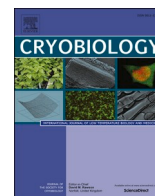




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The systematic development and optimization of large-scale sperm cryopreservation in northern pike (*Esox lucius*)

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ABSTRACT

In our study, a systematic development of a new large-scale sperm cryopreservation protocol was carried out in northern pike (*Esox lucius*). The effect of 2 sugar based (glucose and trehalose) extenders, 3 dilution ratios (1:3, 1:9 and 1:19) 2 vol straws (0.5 and 5 mL) and a 10 mL cryotube, 2 different cryopreservation methods (Polystyrene box-P. box and Controlled Rate Freezer-CRF), as well as 3 different thawing periods (3, 3.5 and 4 min) were investigated on the motility of thawed sperm. The glucose based extender showed significantly higher pMOT (1:3–18 ± 16%, 1:9–20 ± 13%, 1:19–16 ± 12%) at all dilution ratios than in the trehalose based extender (1:3–0.3 ± 1%, 1:9–1 ± 1%, 1:19–4 ± 2%). A similar tendency was recorded in VCL and STR at a ratio 1:3 and 1:9. No significant difference was measured in sperm movement between the P. box and CRF using the 0.5 mL straw. Similarly no significant difference was observed in all motility parameters with 10 mL cryotube frozen in CRF at a ratio 1:3–1:19. An effective and short thawing period (3 min) was experimentally specified for the 10 mL cryotube cryopreserved in the CRF. In all large-scale cryopreservation methods, high pMOT (straw CRF: 57 ± 10%, straw P. box: 50 ± 9%, cryotube CRF: 41 ± 10%), and STR were measured, and no significant difference was recorded in all motility parameters. Our results demonstrate the effectiveness of our newly developed extender and the applicability of 3 different large-scale cryopreservation methods in pike sperm. Our protocols could be new prospective candidates for future exploitation in hatchery practice.

1. Introduction

Fish sperm cryopreservation is an efficient biotechnology method, which enables long term storage of male gametes [8,25]. The application of frozen sperm can simplify the broodstock management, help maintaining valuable lineages of important model species, support the conservation activities in vulnerable wild populations, and increase the genetic variability in farmed broodstocks [13,25]. Numerous protocols have been developed in both freshwater and marine species since one of the first successful sperm cryopreservation in Atlantic herring [9,13]. Protocols focused on commercially interesting and genetically vulnerable species (Acipenseriformes, Cypriniformes, Salmoniformes, Perciformes) [2,13,25]. Despite the increasing scientific interest, only a few published preservation methods were utilized by the aquaculture practice [2]. A lack of standardization and intensification hinder the commercial application of sperm cryopreservation [2,19].

Northern pike has a significant role in maintaining the ecological balance in water systems [3]. The species has a great socio-economic impact as well. Pike is a valuable game fish and is one of the top predators of freshwater systems in Europe [14]. The number of northern pikes is continuously decreasing because of overfishing and the degradation of the environment [14,15]. Its artificial propagation faces with several difficulties [14,15,21]. Commonly, breeders are obtained from wild populations where some individuals reach the maturity later. Males and females need to be synchronized using hormonal treatment. This phenomenon causes individual variation (or reduction) in sperm and egg quality as well [14]. Furthermore, two methods are usually used in males for gamete collection (testicular sperm/squeezing of testis and traditional directly stripping) during propagation. Depletion of the stock using the testicular sperm method is a big disadvantage during the artificial reproduction. On the other hand, direct stripping generally causes low volumes and lower quality of sperm, because is often

Abbreviations: computer assisted sperm analysis, CASA; controlled-rate freezer, CRF; curvilinear velocity, VCL; progressive motility, pMOT; straightness, STR.

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contaminated with urine [20]. Sperm cryopreservation enables the storage of sufficient amount of sperm with the highest quality. The usage of sperm stored on the long term can be designed also in the most convenient time. Cryopreservation of sperm can also help in the optimization of the number of pike males in the broodstock which can reduce the maintenance and production costs [14]. Sperm banks enable also the availability of pike sperm for fertilization, regardless of the synchronization of maturity in breeders [15]. Several studies were published in the last two decades on pike sperm cryopreservation. All methods reached a high fertilization rate in small-scale (0.25–1.2 mL) [3,14,22] etc. Former studies provide a large set of protocols using different extenders, dilution ratios, freezing and thawing protocols (lack of standardization) which makes the hatchery application difficult [14, 26]. Furthermore, according to our knowledge large-scale cryopreservation method of pike sperm optimized for hatchery conditions has not been developed yet.

The aim of our study was to develop a new, efficient extender for pike sperm cryopreservation. Two different types of sugars (glucose vs. trehalose) as well as 3 different dilution ratios (1:3, 1:9 and 1:19) were compared in our experiments. Our new extender (developed in this study) was also tested both in a Polystyrene box and a controlled rate freezer, for small amounts of sperm (0.5 mL straw). In our study, 3 different large-scale freezing techniques (5 mL straw-in polystyrene box and a controlled rate freezer, 10 mL cryotube-in a controlled rate freezer) were tested for the first time. The effective dilution ratio (3 dilutions ranging between 1:3 and 1:19) and thawing time (3 thawing periods in a range of 3–4 min) were also investigated for 10 mL cryotube.

2. Materials and methods

All experiments were conducted in accordance with the Government Decree 40/2013 (II. 14.) on Animal Experiments and the Act XXVIII. Of 1998 of the Hungarian Parliament on the protection and humane treatment of animals. Following consultation with the Scientific Ethical Committee on Animal Experiments of the Ministry of Agriculture, it was concluded that no licences were required for the experiments.

3. Broodstock management and gamete collection

A broodstock of adult pike males ($N = 35$, standard length: 41 ± 8 cm, average body weight: 751 ± 549 g) (reared in earthen ponds, Szegedfish Ltd., Szeged, Hungary) was maintained at the recirculating system at the Department of Aquaculture in Szent István University, Gödöllő, Hungary. Fish were kept in a 1 m^3 plastic tank at an average of 15 ± 2 °C and $8 \pm 1 \text{ mg L}^{-1} \text{ O}_2$ by using mechanical, biological and UV filtration under a 14-h daylight (low light intensity: 10 lux) and 10-h dark photoperiod. Spermiation was hormonally induced using carp pituitary ($3.5 \text{ mg kg bodyweight}^{-1}$) prior to every experiment. Gamete collection was carried out 48 h post-injection. Testis was dissected and was squeezed using a 200 nm mesh. Sperm was stored at 4 °C in 5 mL Eppendorf tubes approximately for up to 30 min prior to the experiments.

4. Motility assessment

Movement of sperm was recorded both before (for control quality) and after cryopreservation using a CASA (Computer-assisted Sperm Analysis) system (Sperm Vision™ v. 3.7.4., Minitube of America, Venture Court Verona, USA). Samples were activated with an ionic solution (100 mM NaCl and 10 mM Tris, pH: 8.0 ± 0.2 , [23]) designed for pike sperm. Motility parameters, such as progressive motility (criteria according to the Sperm Vision™ v. 3.7.4.-straight line distance $>5 \mu\text{m}$, pixel to μm ratio: 151 to 100, pMOT %), curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), and straightness (STR, %) were chosen to evaluate pike sperm movement [6,29]. Measurements were carried out at least in duplicates where spermatozoa were identified ($1\text{--}100 \mu\text{m}^2$) with a digital camera

(JAI CV-A10 CL, Minitube of America, Venture Court Verona, USA) using a frame rate of 60 s^{-1} .

5. Cryopreservation

Sperm was diluted in an extender (75 mM NaCl, 30 mM KCl, 1 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20 mM Tris, and 0.5% BSA, pH: 8.0 ± 0.2) developed in this study based on the seminal fluid composition of muskellunge (*Esox masquinongy*) [24]. Glucose and trehalose (as cryoprotectant) was added to the extender according to the experimental design. Diluted sperm was loaded into 0.5, 5 mL straws and 10 mL cryotubes at a ratio from 1:3 to 1:19 according to the experimental design. Different freezing methods were tested using a Polystyrene box (P. box, internal size of height: 22 cm, width: 25.5 cm, length: 36 cm and the thickness of the walls of 2.5 cm) and a controlled rate freezer (CRF, IceCube 14s, IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria). Frozen sperm was transferred into 48 L storing dewar (VWR XSS 48/10, VWR International Ltd., Debrecen, Hungary). Cryopreserved samples were thawed for various periods (according to the experimental design) in a water bath (Thermo Haake P5, Thermo Electron Corp, Waltham, Massachusetts, USA) at 40 °C. Thawing of 10 mL cryotubes was experimentally investigated. All chemicals were purchased from Reanal (Budapest, Hungary) and Sigma-Aldrich (Budapest, Hungary).

6. Experimental design

6.1. Experiment 1. The comparison of glucose and trehalose based extenders

The above mentioned extender was prepared using 150 mM glucose or trehalose. The sperm from 7 males was diluted in the two extenders at a ratio ranging between 1:3 and 1:19. Sperm was loaded into 0.5 mL straws and cryopreserved in P. box for 3 min at 3 cm above the vapour of liquid nitrogen. Straws were thawed for 13 s [18].

6.2. Experiment 2. the comparison of two freezing methods using 0.5 mL straw

Based on the results of *Experiment 1.*, sperm ($N = 7$) was diluted in the glucose based extender at a ratio of 1:3 and loaded into 0.5 mL straws. Samples were cryopreserved in P. box (mentioned in *Experiment 1.*) and in CRF (Freezing program: from 7.5 °C to -160 °C, cooling rate: 56 °C min^{-1} , [6]). Thawing was performed as mentioned above.

6.3. Experiment 3. the applicability of the 10 mL cryotube at 3 different dilution ratios

Fresh sperm from 7 males was diluted in the glucose based extender at the 3 dilution ratios ranging between 1:3 and 1:19. Samples were frozen in 10 mL cryotubes using the CRF (Freezing program: from 4 °C to -160 °C, cooling rate: $15 \text{ °C minutes}^{-1}$, [11,28]).

6.4. Experiment 4. the comparison of 3 different thawing periods using the 10 mL cryotube

Stripped sperm of 9 males (based on the results of the above mentioned experiments) was diluted in the glucose based extender at a ratio of 1:9. Cryotubes (3 replicates from each male) were cryopreserved in CRF (Freezing program: see above). Samples were thawed for 3, 3.5 and 4 min at 40 °C.

6.5. Experiment 5. the comparison of the 5 mL straw and the 10 mL cryotube frozen in P. box and CRF

Pike sperm ($N = 5$) was diluted in glucose based extender (at a ratio

1:9) and was loaded into 5 mL straw and 10 mL cryotubes. Straws (1-1 straw from each male) were cryopreserved in P. box (for 7 min at 3 cm above the liquid nitrogen [10]) or CRF (Freezing program: identical to Experiment 3-4.). Cryotubes were frozen in CRF. Straws were thawed for 35 s [10] while cryotubes for 3 min.

7. Statistical analysis

Data obtained from CASA measurements was analysed using the statistical software packages SPSS 14.0 (SPSS Inc., Chicago, USA) and GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, California, USA). Kolmogorov-Smirnov test was used to detect normal distribution of data at the significance level of $P \leq 0.05$. Data not showing a normal distribution were transformed using arcsine square-root (pMOT, STR) and logarithm (VCL) function. Fresh (control) and cryopreserved groups were compared using One- and Two-way ANOVA, followed by Tukey's, Dunnett's T3 and Bonferroni post hoc tests (at the significance level of $P \leq 0.05$).

8. Results

8.1. Experiment 1. The comparison of glucose and trehalose based extenders

A significantly higher pMOT was recorded using the glucose based extender (1:3- $18 \pm 16\%$, 1:9- $20 \pm 13\%$, 1:19- $16 \pm 12\%$) at all dilution ratios than in the trehalose based extender (1:3- $0.3 \pm 1\%$, 1:9- $1 \pm 1\%$, 1:19- $4 \pm 2\%$) (Fig. 1). At the dilution ratios of 1:3 and 1:9, a significantly higher VCL was also measured in the glucose group ($77 \pm 29 \mu\text{m s}^{-1}$, $98 \pm 23 \mu\text{m s}^{-1}$) in comparison with the trehalose based extender ($21 \pm 28 \mu\text{m s}^{-1}$, $37 \pm 21 \mu\text{m s}^{-1}$). In STR, the ratio of 1:3 showed significant difference between the two extenders (glucose: $86 \pm 4\%$, trehalose: $38 \pm 48\%$).

8.2. Experiment 2. the comparison of two freezing methods using 0.5 mL straw

No significant difference was recorded between the two freezing methods using the 0.5 mL straw in pMOT (Fig. 2), VCL and STR. A significant difference was measured following thawing in pMOT (P.box: $45 \pm 11\%$, CRF: $42 \pm 17\%$) and STR (P. box: $85 \pm 3\%$, CRF: $86 \pm 4\%$) in comparison with the control (pMOT: $69 \pm 4\%$, STR: $75 \pm 3\%$) group.

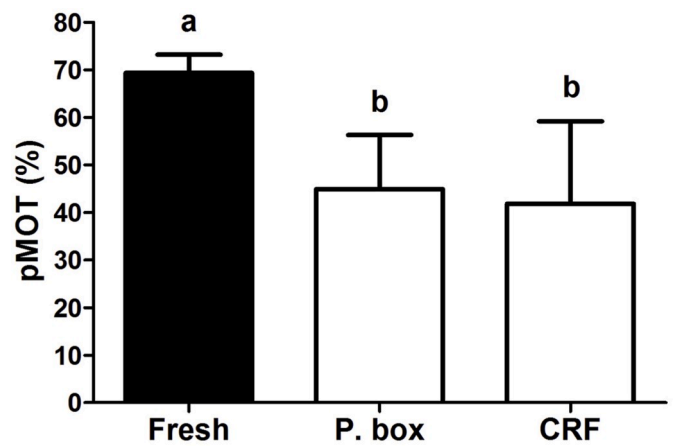


Fig. 2. The progressive motility obtained from the comparison of 2 different cryopreservation methods (P. box, CRF) using 0.5 mL straw (N = 7). Different letters indicate significant difference between the fresh and cryopreserved groups ($p < 0.05$). The columns represent mean and SD.

8.3. Experiment 3. the applicability of the 10 mL cryotube at 3 different dilution ratios

No significant difference was observed in any motility parameters between the dilution ratios of 1:3, 1:9, and 1:19 using the 10 mL cryotube (Fig. 3). A significant reduction was measured however in pMOT (1:3- $20 \pm 10\%$, 1:9- $30 \pm 9\%$, 1:19- $31 \pm 7\%$) and VCL (1:3- $87 \pm 24 \mu\text{m s}^{-1}$, 1:9- $99 \pm 14 \mu\text{m s}^{-1}$, 1:19- $95 \pm 7 \mu\text{m s}^{-1}$) in all cryopreserved groups compared to the control (pMOT: $72 \pm 7\%$, VCL: $140 \pm 6 \mu\text{m s}^{-1}$). STR did not reduce significantly following thawing.

8.4. Experiment 4. the comparison of 3 different thawing periods using the 10 mL cryotube

No significant difference was recorded in any motility parameters between the 3 thawing periods using the 10 mL cryotube (Fig. 4). A significant reduction was measured both in pMOT (3 min: $30 \pm 6\%$, 3.5 min: $29 \pm 6\%$, 4 min: $28 \pm 8\%$) and VCL (3 min: $93 \pm 9 \mu\text{m s}^{-1}$, 3.5 min: $93 \pm 10 \mu\text{m s}^{-1}$, 4 min: $95 \pm 9 \mu\text{m s}^{-1}$) in comparison with the control group (pMOT: $62 \pm 11\%$, VCL: $148 \pm 11 \mu\text{m s}^{-1}$). STR did not show decreasing tendency following thawing.

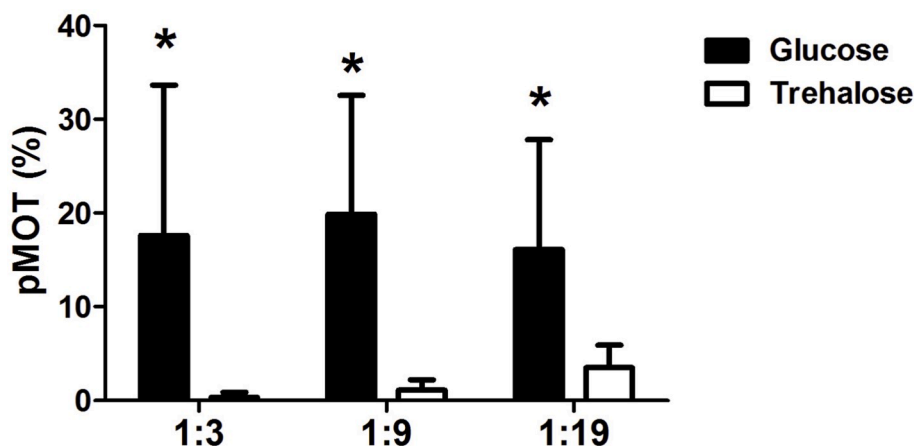


Fig. 1. The progressive motility obtained from the comparison of 2 different extenders (glucose and trehalose based) and 3 different dilution ratios (ranging between 1:3 and 1:19, N = 7). The asterisks indicate significant difference between the 2 extenders at the given dilution ratio ($p < 0.05$). The columns represent mean and SD.

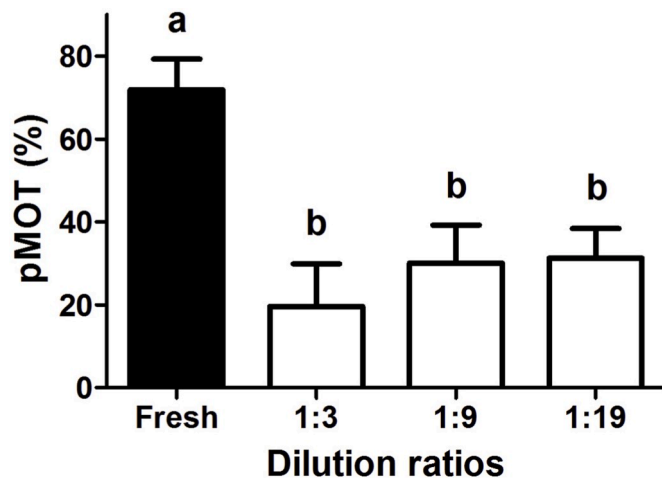


Fig. 3. The progressive motility obtained from the comparison of 3 different dilution ratios (ranging between 1:3 and 1:19) using 10 mL cryotube ($N = 7$). Different letters indicate significant difference between the fresh and cryopreserved groups ($p < 0.05$). The columns represent mean and SD.

8.5. Experiment 5. the comparison of the 5 mL straw and the 10 mL cryotube frozen in P. box and CRF

No significant difference was measured in any motility parameters between the 3 cryopreservation methods. pMOT (straw CRF: $57 \pm 10\%$, straw P. box: $50 \pm 9\%$, cryotube CRF: $41 \pm 10\%$, Fig. 5) and VCL (straw CRF: $86 \pm 14 \mu\text{m s}^{-1}$, straw P. box: $79 \pm 8 \mu\text{m s}^{-1}$, cryotube CRF: $74 \pm 11 \mu\text{m s}^{-1}$) significantly decreased following thawing compared to the control sperm (pMOT: $74 \pm 9\%$, VCL: $136 \pm 7 \mu\text{m s}^{-1}$). STR did not show any significant difference between the fresh and frozen groups.

9. Discussion

In our study the systematic improvement of pike sperm

cryopreservation was carried out, optimized for hatchery practice. A new extender, developed in our work was successfully used for pike sperm cryopreservation. Alavi et al. [1] proved that osmolality above $375 \text{ mOsmol kg}^{-1}$ inhibited the motility of spermatozoa in northern pike. Correspondingly, Lin et al. [24] presented also that an osmolality more than $340 \text{ mOsmol kg}^{-1}$ suppressed totally of muskellunge (*Esox masquinongy*) sperm movement. According to former studies, our new developed glucose-based extender (150 mM glucose, 75 mM NaCl, 30 mM KCl, 1 mM $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$, 1 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 1 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 20 mM Tris, and 0.5% BSA, pH: 8.0 ± 0.2) successfully mimics the composition of the seminal fluid of esocid species where its osmolality ($386 \text{ mOsmol kg}^{-1}$) efficiently prevented the sperm activation. Furthermore, a significantly higher motility was observed using glucose in our new developed extender than with trehalose at 3 different dilution ratios. Babiak et al. [4] already verified the efficiency of the glucose content in the extender (fertilization rate: 6.6–96.0%, depending on the donor male). Furthermore, Dietrich et al. [15] presented a successful application of a glucose methanol based extender during northern pike sperm cryopreservation (hatching rate: 79–89%). Besides, sucrose yielded a high fertilization rate (74.2–84.7%) also using frozen sperm [23]. Contrary to our result, Cejko et al. [14] observed the significant positive impact of trehalose on the efficiency of pike sperm cryopreservation (hatching rate: 73.5%). According to our results and former studies, the sugar content of the extender is a key factor during cryopreservation in northern pike sperm. Our experiment demonstrated that glucose was an effective extracellular cryoprotectant during the freezing process [12].

Northern pike sperm was cryopreserved successfully both with a polystyrene box and a controlled rate freezer (CRF) using 0.5 mL straw. According to our knowledge, the controlled rate freezer has not yet been tested for pike sperm cryopreservation. Nevertheless, successful northern pike sperm cryopreservation was already published by several authors with a similar technique using 0.25–1.2 mL straws [14,15,22,23,30]. Controlled rate freezer enables the optimization of the freezing process and allows a controlled cooling environment. A similar efficiency of both methods was already presented in Eurasian perch (*Perca fluviatilis*) [6]. A high progressive motility was recorded in polystyrene

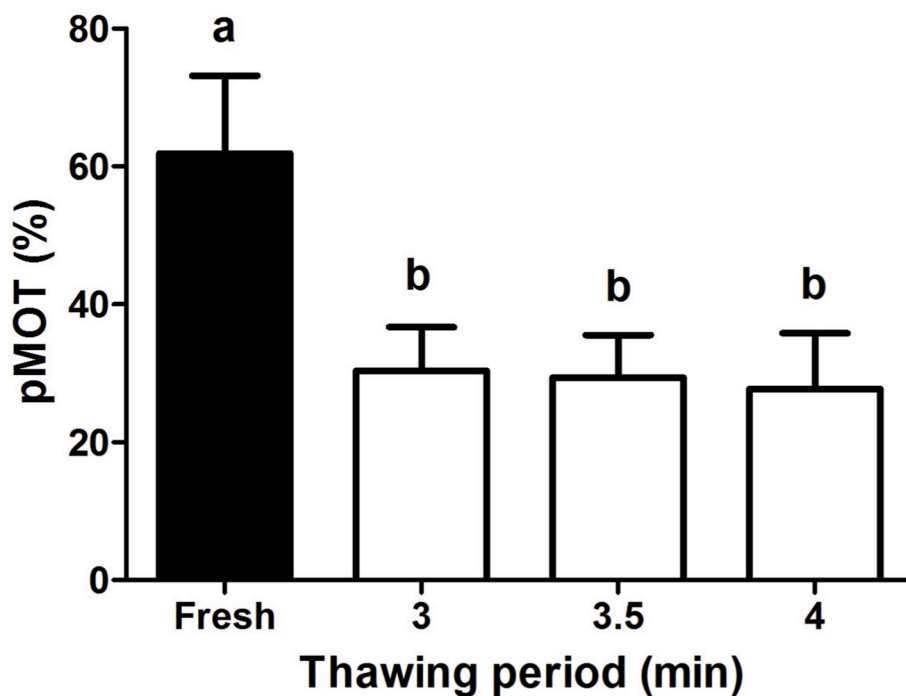


Fig. 4. The progressive motility obtained from the comparison of 3 different thawing periods (ranging between 3 and 4 min) using 10 mL cryotube ($N = 7$). Different letters indicate significant difference between the fresh and cryopreserved groups ($p < 0.05$). The columns represent mean and SD.

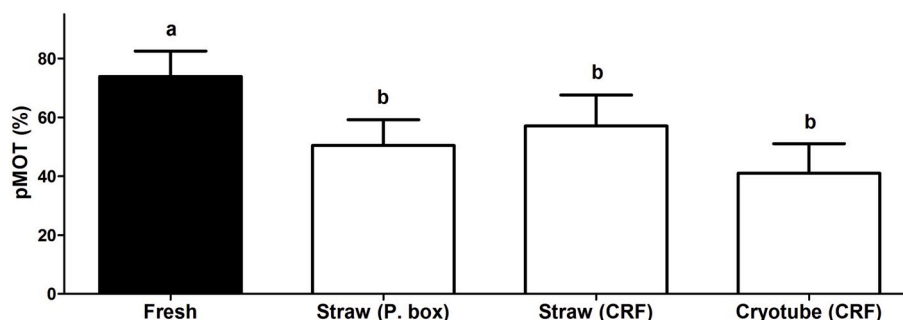


Fig. 5. The progressive motility obtained from the comparison of 3 different cryopreservation methods (5 mL straw in P. box and CRF, 10 mL cryotube in CRF, $N = 5$). Different letters indicate significant difference between the fresh and cryopreserved groups ($p < 0.05$). The columns represent mean and SD.

box ($62 \pm 15\%$) as well as in CRF ($72 \pm 15\%$). Bernáth et al. [7] proved the applicability of large-scale (57 straws) cryopreserved perch sperm using a controlled rate freezer and the similar cooling rate ($56^\circ\text{C min}^{-1}$) during out-of season propagation (fertilization rate: $72 \pm 14\%$) as well. Our results indicate that the new tested freezing method (using the controlled rate freezer) is suitable for pike sperm cryopreservation. The cooling program established in Eurasian perch was successfully adapted to northern pike.

The 10 mL cryotube was efficiently used for the first time in pike sperm cryopreservation. According to our knowledge the highest volume preserved in pike sperm was 1.2 mL so far. Lahnsteiner et al. [23] presented high fertilization rate ($74.2 \pm 0.6\%$) using the mentioned volume of frozen sperm (cryopreserved at -150°C , and at 0.5 cm in the vapour of liquid nitrogen). The method applied in our experiment (Freezing program: from 4°C to -160°C , cooling rate: $15^\circ\text{C min}^{-1}$) was already tested in common carp (*Cyprinus carpio*) [28] and wels catfish (*Silurus glanis*) [11]. The 10 mL cryotube showed a similarly high progressive motility ($27 \pm 6\%$) in common carp than in pike sperm using the same cooling program and extender [28]. Furthermore, the freezing method achieved a similar hatching rate (10 mL cryotube: $66 \pm 6\%$) than the control fresh sperm ($68 \pm 4\%$) in wels catfish at hatchery conditions [11]. Former studies and our results showed that the sperm cryopreservation in 10 mL cryotube is adaptable for different commercially important species. The dilution ratios (1:3–1:19) that were compared in 10 mL cryotube did not affect the motility parameters following thawing. Former studies proved that lower dilutions (1:1–1:5) of sperm and extender can enhance the efficiency of cryopreservation in northern pike sperm [3–5,15–17,22,23,30]. Similarly to our study, Cejko et al. [14] also successfully used a sperm to extender (and cryoprotectant) ratio of 1:9 during pike sperm preservation which indicates the applicability of higher dilution in comparison with the above mentioned studies. Lahnsteiner [22] suggested, that too high dilution using the 0.5 mL straw is inefficient during fertilization (more straws are needed) in salmonid species. However, at higher cell concentration, post-thaw fertility of sperm significantly decreased because of the cell compression (limited cellular space). In our experiment, the 10 mL cryotube contained sufficient amount of pike sperm (at higher dilution as well) which allows an optimized insemination even at hatchery practice in the future.

Thawing periods (ranging between 3 and 4 min) did not show any negative effect on the investigated motility parameters. Thawing rate needed to be optimized species specifically, according to the size of the sperm sample [27]. In the last 25 years, several volumes of straws and pellets were tested in northern pike sperm cryopreservation. Glogowski et al. [17] applied a rapid thawing for 0.07 mL of pellets at 30°C where 0.5 mL straws were thawed for 10–12 s at 40°C . Contrary, Lahnsteiner et al. [23] suggested 30 s at 25°C for the 0.5 mL straw and 30 s at 30°C for the 1.2 mL straw. Cejko et al. [14] tested 0.25 mL straw for pike sperm cryopreservation. Straws were thawed efficiently for 5 s at 40°C . Former studies showed a high variability in the different thawing

techniques according to the size of the container or the freezing method. Our study focused on the improvement of the appropriate thawing rate for the first time for the 10 mL cryotube in northern pike. The results suggest that cryopreserved pike sperm could tolerate even the rapid thawing process if cryopreserved in large amount. From the point of the hatchery practice, the fast thawing process (3 min) can be the most suitable prior to the fertilization.

No significant difference was observed between the 3 tested cryopreservation methods. In this study, 5 mL straw and 10 mL cryotube were tested for the first time (according to our knowledge), using a polystyrene box and a controlled rate freezer. The efficiency of the applied protocols was already presented in common carp [28]. A high progressive motility was recorded in all frozen groups (5 mL straw polystyrene box: $64 \pm 8\%$, 5 mL straw CRF: $37 \pm 5\%$, 10 mL CRF: $27 \pm 6\%$). Furthermore, the mentioned methods were successfully adapted for large-scale wels catfish sperm cryopreservation. Thawed sperm showed a similar hatching rate (5 mL straw polystyrene box: $75 \pm 5\%$, 5 mL straw CRF: $72 \pm 3\%$, 10 mL CRF: $66 \pm 6\%$) compared to the control group ($68 \pm 4\%$) during fertilization of 200 g of eggs [11]. Former studies and our results indicate that the first ever tested 3 cryopreservation methods can be a new effective candidate for large-scale pike sperm cryopreservation at hatchery conditions.

10. Conclusion

In conclusion, a new effective extender was developed and tested in pike sperm cryopreservation. A new large-scale cryopreservation method (10 mL cryotube) was successfully adapted for pike sperm where an effective thawing rate was also investigated. In our study, all 3 different large-scale cryopreservation methods were applicable at hatchery conditions. Our new methods can be potentially tested during fertilization of large egg batch.

Declaration of competing interest

Authors declare no conflicts of interest.

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