of the sample in the microplate reader.

The adjustment of sperm concentration before cryopreservation can have an effect on the fertilizing capacity. This can be caused by the fact that cryopreserving more cells, more will be alive after thawing. In the researches of the dissertation we only found significant difference in the linearity working with variable densities. This means that for getting higher fertilization rate adjusting higher density prior to cryopreservation can be considered.

The origin from fresh or cryopreserved sperm had not significantly affected the density of the sperm and the fertilization capacity neither in zebrafish nor in carp. In zebrafish, sperm motility parameters were not significantly different in the two groups. However, in the first generation of carps the post-thaw progressive motility was significantly different between the two groups. Although the sampling date had also affected the post-thaw progressive motility, this difference did not show in the second generation, which can be caused by the variability of the individuals. This means that it cannot be stated that the origin of the fish definitely affects post thaw motility parameters. Another factor can be the quality of sperm which can be different by sampling the same individual, also. This means that the monitoring of sperm motility parameters can be useful prior to cryopreservation.

In the second generation of carps there was a morphological difference between the fish originated from fresh and cryopreserved sperm. Since all of the fish originated from fresh sperm were different from the fish originated from cryopreserved sperm we found that the cryopreservation of the gametes can have an affect on the morphology of the offspring. In this case the cryopreservation caused a smaller head, lower back and narrower caudal peduncle. It can be stated that in the cryopreservation process spermatozoa are being selected. That should be considered in gene banking where the preservation of the genetic diversity is important.

Further investigations are needed to measure the impact of the cryopreservation on the pheno- and genotypes of the offspring. This

differences cannot always be measured by general methods like lenght measurements. In those species where the morphometry of the fish is important, cryopreservation can be used as a tool to create the suitable phenotypes. For example in case of *Clarias gariepinus* where the big head can be a problem, guided selections can offer a solution. It would be also important to measure the cryopreservation effect on the skeleton and the organs in the offspring.

The cryopreservation of sperm had its advantages and disadvantages also. In the dissertation we found that sperm density and fertilizing capacity were not affected by the origin of males. The only exepcion was in the post-thaw progressive motility of the sperm in the first generation in carps. This means that cryopreserved sperm can be used to create other generations because it does not affect the offspring's sperm parameters. On the contrary, in the second generation of carps, the morphology of the fish was influenced by the origin. This means that it cannot be stated that cryopreservation of sperm can be freely used because it does not have an affect on the offspring.

In case of *Salmo salar* where the supply and demand are similar and stable there is a need for guaranteed fertilization. In these conditions the farmers can afford to buy cryopreserved sperm to stabilize the fertilization rate. This also requires a reliable company. This is the reason cryopreservation of fish sperm could spread in the northern countries. On the contrary, in Hungary the demand for the fish meat is seasonal. However the African catfish could have a potential because for the breeding the rare *Heterobranchus longifilis* is needed. In this species further studies about the offspring hatched from fertilization with cryopreserved sperm would be required.

6 New scientific results

1. In zebrafish there was no significant difference in sperm motility parameters, density and fertilization capacity between the groups originated from fresh and cryopreserved sperm. In the first generation of carps significant difference was found in post-thaw progressive motility between the males originated from fresh and cryopreserved sperm, however other motility parameters, density and fertilization capacity had not been affected. I found that in the case of carps the sperm motility parameters can be affected by the origin of the sperm (cryopreserved, fresh).
2. I found that the origin of the males (hatched from fertilization with fress or cryopreserved sperm) can affect the morphology of the second generation in carps (smaller head, lower back and narrower caudal peduncle).
3. I found linear correlation between the absorbance of the microplate reader, the measuring of the CASA and the manual cell counting in Bürker-Türk type hemocytometer. This can be described in the case of the microplate reader as $y = 1.363 \times 10^{11}x + 1.576 \times 10^{9}$ and in the case of CASA as $y = 0.7317x + 8.555 \times 10^{8}$. With those equations the sperm density can be easily and fastly calculated.
4. In carps the adjustment of sperm density prior cryopreservation only caused a significant difference in linearity. The other motility parameters were not affected.
5. In carps the adjustment of sperm density prior cryopreservation caused a significant difference between the 1:9 dilution ratio and the 4×10^{9} spermatozoa/ mL. I found that the adjustment of sperm density prior cryopreservation can affect the fertilization capacity of the sperm. The higher density can result higher fertilization.

7 Publications related to the topic of the dissertation

7.1 Conference proceedings

2019: **Bernadett Pataki**, Tímea Kollár, Zoran Marinović, Jelena Lujić, Gyöngyi Gazsi, Roberta Izabella Berta, Béla Urbányi, Ákos Horváth. Zebrafish *(Danio rerio)* sperm cryopreservation. 54th Croatian & 14th International Symposium on Agriculture, Vodice-Croatia, February 17 - 22, 2019

2019: **Bernadett Pataki**, Tímea Kollár, Zoran Marinović, Jelena Lujić, Gyöngyi Gazsi, Roberta Izabella Berta, Béla Urbányi, Ákos Horváth. Inheritance of sperm cryoresistance in zebrafish *(Danio rerio)*. 7th International Workshop on the Biology of Fish Gametes, Rennes-France, September 2- 6, 2019

2019: **Bernadett Pataki**, Logan Andrew Goddard, Béla Urbányi, Tímea Kollár, Ákos Horváth. Investigation of sperm agglutination and a new method for measuring sperm concentration in common carp *(Cyprinus Carpio)*. Aquaculture Europe 2019. Berlin-Germany, October 7-10, 2019

2019: **Pataki Bernadett**, Berta Izabella Roberta, Gazsi Gyöngyi, Goddard Andrew Logan, Marinović Zoran, Lujić Jelena, Urbányi Béla, Kollár Tímea, Horváth Ákos. Mélyhűthetőség öröklődésének vizsgálata zebradánióban *(Danio rerio)*. 25. Szaporodásbiológiai Találkozó. Balatonkenese, November 08-09.

2020: **Pataki Bernadett**, Urbányi Béla, Kollár Tímea, Horváth Ákos. Három különböző módszer összehasonlítása a pontysperma *(Cyprinus carpio)* koncentrációjának méréséhez. XLIV. Halászati Tudományos Tanácskozás. Szarvas, Szeptember 23-24.

2021: **Bernadett Pataki**, Tímea Kollár, Roberta Izabella Berta, Béla Urbányi, Ákos Horváth. Inheritance of sperm cryoresistance in zebrafish *(Danio rerio)*. The 56th Croatian & 16th International Symposium on Agriculture. Vodice, Horvátország, Szeptember 20-25.

2021: **Bernadett Pataki**, Béla Urbányi, Tímea Kollár, Ákos Horváth. Inheritance of sperm cryoresistance in common carp *(Cyprinus carpio)*. Aquaculture Europe 2021. Funchal, Madeira, Október 4-7.

2021: **Pataki Bernadett**, Urbányi Béla, Kollár Tímea, Horváth Ákos. Mélyhűthetőség öröklődésének vizsgálata pontyban *(Cyprinus carpio)*. 26. Szaporodásbiológiai Találkozó. Balatonkenese, November 5-6.

2022: **Pataki Bernadett**, Staszny Ádám, Mészáros Gergely, Kitanović Nevena, Ács András, Hegyi Árpád, Molnár József, Csorbai Balázs, Urbányi Béla, Horváth Ákos. Morfológiai változások pontyban *(Cyprinus carpio)*: befolyásolja-e az utódok külalakját a mélyhűtött spermával történő termékenyítés? XLVI. Halászati Tudományos Tanácskozás. Szarvas, Május 25-26.

2022: **Bernadett Pataki**, Ádám Staszny, Gergely Mészáros, Nevena Kitanović, András Ács, Árpád Hegyi, József Molnár, Balázs Csorbai, Béla Urbányi, Ákos Horváth. Morphological changes in common carp (Cyprinus carpio) progeny induced by the use of cryopreserved sperm. 57th Croatian & 17th International Symposium on Agriculture. Horvátország, Vodice, Június 19 – 24

7.2 Publications in scientific journals

Uros Ljubobratovic, Géza Péter, Ferenc Zoltán Demény, Nándor Kugyela, Akos Horvath, **Bernadett Pataki**, Zoltán Horváth, Zsuzsanna J. Sandor, Andras Rónyai. Evaluation of the optimal oocyte diameter for artificial reproduction in virgin pikeperch *(Sander lucioperca* L.*)* in fully controlled conditions relating to the different dietary levels of arachidonic acid. Aquaculture Reports, Volume 18, November 2020. DOI: 10.1016/j.aqrep.2020.100430

Bernadett Pataki, Ákos Horváth, Gergely Mészáros, Nevena Kitanović, András Ács, Árpád Hegyi, József Molnár, Balázs Csorbai, Béla Urbányi. Adjustment of common carp sperm concentration prior to cryopreservation: Does it matter? Aquaculture Reports, Volume 24, June 2022. DOI: 10.1016/j.aqrep.2022.101109

8 Publications not related directly to the topic of the dissertation

8.1 Conference proceedings

2020: Nguyen Quyen, **Pataki Bernadett**, Nevena Kitanović, Horváth Ákos, Havasi Máté Keszte Szilvia, Urbányi Béla, Harmut Greven, Müller Tamás. Kísérletek az afrikai harcsa természetes ívási viselkedésének részletes feltárására In: Biró Janka (Bíró Janka Haltakarmányozás) (Ed.) Halászatfejlesztés 37: A XLIV. Halászati Tudományos Tanácskozás kiadványa. Konferencia helye, ideje: Szarvas, Magyarország 2020.09.23. - 2020.09.25.

8.2 Publications in scientific journals

Bernadett Pataki, Berta Izabella Roberta, Gyöngyi Gazsi, Béla Urbányi, Tímea Kollár & Ákos Horváth. Effect of age on the mercury sensitivity of zebrafish *(Danio rerio)* sperm. FISH PHYSIOLOGY AND BIOCHEMISTRY Volume 46 Issue 5, October 2020. DOI: 10.1007/s10695-020-00875-9

Uros Ljubobratović, Ferenc Zoltán Demény, Géza Peter, Oleksandr Malinovskyi, Maciej Kwiatkowski, **Bernadett Pataki**, Ákos Horváth. Can artificial reproduction strategies (hormonal type and dose/thermal regime) affect gamete quality in indoor-reared pikeperch *(Sander lucioperca)*? Aquaculture Reports, Volume 23, April 2022. DOI: 10.1016/j.aqrep.2022.101032