SZENT ISTVÁN EGYETEM

PRACTICAL IMPROVEMENT OF FISH SPERM QUALITY ASSESSMENT METHODS

Theses

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1. INTRODUCTION

Cryopreservation is an assisted reproductive technology that can be very useful both in fish farming and in conservation work. The cryogenic storage of fish sperm could efficiently support the synchronization of spermiation and ovulation during the spawning season, preservation and trade of high-quality gametes, gene banking for the conservation of endangered species, and simplification of broodstock management (Cabrita et al. 2010). Initial success in sperm cryopreservation occurred at about the same time for aquatic species and livestock. However, in the 50-plus years since then, cryopreserved sperm of livestock has grown into a billion-dollar global industry, while cryopreserved sperm of aquatic species remains a research activity with little commercial application (Tiersch 2008).

The applicability and cost efficiency of cryopreservation tecnology can be increased with the development of protocols (Tiersch 2008). The lack of standardization resulted in a high variabilty of protocols which prevented the involvement of sperm manipulation techniques in the aquculture industry (Cabrita et al. 2010, Tiersch 2008). Information regarding fish reproduction biology and genetics needs to be collected into a systematically developed database. Selective and long-term storage, as well as gamete quality assessment, are important parts of the system. The development of the databases and the infrastructure (cryopreservation stations) makes fish sperm cryopreservation available as a service. A remarkable step in the improvement of cryopreservation methods are the scientific work and the education of experts. Financial support is needed for the involment of sperm preservation in the aquaculture practice. International funds can enhance the standardization process (AQUAGAMETE Cost Action 2015, Tiersch 2008).

1.1. Aims

In my study, the following topics were investigated:

1. Post-thaw quality of cryopreserved sperm in two endangered Acipenseriform species (Siberian sturgeon-*Acipenser baerii* and Russian sturgeon-*Acipenser gueldenstaedtii*).
2. Optimization of sperm quality assessments and cryopreservation protocols in Eurasian perch (*Perca fluviatilis*).
3. Standardization of sperm cyropreservation in the common carp (*Cyprinus carpio*).
4. Investigation of short-term and post-thaw storage methods, and the cryopreservation of sperm in different goldfish lines.
5. Post-thaw quality of brown trout (*Salmo trutta* m. *fario*) and marble trout (*Salmo marmoratus*) sperm. Comaprison of methanol and DMSO as cryoprotectants during sperm cryopreservation. Investigation of the effective sperm to egg ratio and comparison of 3 different freezing protocol in the Adriatic grayling (*Thymallus thymallus*).

2. EXPERIMENTS, RESULTS AND DISCUSSION IN THE INVESTIGATED FISH TAXA

2.1. General methodology

During stripping, spawners were handled using a wet towel and gametes were obtained via abdominal massage. Sperm was stored at 4 °C whereas eggs were stored at a temperature according to the spawning season of the species (cold water spaswning: 4 °C, warm water spawning-hatchery conditions) Fresh and thawed sperm was investigated using two different CASA systems (in Hungary and Slovenia: Sperm VisionTM v. 3.7.4., Minitube of America, Venture Court Verona, Poland: Sperm Class Analyzer v. 4.0.0., Microptic S.L., Barcelona). Sperm was activated with different solutions according to the experimental design using a Makler chamber (Sefi-Medical Instruments, Israel). To avoid sticking cells in the chamber, activating solutions were mixed with 0.01 g/mL bovine serum albumin (BSA). Measurements were carried out at least in duplicates. The parameres [progressive motility (pMOT), curvilinear velocity (VCL) and straightness (STR)] describing different traits of sperm movement were chosen according to the literature (Rurangwa et al. 2004, WHO 2010a, Wilson-Leedy és Ingermann 2007). Sperm density was measured using a Bürker chamber (Marienfield Superior, Paul Marienfield GmBH) and a light microscope (Nikon Eclipse E600, Auroscience Ltd., Budapest). For cryopreservation of sperm, 0.5 mL straws were used. Samples were immobilized in different solutions and extenders (according to the experimental design) and were mixed with 10% methanol as cryoprotectant. In my study, two cryopreservation methods were used. According to the experimental design, a controlled-rate freezer (IceCube 14s, IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf) with a cooling program (from 7.5 ºC to -160 ºC, cooling rate: 56 ºC/min) or a styrofoam box with a floating polystyrene frame (straws frozen 3 cm above the surface of liquid nitrogen for 3 minutes) were used. The cooling program was established experimentally. Straws were stored in 10 (Bio 20, Statebourne Cryogenincs, UK) and 35 L

(VWR XSS 48/10, VWR Inernational Ltd., Debrecen) dewars. Straws were thawed for 13 seconds in a water bath (ThermoHaake P5, Thermo Electron Corp, Waltham) at 40ºC (Horváth et al. 2012). Chemicals were purchased from Reanal (Budapest), POCH (Gliwice) and Sigma-Aldrich (Budapest). Fertilization rate was calculated using a stereomicroscope (Leica MZ16.5, Wetzlar, or Carl Ceiss Jena Technival, Carl Zeiss). Data were analized using Microsoft Excel (Microsoft Corporation, Redmond) and SPSS 14.0 statistical software (SPSS, Chicago) and GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla). Normal distribution of data was verified using a Kolmogorov-Smirnov, Shapiro-Wilk, and Jarque-Berraval test at a significance level of $P \leq 0.05$. Data were logarithmized (VCL) or transformed using arcsine square root (pMOT, STR, fertilization rate) if distribution was not normal. To calculate the differences among groups, One-way ANOVA, Multi-way ANOVA, Kruskal-Wallis, Welch, Student's unpaired t-test, Mann-Whitney tests followed by post-hoc Tukey, Bonferroni, Dunn's, Dunnett's T3 post-hoc test were used at the significance level of $P \leq 0.05$. The correlation among variables was analyzed by full factorial Generalized Linear Model and standard Multi-way regression (significance level $P \leq 0.05$).

Changes from the above mentioned methods were presented in the experimental design belonging to different fish species.

Table 1. Abbreviations.

Complete name	Abbreviation in text
Bovine serum albumin	BSA
Computer-assisted Sperm Analysis	CASA
Controlled-rate freezer	CRF
Curvilinear velocity	VCL
Distilled water	Dw
Hank's Balanced Salt Solution	HBSS
Horváth et al. method (Horváth et al. 2012)	Hm
Lahnsteiner et al. method (Lahnsteiner et al. 1996)	Lm
Common activating solution for perch (Lahnsteiner 2011)	Ca
Modified Lahnsteiner's activating solution (Lahnsteiner 2011)	La
Modified Lahnsteiner's immobilizing solution (Lahnsteiner 2011)	Li
Modified Grayling extender	MGe
Modified Tanaka extender (Szabó et al. 2005)	Te
Nynca et al. method (Nynca et al. 2015)	Nm
Grayling extender (Horváth et al. 2012)	Ge
Seminal fluid	Sf
Activating solution for cyprinids (Saad et al. 1988)	As
Progressive motility	pMOT
Propidium-iodide	PI
Straightness	STR
Woynárovich solution (Woynárovich and Woynárovich 1980)	Ws

2.2. Experiments in Acipenserids

Table 2. General methodology in Acipenserids.

Species	Siberian and Russian sturgeon
Motility assesments	Visual observation (light microscope) and CASA (after thawing), activation: salt solution (Horváth personal communication)
Cryopreservation	Modified Tsvetkova's extender (Glogowski et al. 2002), 1:1 dilution ratio

Effect of post-thaw storage on the motility of cryopreserved sperm (*N*=10)

Thawed sturgeon sperm was stored for up to 12 hours (pMOT: 1 hour interval).

Effect of post-thaw storage on viability of cryopreserved sperm (*N*=10)

Thawed sturgeon sperm was stored for 12 hours and was stained every 3 hours (*SYBR green*-intact and PI-damaged).

2.2.1. Results in Acipenserids

Thawed sperm of Siberian sturgeon showed a significant reduction in pMOT after 6 to 12 hours in comparison to 0 hours (50±24%). The viability did not show a significant decrease. A significantly lower pMOT was recorded in Russian sturgeon after 2 to 12 hours in comparison to 0 hours (32±10%). The above mentioned phemonenon was observed during the membrane integrity test in this species.

2.2.2. Discussion in Acipenserids

The sperm could be stored for a long period following thawing in both species. Milt seems to be more sensitive for storage time in Russian storgeon than in Siberian.

2.3. Experiments in Eurasian perch

Table 3. General methodology in Eurasian perch

Sampling	120 males and 36 females
Motility assesment	2 kinds of CASA (fresh and thawed sperm, Chapter **2.1.**)
Solutions	Immobilizing solutions/extenders: Ge Te, Li Activating solutions: As, Ca, La, Ws
Cryopreservation	Styrofoam box and CRF (Chapter **2.1.**)

2.3.1. Optimization of sperm quality assesment and cryopreservation in Eurasian perch

In the cryopreservation experiments a Styrofoam box was used.

The best immobilizing solution (*N*=10)

Fresh sperm was diluted in Te, Ge and Li and stored for 6 hours. (pMOT: at 2-hour intervals).

Best activating soulution I. (*N*=10)

Fresh sperm was activated with As, Ca, La and Ws. (pMOT: at 10, 30, 60, 90 and 120 seconds following activation).

Best activating soulution II. (*N*=10)

The above mentioned activators were used in the experiment. (pMOT: at 10 and 20 seconds following activation)

Short-term storage of fresh sperm (*N*=10)

Undiluted sperm was stored for 6 hours (pMOT: 1-hour interval).

The best extender for cryopreservtion (*N*=7)

Sperm was cryopreserved using Te and Li at a dilution ratio 1:40.

The best dilution ratio for cryopreservation (*N*=10)

Sperm was cryopreserved with Te using the dilution ratios 1:1, 1:5, 1:10, 1:20, 1:30, and 1:40.

Post-thaw storage (1 or 6 hours) of perch sperm (*N*=10)

Thawed sperm (Te, 1:20) was stored for 1 or 6 hours. (pMOT: at 15- and 120-minute intervals).

Fertilizing capacity of thawed sperm (*N*=9)

Thawed sperm (Te, 1:20) was used for fertilization of eggs.

2.3.2. The comparison of two cryopreservation methods in Eusrasian perch

Comparison of the two methods (*N*=10)

To set up a cooling program for the CRF, the cooling rate was measured 3 cm above the liquid nitrogen for 3 minutes using a thermocouple attached to a digital thermometer. Sperm was cryopreserved using both Styrofoam box and CRF.

The best dilution ratio for CRF (*N*=4)

Sperm was cryopreserved (Te, CRF) at the dilution ratios 1:5, 1:10, 1:20.

2.3.3. Increased-scale cryopreservation in off-season collected Eurasian perch sperm

The effect of equilibration time on the quality of fresh-diluted and thawed sperm (N=5)

The motility of diluted and thawed (Te, Styrofoam box, 1:20) sperm was recorded following an equilibration of 0, 30 or 60 minutes.

Correlation between dilution ratio, cell concentration and post-thaw quality (*N*=5)

The correlation among thawed pMOT (CRF, Te), sperm density (Chapter **2.1.**) and dilution ratios (1:5, 1:10, 1:20) was investigated.

The post-thaw motility duration with 4 different activating solutions (*N*=6)

Thawed sperm was activated with As, Ca, La and Ws. (pMOT: at 10, 30, 60, 90 and 120 seconds following activation).

Increased-scale cryopreservation (*N*=6)

Sixty-seven straws were cryopreserved using Th, 1:10, CRF. Ten straws were used for fertilization whereas pMOT was recorded in the remaining 57.

2.3.4. Results in Eurasian perch

The highest pMOT was obtained at 0, 2, 4 and 6 hours of storage time using Li. After 30 seconds pMOT decreased below 5% with all tested activating solutions. The lowest reduction in progressive movement was recorded with La at 20 seconds. Following thawing, no significant difference was recorded in pMOT, VCL and STR with the 4 tested activators at 10 to 120 seconds. pMOT decreased significantly after 2 hours in stored fresh sperm. Te showed higher pMOT (54±14 %) during cryopreservation than Li (19±17%). The highest progressive motility was recorded with cryopreserved sperm using dilution ratios 1:10 and 1:20. Significant reduction was recorded in pMOT after 2 hours following thawing. Both cryopreservation techniques (CRF and Styrofoam box) resulted in similar pMOT, VCL and STR. No significant difference was recorded in pMOT and STR in diluted sperm following a 60-minute equilibration. Similar motility parameters were recorded in thawed sperm using 0, 30 or 60 minutes of equilibration. Sperm cryopreserved in a Styrofoam box showed a high fertilizing capacity (75±6%) and pMOT (58±25%). Optimal progressive motility (37±7%), VCL (92±10 μm/s), STR (89±3%), and a similarly high fertilization rate (72±14%) was observed with thawed sperm using CRF.

2.3.5. Discussion in Eurasian perch

The results of 6 hours dilured storage showed that the most effective immobilizing solution was Li. There is a narrow time interval for CASA measurements (10-20 seconds) in Eurasian perch. La can be used effectively as activator. In the long run, a similar decreasing tendency was observed both in fresh and thawed sperm. The simple mixture of NaCl and water (Ca) can be used both in sperm experiments and in the hatchery practice (fertilization) as an activator using cryopreserved sperm. Both fresh and thawed sperm showed sensitivity already at 2 hours of storage. Tanaka extender originally described for the sperm of the European eel (*Anguilla anguilla*) (Szabó et al., 2005) can be successfully applied to the cryopreservation of perch sperm. Sperm showed higher tolerance against cold shock using dilution ratios of 1:10 és 1:20. The Styrofoam box and CRF can be used for cryopreservation of low and high number of straws with a high efficiency. In effect, the findings of our study allow recommending one hour as a secure equilibration time. This allows the loading and handling of numerous straws before cryopreservation and thus creates the basis for commercialization of cryopreservation in this species. Cryopreserved sperm can be applied for fertilization in Eurasian perch (Styrofoam box: 75%, CRF: 72%).

2.4. Experiments in Cyprinids

Table 4. General methodology in Cyprinids.

Species	Common carp	Goldfish
Motility assessment	CASA (fresh and thawed sperm)	
Solutions	Activating solutions: As, Dw Extender: Ge, MGe (less glucose, more KCl), Sf, HBSS	Activating solution and Extender: AS and Ge
Cryopreservation	Styrofoam box CRF (Chapter **2.1.)**	Styrofoam box

2.4.1. Experiments in common carp

The effect of equilibration time on the quality of fresh-diluted and thawed sperm (*N*=6)

The motility of diluted and thawed (Ge, Styofoam box, 1:9) sperm was recorded following 0, 30 or 60 minutes of equilibration.

The comparison of the two methods (*N*=4)

Sperm was cryopreserved using both Styrofoam box and CRF (Ge, 1:9).

Selection of extender (*N*=5)

Sperm was cryopreserved (CRF, 1:9) using three different extenders (Ge, Sf, HBSS).

Carp sperm cryopreservation in 2 different grayling extenders and at 4 different dilutions (*N*=6)

Cryopreservation (CRF) of sperm was carried out with Ge and MGe at dilution ratios of 1:1, 1:5, 1:9 or 1:20.

The post-thaw motility duration with 2 different activating solutions (*N*=6)

Thawed sperm (CRF, Ge, 1:9) was activated with As and Dw. (pMOT: at 10, 30, 60, 90 and 120 seconds following activation

Post-thaw storage (1 and 6 hours) of perch sperm (*N*=6)

Thawed sperm (CRF, Ge, 1:9) was stored for 1 or 6 hours. (pMOT: at 15- or 120-minute intervals).

Increased-scale cryopreservation of carp sperm (*N*=6)

Pooled sperm (104 straws) (CRF, Ge, 1:9) was cryopreseved and tested for fertilization following thawing.

2.4.2. Experiments in goldfish

Chilled storage of goldfish sperm (preliminary experiment, *N*=5)

Sperm was stored at 4 °C and 20 °C for 10 hours (pMOT: at 1-hour intervals).

Chilled storage of sperm in Black moor (*N*=4), Oranda (*N*=3) and Calico(*N*=3) goldfish

Samples were stored at 4 °C for 48 hours (pMOT: at 6-hour intervals).

The comparison of sperm concentration in the 3 goldfish types

The density was recorded in the samples obtained from the above mentioned experiment. Sperm was diluted in Ge (1 µl sperm and 999 µl Ge, Chapter **2.1.**)

Post-thaw storage (6 hours) of sperm in the three goldfish types (*N*=5)

Thawed (Styrofoam box, Ge, 1:9) sperm from all goldfish varieties was stored for 6 hours (pMOT: at 2-hour intervals).

Fertilizatin test in Oranda (*N*=5) and Calico (*N*=5) types

Cryopreserved sperm (Styrofoam box, Ge, 1:9) of Oranda and Calico goldfish was used for fertilization.

2.4.3. Results in Cyprinids

An agglutination phemonemon (50%) was observed after thawing in the sperm samples of carp and goldfish cryopreserved using different grayling extenders.

pMOT, VCL and STR did not reduce significantly neither in diluted nor thawed sperm during 60 minutes of equilibration. Similar motility parameters were recorded using the two cryopreservation methods. Significantly higher pMOT and VCL were observed using Ge than Sf and HBSS. The highest pMOT and VCL was recorded using Ge at a dilution 1:9 (pMOT: 52±12%, VCL: 76±9 µm/s) and 1:20 (pMOT: 49±8%, VCL: 76±6 µm/s). Using As, movement was recorded even after 120 seconds. Motility

parameters following thawing did not reduce significantly during 1 or 6 hours of storage. Increased-scale cryopreserved sperm showed an optimal pMOT (47±5%) VCL (62±9 µm/s) and STR (91±1%) and 32±6% fertilization rate.

In the preliminary study, pMOT of goldfish sperm stored at either temperatures did not reduce significantly for 10 hours storage. However in the group stored at 4 °C, progressive motility (64±15%) was recorded still after 27 hours storage. pMOT and VCL decreased significantly after 48 hours in Oranda compared to the control group (0 hour). Sperm density was similar in all goldfish types. pMOT reduced significantly after 6 hours of storage following thawing in Calico. A low fertilization rate was observed in Oranda and Calico using thawed sperm.

2.4.4. Discussion in Cyprinids

The 60 minutes equilibration did not have any effect on diluted and fresh carp sperm. Both techniques are capable for carp sperm cryopreservation. The one-step cooling program with a relatively high cooling rate (56 °C/min) can result in a high pMOT following thawing. According to my study, a dilution of 1:9 and the Ge (with higher sugar content) is recommended to be used for carp sperm cryopreservation. For fertilization, the use of the cyprinid activating solution is recommended as in this case, spermatozoa have a longer possible longevity. pMOT remained high still after 6 hours of storage in thawed sperm which can enhance its applicability time for fertilization. The reduction in fertilization was associated with the agglutination of thawed sperm.

According to my findings, Oranda was more sensitive for chilled and Calico goldfish was more sensitive for thawed storage. The decreased fertilization rate was caused by the effect of egg storage during transportation and the agglutination of thawed sperm.

2.5. Experiments in Salmonids

Table. 5. General methodology in Salmonids.

Species	Brown trout	Marble trout	Adriatic grayling
Motility assessment	CASA (fresh and thawed sperm, Chapter **2.1.**)	Light microscope, CASA (fresh and thawed sperm, Chapter **2.1.)**	
Solutions	Activating solution: Billard's activationg solution (Billard 1977)		
	Extender: Ge		See in experimental design
Cryopreservation	Styrofoam box, 1:4		Styrofoam box, See in experimental design

2.5.1. Experiments in brown trout

Motility during 60 minutes of post-thawing storage (*N*=6)

pMOT of cryopreserved (1:4) sperm was recorded at 0, 10 or 60 minutes following thawing.

Fertilization test (*N*=6)

Fertilization was carried out with the above mentioned ssmples 0, 10 or 60 minutes following thawing.

2.5.2. Experiments in marble trou

Motility during 60 minutes of post-thawing storage (*N*=5)

Sperm was cryopreserved (Ge, 1:4) using 10% methanol or dimethyl sulfoxid (DMSO). pMOT of cryopreserved sperm was recorded 0, 10 or 60 minutes following thawing.

Fertilization test (*N*=5)

Fertilization was carried out with the above mentioned samples (with both cryoprotectants) 0, 10 or 60 minutes following thawing.

2.5.3. Experiments in Adriatic grayling

Sperm concentration measurements

In my studies (in the years 2014 and 2015) sperm density was measured (1:99 or 1:999, Ge, method: Chapter **2.1.**).

Cryopreservation (*N*=5)

In 2014 (comparison of different sperm-to-egg ratios), Hm (Ge, 1:1) was used. In 2015, 3 published methods were compared (Hm, Lm (ion based extender, 1:3), Nm (glucose extender, 1:5)).

Fertilization test (*N*=5)

In 2014, 4 sperm-to-egg ratios (10^3, 5×10^3, 10^4, 5×10^4 spermatozoon/egg) was tested using cryopreserved sperm. In 2015, sperm cryopreserved with the above mentioned methods was used for fertilization.

2.5.4. Results in Salmonids

The highest pMOT (56±15%) and fertilization rate (45±22%) in brown trout was recorded at 10 and 60 minutes after thawing. In the marble trout, sperm cryopreserved with methanol showed a significantly higher fertilization and hatching rate at 0 and 10 minutes following thawing than using DMSO. A significant reduction was recorded in fertilization and hatching rates between 0 and 60 minutes using methanol. A similar tendency was observed with DMSO between 0 and 10 minutes, as well as 0 and 60 minutes. In the Adriatic grayling, the highest fertilization (60±7%) and hatching rate (53±7%) was obtained using a ratio $5x10^4$:1 (spermatozoon/egg) which was similar to the control. Lm showed significantly lower pMOT and fertilization rate than Hm. Hm and Nm resulted similar pMOt, fertilization and hatching rate.

2.5.5. Discussion in Salmonids

According to the results, thawed sperm showed a high tolerance against storage time whereas marble trout sperm was more sensitive for the 60-minute storage following thawing. Methanol is a

more effective cryoprotectant for sperm cryopreservation in marble trout than DMSO. The results showed that a lower sperm-to-egg ratio (5×10^4:1) than 1.5×10^5 (Nynca et al. 2015) can be applied for fertilization. Hm and Nm showed a similarly high fertilization rate as the fresh control. Hm (0.5 mL) seems to be more beneficial for hatchery use than Nm (0.25 mL) because of its higher straw volume.

2.6. New scientific results

1. The effective storage time for cryopreserved sperm following thawing was defined in Siberian (6 hours) and Russian (2 hours) sturgeons.
2. An optimal sperm quality assessment (modified Lahnsteiners immobilizing and activating solution, common activating solution for perch) and 2 cryopreservation methods [modified Tanaka extender, 1:10 and 1:20 dilution ratios, 3 cm above the surface of liquid nitrogen for 3 minutes or controlled rate freezer (from 7.5 ºC to -160 ºC, cooling rate: 56 ºC/min)] was established in the Eurasian perch. A high fertilizing capacity was recorded using small and increased-scale cryopreserved sperm.
3. Parameters (Grayling extender, 1:9 dilution ratio) of carp sperm cryopreservation were optimized. The method was successfully tested in the controlled rate freezer (from 7.5 ºC to -160 ºC, cooling rate: 56 ºC/min) for increased-scale sperm cryopreservation.
4. An effective time frame was observed for post-thaw storage time in brown (60 minutes) and marble trout (10 minutes) sperm. In the marble trout a clear evidence was obtained that methanol is more efficient for sperm cryopreservation than DMSO.
5. A small sperm to egg ratio (5×10^4:1) was successfully used for fertilization with cryopreserved sperm in the grayling. An efficient and beneficial method for grayling sperm cryopreservation was experimentally choosen.

2.7. Proposals

According to my results, I would like to make the following suggestions to improve the applicability of the methods in scientific and practical use:

1. I suggest the application of cryopreserved sperm in Siberian sturgeon sperm at most until 6 and 2 hours following thawing.
2. For perch sperm quality assessment, I suggest to use modified Lahnsteiner's immobilizing and activating solution or common activating solution for perch.
3. For perch sperm cryopreservation I suggest the application of 1:10 dilution ratio, modified Tanaka extender, and the controlled-rate freezer.
4. I suggest for cryopreservation in common carp sperm the grayling extender, 1:9 dilution ratio and the controlled-rate freezer.
5. I suggest the application of brown and marble trout sperm at most 1 hour following thawing.
6. In the Adriatic grayling, I suggest to use the method published by Horváth et al. (2012) and a sperm-to-egg ratio of 5×10^4:1.

Publications related to the topic of the dissertation

Publications in scientific journals

1. Horváth, Á., Jesenšek, D., Csorbai, B., Bokor, Z., Raboczki, É., Kaczkó, D., **Bernáth,G.,** Hoitsy, G., Urbányi, B., Bajec, S.S., Snoj, A. (2012). Application of sperm cryopreservation to hatchery practice and species conservation: A case of the Adriatic grayling (Thymallus thymallus). Aquaculture *358–359*, 213–215.
2. Żarski, D., Horváth, Á., **Bernáth, G**., Palińska-Żarska, K., Krejszeff, S., Müller, T., and Kucharczyk, D. (2014). Application of different activating solutions to in vitro fertilization of crucian carp, *Carassius carassius* (L.), eggs. Aquacult Int *22*, 173–184.
3. Horváth, Á., Bokor, Z., **Bernáth, G**., Csenki, Z., Gorjan, A., Herráez, M.P., Urbányi, B., and Jesenšek, D. (2015). Very low sperm–egg ratios result in successful fertilization using cryopreserved sperm in the Adriatic grayling (*Thymallus thymallus*). Aquaculture *435*, 75–77.
4. Horváth, Á., Labbé, C., Jesenšek, D., Hoitsy, G., **Bernáth, G.**, Kaczkó, D., Bokor, Z., and Urbányi, B. (2015). Post-thaw storage of sperm from various salmonid species. J. Appl. Ichthyol. *31*, 119–124.
5. **Bernáth, G.,** Bokor, Z., Kása, E., Várkonyi, L., Hegyi, Á., Kollár, T., Urbányi, B., Żarski, D., Radóczi Ifj., J., and Horváth, Á. (2015). Comparison of two different methods in the cryopreservation of Eurasian perch (*Perca fluviatilis*) sperm. Cryobiology *70*, 76–78.
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8. **Bernáth, G.**, Bokor, Z., Urbányi, B., Horváth, Á. (2015). Post-thaw quality of cryopreserved sperm in two endangered acipenseriform species. Hungarian Journal of Aquaculture and Fisheries, Vol. 108 (1) pp. 32-36. (in Hungarian)
9. Bokor, Z., **Bernáth, G.**, Kása, E., Várkonyi, L., Hegyi, Á., Kollár, T., Urbányi, B., Zarski, D., Radóczi, J. Ifj., Horváth, Á. (2015). The effectiveness of two different methods in the cryopreservation of Eurasian perch (*Perca fluviatilis*) sperm. Hungarian Journal of Aquaculture and Fisheries, Vol. 108 (3). pp. 25-28. (in Hungarian)

Conference proceedings

1. **Bernáth, G.**, Kaczkó, D., Hoitsy, Gy., Dušan, J., Horváth, Á. (2012). Application of sperm cryopreservation to the conservation of two endemic European salmonid fish. XXXVI. Scientific Symposium of Fisheries, Szarvas, Hungary, may 23-24. 2012., Book of abstracts p. 42. (In hungarian)
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