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Cryopreservation of Danube barbel *Barbus balcanicus* sperm and its effects on sperm subpopulation structure

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Abstract: The objective of this study was to develop a successful freezing protocol for cryopreservation of Danube barbel sperm, and to identify the presence of different spermatozoa subpopulations. By testing different concentrations of different cryoprotectants, we determined that the use of 5% dimethyl sulfoxide (DMSO) yielded the highest total motility of ~25%. Cooling rates influenced by frame height and cooling time in liquid nitrogen vapor showed that a frame height of 3 cm and cooling time of 2 min yielded the highest post-thaw motility. Supplementation of cryomedia with 0.1 M of sugars led to an increase in the total post-thaw motility by ~50%, while protein supplementation lowered post-thaw motility. Motile spermatozoa hierarchically clustered according to their motility parameters, displaying a four-subpopulation (SP1-SP4) structure. SP1 was defined by low values of velocity but high overall linearity; SP2 was comprised of fast non-linear spermatozoa, that had high velocity values but low linearity; SP3 was characterized by fast linear spermatozoa, and SP4 by slow non-linear spermatozoa. Protocols developed in this study will lead to the creation of new and enhanced conservation strategies for this species.

Keywords: Balkan barbel; spermatozoa; fish conservation; sperm subpopulation

INTRODUCTION

The Danube barbel, *Barbus balcanicus* [1] is a small rheophilic cyprinid fish that is widespread throughout the mountainous regions of the Danube River basin and several adjacent drainages [2,3]. It inhabits fast or moderate flowing streams and small rivers, which are sensitive ecosystems, where even small environmental changes can lead to changes in the structure of biocenoses. In recent years, native populations of this species are under threats of anthropogenic or natural origin, such as fragmentation, pollution, diseases, flash floods and droughts. It is therefore necessary to take the appropriate action to protect the genetic biodiversity of particular populations [4]. As rheophilic cyprinids are not economically significant in aquaculture, their reproduction has not been sufficiently studied, and to date, very little is known about their reproductive biology.

Cryopreservation is a widely used method in reproductive management, and is especially valuable for the conservation of genetic resources of a wide range of species [5-7]. As protocols for sperm cryopreservation in fish are generally species-specific, their development is essential to preserve unique genetic resources of natural populations. Several sperm cryopreservation methodologies have been successfully tested for cyprinid species; however, there is no available information on the use of this technique for *B. balcanicus*.

The success of fish sperm cryopreservation depends on various factors, the most crucial being the right choice of cryoprotectant and extender, as well as the freezing protocol [8]. To improve the quality of gametes after cryopreservation, these factors must be fine-tuned and sometimes they need to include supplementation with certain compounds [6,7,9].

One of the most reliable parameters of sperm quality assessment in fish is sperm motility. The existence of spermatozoa with different motility parameters in the same sample and analysis of sperm subpopulations is a powerful tool for the evaluation of male fertility.

In order to develop a successful freezing protocol for the cryopreservation of Danube barbel (*B. balcanicus*) sperm, the aim of this study was to experimentally investigate the effects of different cryoprotectants and their concentrations, different cooling rates, and also the effects of sugar and protein supplementation. A further goal of this work was to identify sperm subpopulations with distinct motility characteristics and to assess the impact of cryopreservation on their distribution post-thaw.

MATERIALS AND METHODS

Broodstock management

A total of 20 *B. balcanicus* (weight: 8.6-15.5 g; total length: 9.5-12.3 cm) were caught in the Jasenica River (Danube River basin, central Serbia) during June 2016 by standard electrofishing equipment (AquaTech IG 1300, Austria). Individuals were transferred to the experimental aquaria of the Faculty of Science, University of Kragujevac, Serbia, and kept at 20±2°C with a constant air supply. All male individuals were in their natural spawning period, which was evidenced by milt stripping without prior hormonal treatment. All trials were conducted within the first week of capture to ensure the natural production of gametes. Fish were not fed during the experimental period.

Sperm collection

Before handling, all fish were anesthetized in a 0.03% clove oil solution (Eugenol; Sigma-Aldrich Ltd., MO, USA) [10]. Immediately before stripping, the genital papilla was wiped dry to prevent contamination by water or mucus, and sperm was collected into 2-ml syringes by applying abdominal pressure. To prevent fast agglutination occurring in barbel sperm, stripped sperm was diluted in a glucose-based extender (GBE; 40 mM KCl, 30 mM Tris, 200 mM glucose [11,12]), supplemented with 20 μg/mL DN*ase* I (PanReac AppliChem, Spain, Germany, Italy). Samples were kept on ice until further analysis (maximum duration 20 min).

Sperm quality evaluation

Sperm motility parameters (total motility [TM, %], curvilinear velocity [VCL, μm/s], and linearity [LIN, %]) were used as the principal quality markers of fresh and frozen/thawed spermatozoa. They were assessed using a computer-assisted sperm analysis software (CASA; ISASv1, Proiser R+D, S.L., Spain) by activating about 0.5-5 μL sperm with 20-30 μL of common carp activating solution (45 mM NaCl, 5 mM KCl, 30 mM Tris, pH 8.0 [13]) supplemented with 1% bovine serum albumin (BSA) in a Makler chamber. Spermatozoa were visualized under a phase-contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) with a 20×negative phase contrast objective with digital camera (XIMEA C13-ON). CASA settings at default for fish. Motility parameters were recorded within a maximum of 5 s after activation. Three activations were performed per sample. For each experiment, samples from three males with the highest total motility values (>80%) were chosen and pooled for further analysis. The motility of cryopreserved spermatozoa was presented as the recovery rate of cryopreserved sperm total motility compared to the total motility measured from fresh control samples.

Cryopreservation procedure

Cryoprotective media containing GBE (formulation described above) and appropriate cryoprotectant as follows: methanol (MetOH), dimethyl sulfoxide (DMSO), ethylene glycol (EG), 1,2-propylene glycol (PG), and 2-metoxyethanol (ME) at concentrations of 5%, 10% and 15%, was prepared. Pooled sperm was diluted in the cryomedia at a 1:9 ratio, equilibrated at room temperature for 15 min and loaded into 0.25-ml straws. Styrofoam boxes were filled with liquid nitrogen to a height of about 10 cm, and the covers were closed for 10 min to allow vapor to stabilize. The covers were then removed and the samples were placed on a 3-cm polystyrene frame and placed on the surface of liquid nitrogen for 2 min, unless otherwise specified. The straws were then plunged into liquid nitrogen and stored in a storage canister until thawing (minimum duration 3 h). Samples were thawed in a 40°C water

bath for 5 s. Three additional motility measurements of the fresh pooled sample were used as the control.

Modifications used during protocol optimization

In each experiment, one cryopreservation parameter (cryoprotectant type, concentration, freezing rate, etc.) was modified at a time and the effects of that modification on sperm motility parameters were assessed. Parameters yielding the highest post-thaw motility rates were used in each sequential experiment.

Experiment 1: Varying cryoprotectants

The effects on sperm motility parameters of five different cryoprotective agents, MetOH, DMSO, EG, PG and ME at a concentration of 10%, were assessed. The pooled sample was equilibrated in the cryomedia, loaded into 0.25-ml plastic straws and frozen as described above.

Experiment 2: Cryoprotectant concentrations

Two cryoprotectants with the highest motility recovery (DMSO and ME) were selected for a further trial on the effects of three different concentrations (5%, 10%, and 15%) on sperm motility. The samples were frozen as described above.

Experiment 3: Different cooling rates

After identifying a suitable cryoprotectant and its concentration (5% DMSO), we tested different cooling rates by comparing the distances of 2, 3 and 5 cm above the surface of liquid nitrogen. We also tested the different times (1, 2 and 3 min) that the samples spent in a vapor of liquid nitrogen before the plunging.

Experiment 4: Sugar supplementation

In light of reports on the beneficial effects of sugars on sperm motility parameters [12], we tested the additional supplementation of three sugars: glucose (Glu), sucrose (Suc) and trehalose (Tre) at two concentrations, 0.1 M and 0.3 M (v/v). The control group contained no additional sugar supplementation. As in the previous experiment, 5% DMSO was used as the cryoprotectant, while the freezing and thawing methodologies were the same as in Experiments 1 and 2.

Experiment 5: Protein supplementation

Supplementation of extenders with 5% and 10% of either BSA or fetal bovine serum (FBS) was tested. Similarly to the previous experiment, 5% DMSO was used as a cryoprotectant, and the cryomedia was additionally supplemented with 0.1 M glucose. The additional control group was made by cryopreserving semen without protein supplementation. Freezing and thawing methodologies were the same as in the previous experiment.

Sperm subpopulation identification

For sperm subpopulation analysis, eight motility parameters were measured by the CASA system for each spermatozoon: curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), average path velocity (VAP, μm/s), straightness (STR, %), linearity (LIN, %), wobble coefficient (WOB, %), mean amplitude of lateral head displacement (ALH, μm) and frequency of head displacement (BCF, Hz). The selected parameters are described elsewhere [14]. Data from all motile spermatozoa obtained in evaluations of fresh and frozen/thawed samples were imported into a single dataset, which represented a total of 3055 spermatozoa.

Prior to multivariate cluster analysis, principal component analysis (PCA) was conducted to reduce the number of variates while retaining as much information from the original dataset as possible. The number of (PCs) selected for hierarchical clustering was chosen according to Kaiser's criterion (selecting only PCs with eigenvalues higher than one) [15]. Individual spermatozoa were assigned to specific subpopulations based on Euclidean distances, where spermatozoa close to each other were assigned to the same subpopulation. In contrast, distant spermatozoa were assigned to different subpopulations.

Statistical analysis

Percentage motility data were arcsine-transformed while the velocity parameters were ln-transformed prior to statistical analysis. During protocol optimization, parameters in each experiment were compared by one-way ANOVA followed by Tukey's HSD test except in Experiment 3, where a two-factor ANOVA

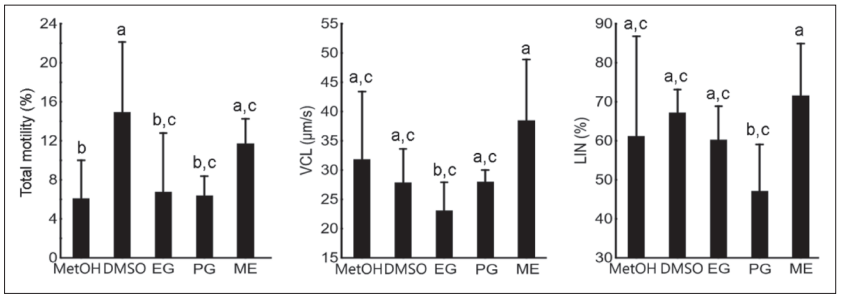

Fig. 1. Effects of methanol (MetOH), dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG) and 2-metoxyethanol (ME) on Danube barbel post-thaw sperm motility. All values are presented as the mean±SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD, P<0.05).

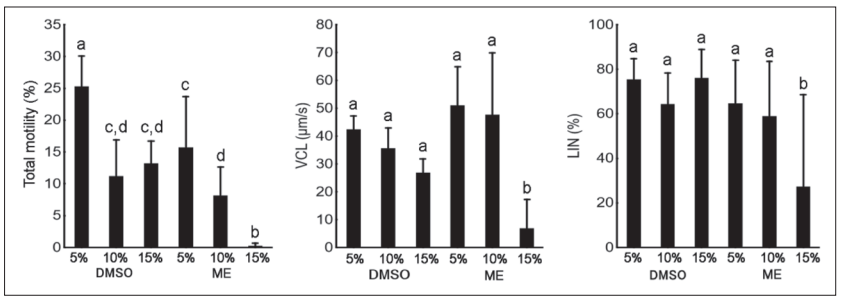

Fig. 2. Effect of different concentrations (5, 10 and 15%) of dimethyl sulfoxide (DMSO) and 2-metoxyethanol (ME) on Danube barbel post-thaw sperm motility. All values are presented as the mean±SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD, P<0.05).

(distance from liquid N_2 and cooling time in liquid N_2 vapor set as factors) was used. For comparison of the contribution of sperm subpopulation in fresh and frozen/thawed semen, we used the χ^2 test. All statistical analyses were conducted in STATISTICA v12 software (StatSoft Inc., Tulsa, OK, USA).

RESULTS

Cryopreservation protocol optimization

Experiment 1: Varying cryoprotectants

In general, cryopreservation decreased total sperm motility detected in the fresh sample (81.34 ± 3.40%). Cryoprotectants had a significant effect on sperm total motility ($F_{(4,40)}$=6.44, P<0.001). DMSO and ME displayed significantly higher total motility compared to other cryoprotectants (Fig. 1; Tukey's HSD, P<0.05) and retained ~18% and ~14% of fresh motility, respectively. Since there were no significant differences, the effects of concentration on post-thaw motility were assessed with both cryoprotectants. The decrease in VCL was also detected in cryopreserved groups compared to fresh sperm (111.80±14.64 μm/s), while there was no obvious change in LIN (fresh sperm: 61.24±1.48%). One-way ANOVA did indicate that there were significant differences caused by the cryopreservation process in both treatments for both parameters (P<0.05); however, Tukey's HSD did not display significant between-group variance.

Experiment 2: Cryoprotectant concentrations

When comparing the effects of three different concentrations of DMSO and ME on sperm post-thaw motility, the concentration of 5% yielded higher motility in both cryoprotectants. The group cryopreserved with 5% DMSO had significantly higher total motility compared to all other groups (Fig. 2; Tukey's HSD, P<0.05), and retained ~32% of fresh sperm motility. When looking at inter-group variation in VCL and LIN, only 15% of the ME group displayed significantly lower mean values (Tukey's HSD, P<0.05). According to these results, 5% ME was used as a cryoprotectant in subsequent trials.

Experiment 3: Different cooling rates

Both frame height ($F_{(2, 72)}$=25.35, P<0.001) and cooling time in liquid N_2 vapor ($F_{(2, 72)}$=43.93, P<0.001) had a significant effect on post-thaw motility. The frame height of 3 cm with a liquid N_2 vapor cooling time of 2 min seemed to be the most favorable method, retaining ~34% of fresh sperm motility; however, there were no significant differences between this group and the 2-cm 2-min and 2-cm 3-min groups (Fig 3.; Tukey's HSD, P>0.05). Similarly, both height and exposure time had a significant effect on VCL and LIN; however, significantly lower values were only detected in the

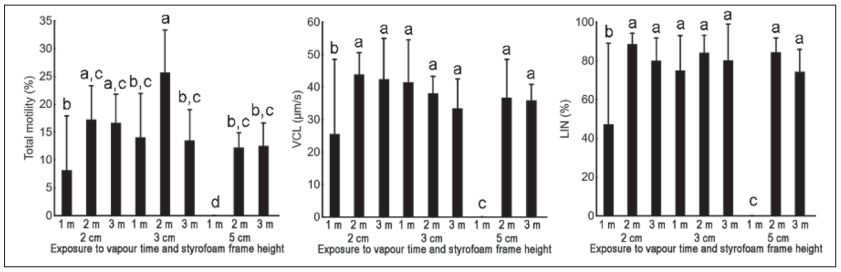

Fig. 3. Effect of frame height (2, 3 and 5 cm) and cooling time (1, 2 and 3 min) in liquid N_2 vapor on Danube barbel post-thaw sperm motility. All values are presented as the mean±SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD, P<0.05).

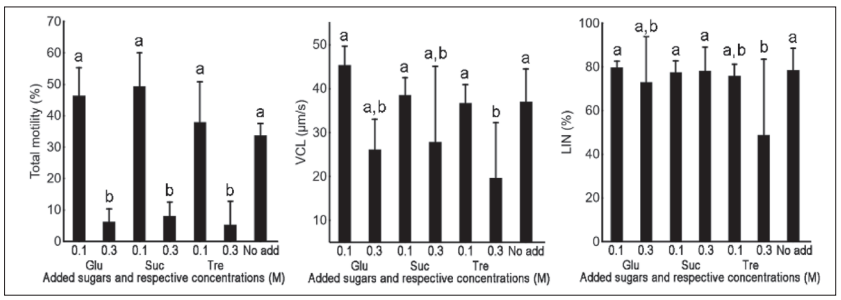

Fig. 4. Effect of additional sugar supplementation (0.1 and 0.3 M of glucose (Glu), sucrose (Suc) and trehalose (Tre)) on Danube barbel post-thaw sperm motility. All values are presented as the mean±SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD, P<0.05).

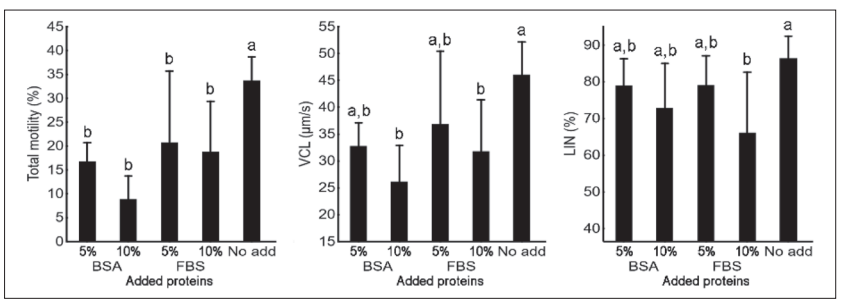

Fig. 5. Effect of protein supplementation (5 and 10% of bovine serum albumin (BSA) and fetal serum albumin (FBS)) on Danube barbel post-thaw sperm motility. All values are presented as the mean±SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD, P<0.05).

2-cm 1-min and 5-cm 1-min groups (Fig. 3; Tukey's HSD, P>0.05). According to these results, the frame height of 3 cm and 2 min of exposure to liquid N_2 vapor were used in all subsequent trials.

Experiment 4: Sugar supplementation

In general, additional supplementation of cryomedia with 0.1 M of sugars led to an increase in post-thaw motility, while supplementation with 0.3 M of sugars led to a significant reduction in post-thaw motility (Fig. 4; Tukey's HSD, P<0.01). Even though the increase was not statistically significant, the addition of 0.1 M glucose and 0.1 M sucrose increased recovery of fresh sperm motility to ~49% and ~53%, respectively, compared to the control group with no additional supplementation (~36%). Therefore, for further trials, we supplemented the cryomedia with an additional 0.1 M glucose, making the total concentration of glucose in the medium 0.3 M (new cryomedia named 0.3 GBE). Similar observations were made for VCL, while there were no differences in LIN (Fig 4).

Experiment 5: Protein supplementation

Contrary to sugar supplementation, protein supplementation lowered the post-thaw motility in all cases ($F_{(4, 40)}$=7.59, P<0.001). The group with no added protein yielded the highest post-thaw motility (Fig 5; Tukey's HSD, P<0.05) and had the highest recovery of fresh sperm motility with ~40%. Similar observations were made for VCL, while there were mostly no differences in LIN (Fig. 5).

Subpopulation structure

Motile spermatozoa hierarchically clustered according to their motility parameters, displaying a four-subpopulation (SP1-SP4) structure. Subpopulations could be distinguished and classified according to two main motility parameters, VCL and LIN. SP1 was defined by low values for velocity (low VCL, VAP), but high overall linearity made these spermatozoa slow-linear. SP2 was comprised of fast-non-linear spermatozoa, which had high velocity values, but low linearity. On the other hand, SP3 was characterized by fast but linear spermatozoa, which

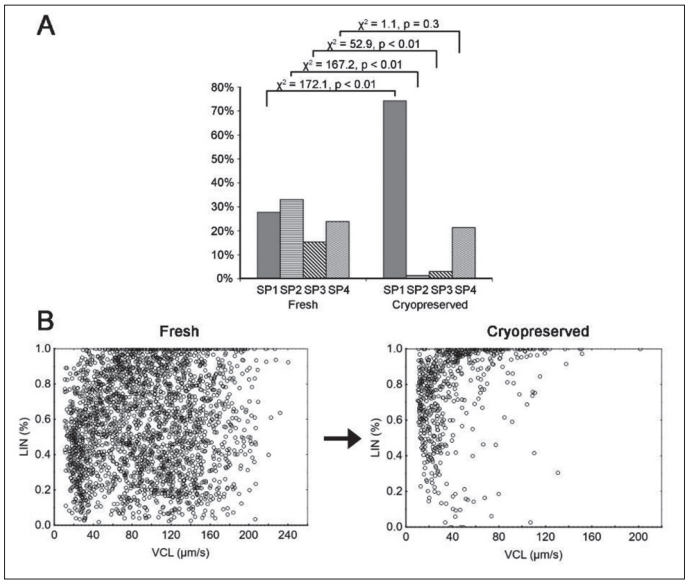

Fig. 6. A – Contribution of each sperm subpopulation (SP1–SP4) in fresh and cryopreserved ejaculates of Danube barbel. **B** – Scatter plot of sperm linearity (LIN) and curvilinear velocity (VCL) displaying distribution of individual spermatozoa before and after cryopreservation.

displayed both high velocity and linearity values. Lastly, SP4 was comprised of slow-non-linear spermatozoa, which had both low velocity and linearity values.

In fresh sperm, SP2 had the highest proportion of spermatozoa (33%), while SP3 was characterized by fast linear movement and had the lowest contribution of 15%. After cryopreservation, the subpopulation structure markedly changed where all subpopulations except SP4 had a significantly different percentage contribution (Fig. 6A). The most obvious was the increase in percentage contribution of SP1 at the expense of SP2 and SP3, indicating that spermatozoa lost their velocity after thawing. A similar characteristic was observed in the scatterplot showing changes in VCL and LIN after cryopreservation for each spermatozoon (Fig 6B). While in fresh sperm, the spermatozoa were distributed evenly throughout the scatterplot, after cryopreservation, most spermatozoa had VLC values below 40 μm/s with varying percentages of LIN.

DISCUSSION

In this study, we developed for the first time a successful protocol for the cryopreservation of Danube barbel *B. balcanicus* sperm. The cryomedium containing DMSO and a glucose-based extender (GBE) was the most suitable combination based on recovery rates up to 50% of fresh sperm motility. Among the tested cryoprotectants, DMSO proved to be the optimal cryoprotectant for cryopreservation of *B. balcanicus* spermatozoa. In this regard, different authors reported successful use of ME for freezing sperm in carp [5], silver carp [16], rainbow trout [17], northern pike [18], and European eel [19]. The success of DMSO can be explained by its ability to penetrate the spermatozoa quickly and by its interactions with the phospholipids of the sperm membrane, creating a rapid balance between the intracellular and extracellular media [20,21]. On the other hand, Horváth et al. [22] reported that sperm of carp cryopreserved with MetOH exhibited significantly higher post-thaw motility than sperm cryopreserved with DMSO. MetOH has been used successfully for sperm cryopreservation in other cyprinid species such as tench [12] and zebrafish [23]. The appropriate cryoprotectant varies among fish species and interactions between extender and cooling rate [24]. In addition, DMSO and ME provided sufficient cryoprotection in *B. balcanicus* sperm, whereas other cryoprotectants (EG, PG) were either toxic to spermatozoa before freezing or decreased post-thaw viability. As for the used concentrations, the lower cryoprotectant concentration of 5% gave higher post-thaw motility rates than the higher concentrations (10% and 15%), especially in the case of ME, where sperm cryopreserved with 15% ME showed hardly any movement. These results point to the toxic effect of cryoprotectants, and that lower concentrations are more favorable for the cryopreservation of *B. balcanicus* sperm. The cooling rate affects the osmotic balance of intracellular and extracellular solutions during freezing, and this is an important factor in sperm cryopreservation [23]. If the cooling rate is too slow, an excess of water leaves the cell and dehydration causes cell death; however, if the freezing rate is too fast, an insufficient amount of water leaves the cell and large intracellular ice crystals form, causing the cell to rupture [25,26]. In the present study, we tested the effects of different cooling rates on cryopreservation outcome by freezing straws on Styrofoam frames floating on the surface of liquid nitrogen. Even though programmable freezers can improve cryopreservation effectiveness, these machines

are not readily available, especially in field conditions. For the Danube barbel, results from the present study indicated that a combination of 3-cm frame height and 2 min of exposure to liquid N_2 provided optimal cell protection during cryopreservation.

Sugars have been used extensively as supplements to extenders to improve sperm motility during sperm cryopreservation in several fish species such as African catfish [27], common carp [28], brown trout [29], northern pike and whitefish [4]. It has been suggested that sugars provide cryoprotection through direct interaction with the polar groups of phospholipids by increasing fluidity [30]. Sugars also act as external osmoregulators because they do not enter the cell but alleviate the swelling of cells during thawing/warming as water remains extracellularly to dilute these solutes. Post-thaw sperm motility of the Danube barbel is influenced by sugar supplementation and concentrations of added sugars. Generally, supplementation of cryomedia with 0.1 M of sugars significantly improved post-thaw motility, while supplementation with 0.3 M of sugars caused dramatic decreases in sperm motility after thawing. The mechanism causing deterioration of sperm motility at a high concentration of sugars is unknown [31]. Our study revealed that glucose and sucrose had similar results, suggesting that the mechanisms underlying the cryoprotective effects of monosaccharides and disaccharides are similar for this species. This is consistent with the finding [32] where it was reported that glucose and trehalose secured the highest post-thaw sperm motility after thawing in several salmonids.

The addition of specific proteins protects the membrane against cryodamage, and several protocols developed for fish sperm incorporate this compound in the freezing medium [33]. For these purposes, FBS and BSA have been often used for sperm cryopreservation, due to their function in reducing solution toxicity and buffering osmotic pressure [34]. Contrary to expectation, our results indicate that they did not have beneficial effects on sperm motility. Similar cases have been reported for grayling when the addition of BSA to the extender had no influence on the structural stability of the spermatozoa [35]. De Leeuw et al. [36] suggested that BSA protection is partial but that it has a synergetic effect when used in combination with lipid vesicles because of its emulsifying properties. Although in the present study BSA and FBS showed inferior potential for freezing of *B. balcanicus* sperm, they have been successfully used in sperm cryopreservation of other freshwater fish [37-39].

Our study demonstrated that sperm subpopulations with distinct motility characteristics coexist within the *B. balcanicus* milt. These subpopulations can be identified by measuring spermatozoa with the CASA system and through subsequent multivariate cluster analysis. Similar sperm subpopulation structures, with four subpopulations, have been identified in boar [40], bulls [41,42], dogs [43], stallions [44] and Senegalese sole [45].

Spermatozoa contributing to different subpopulations are in different physiological states, which is reflected in their swimming behavior. Spermatozoa that contribute most to the fertilization population of the ejaculate are the fast-linear spermatozoa (SP3). Subpopulations that could also contribute to fertilization but most likely to a lesser extent, are SP1, comprised of slow-linear spermatozoa, and SP2, comprised of fast-non-linear spermatozoa. As demonstrated by Gallego et al. [46] in tambaqui and by Beirão et al. [47] in Senegalese sole, only high-velocity spermatozoa, regardless of their linearity, are correlated with fertilization success. Therefore, SP2 and SP3 predominantly contributed to the fertilization success of Danube barbel ejaculates, accounting for a total of 48% of spermatozoa. SP4 was characterized by slow non-linear spermatozoa that were metabolically compromised and would most likely soon lose their motility completely [43]. The exact cause of defective kinematic patterns is unknown; it might stem from irregular spermatogenesis resulting in membrane weakness, defective membrane-associated enzymatic activity, low glycolytic activity, low mitochondrial respiration, or they could represent a sperm population that has not completely matured but was released by the stripping technique [45].

As demonstrated by mean motility parameter values, cryopreservation had a significant effect on sperm motility parameters. Similar trends were observed in the sperm of many cyprinid, salmonid, and acipenserid species [8]. Mean motility parameter values indicate that there is an overall loss of motility and an overall loss of velocity during spermatozoa movement. However, it is unclear whether these changes affected

one specific sperm population in the ejaculate, or the ejaculate population as a whole. Observation of sperm subpopulation dynamics in fresh and frozen/thawed ejaculates indicates the frequency in which sperm with specific kinematic properties are affected. In subpopulations, we can clearly see percentages of spermatozoa with high velocity and spermatozoa with a low velocity in ejaculates after cryopreservation, and thus, we can precisely assess the impact of manipulation on the sample.

In the current study, sperm subpopulation dynamics reflected the observed changes in mean motility parameter values. SP2 and SP3 were characterized by high motility values and decreased significantly in the cryopreserved groups compared to the fresh control. This was compensated by a significant increase in the frequency of SP1, characterized by slow-linear spermatozoa, whereas the contribution of SP4 (slow non-linear spermatozoa) remained similar. A significant decrease of high-velocity spermatozoa in favor of low-velocity spermatozoa was also demonstrated in tambaqui [46], Senegalese sole [47], donkeys [40] and bulls [41,42]. These results indicated that cryopreservation most likely had a negative effect on mitochondrial metabolism, leading to an overall decrease in sperm velocity.

CONCLUSIONS

Our research represents the first successful cryopreservation of Danube barbel sperm. Freezing of sperm using 5% DMSO as a cryoprotectant with a cooling time on liquid N_2 vapor of 2 min and a frame height of 3 cm resulted in the highest post-thaw motility. The results of the current study also revealed the existence of four subpopulations of motile spermatozoa. Further studies are required to explore the fertilization capacity of Danube barbel sperm and the importance of different subpopulations.

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Conflict of interest disclosure: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the reported research.

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