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Optimization of the process of cryopreservation of reproductive cells in male pikeperch, *Sander lucioperca*

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ABSTRACT

The process for cryopreservation of pikeperch sperm was studied as part of the research. The purpose of these studies is to assess the effect of different concentrations of DMSO cryoprotectant during cryopreservation of pikeperch sperm on the sperm quality indicators. The study of sperm quality was carried out according to generally accepted methods, in which the following indicators were determined: the concentration of fresh sperm, the motility of spermatozoa of fresh and defrosted sperm, and the sperm activity time. In the course of the research, we studied the effect of cryoprotectant concentration on the motility of spermatozoa, in which good results were obtained when using DMSO with a concentration of 10%, which was 68.2 ± 2.24% in the first group and 58.8 ± 2.16% in the second group comparing to DMSO at 20% ($p < 0.01$). The highest motility rate of spermatozoa was observed by sperm freezing with equilibration of 68.2 ± 2.24% in the first group and 58.8 ± 2.16% in the second group compared to equilibration ($p < 0.01$). When studying the sperm activity time in two groups, high results were observed when frozen with DMSO 10% cryoprotectant at 883.4 ± 15.12 s and 799.4 ± 10.32 s compared to DMSO 20% cryoprotectant, respectively ($p<0.05$). Of the two groups studied, the group with hormonal stimulation showed the best results in terms of sperm quality. Thus, when studying the protocol for cryopreservation of pikeperch sperm, we found that DMSO cryoprotectant at a concentration of 10% has a positive effect on the quality of deforested sperm.

Keywords: Cryopreservation, Cryoprotector, Reproductive cells, Pikeperch, Sperm.
Article type: Research Article.

INTRODUCTION

Cryopreservation of fish sperm is an effective method of preserving and restoring the gene pool of not only rare and endangered species, but also aquaculture objects, making it possible to solve many environmental, breeding and other scientific problems. Creating banks of genomic resources can lead to an increase in the potential size of the breeding population and minimization of inbreeding to ensure that proper genetic combinations are obtained (Martinez-Paramo *et al*. 2016). Despite a number of advances in the cryobiology of fish sperm, research continues to optimize methods for cryopreserving fish sperm (Kristan *et al*. 2013; Hermelink *et al*. 2017; Khendek *et al*. 2018). These usually include semen collection, sperm quality determination, semen dilution with species-specific protector solutions, freezing of the resulting suspension according to a specific program, storage in liquid nitrogen, thawing under optimal conditions, and evaluating the result by motility of thawed sperm (Asturiano *et al*. 2017; Martínez-Paramo *et al*. 2017). The success of cryopreservation, i.e. preservation of viability of fertilizing ability by frozen cells depends on many factors: the quality of native sperm, the selection that is optimal for a given fish

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species, the composition of the cryoprotective medium, the ratio of sperm dilution with the medium, freezing and thawing modes, the method of activating thawed sperm and other technical details, so the need for optimization and improvement of technology always remains relevant. Hormonal stimulation also plays an important role in improving the quality of fresh semen and semen after thawing (Ciereszko *et al*. 2020). Activation of thawed sperm also affects the success of the cryopreservation process and the quality of thawed sperm (Judycka *et al*. 2021). In most fish species, spermatozoa suspended in seminal plasma are immobile and activated only upon contact with water (Billard *et al*. 1986; Cosson 2004). In any method of cryopreservation of fish sperm, in order to achieve a successful result, it is very important to select a suitable protective environment, which should be non-toxic for sperm, slightly hypertonic due to precise concentrations of solutes, and having a certain composition of ions. Among the components of the medium, the main role in cell protection is given to cell-penetrating (intracellular) cryoprotectants. The action of intracellular cryoprotectants (low molecular weight sugars, alcohols, dimethyl sulfoxide, amino acids) is associated with a slow decrease in temperature by the prevention of excessive dehydration of the cell (osmotic shock), with the prevention of salting out and denaturation of proteins due to interaction with them, with a rapid decrease in temperature - with the prevention of intracellular crystallization after by lowering the freezing point. In our studies, we used demethyl sulfoxide. Pikeperch is a new species in freshwater aquaculture and is characterized by excellent meat quality, fast growth and high commercial value (Policar *et al.* 2019). Management of the broodstock of this species is a prerequisite for promising artificial breeding (Khendek *et al*. 2018). To date, the results of cryopreservation of pikeperch sperm using a glucose-methanol filler are known (Judycka *et al*. 2021). These developments provide a successful technology for cryopreservation of pikeperch sperm, at a concentration of glucose in the range of 0.11-0.15 M in 7.5% methanol, which allowed obtaining good results in the percentage of motility after frozen/thawed spermatozoa, where the highest values were 0.13 M (44 ± 12%; Judycka *et al*. 2021). The aim of our research is to study the effect of different concentrations of cryoprotectants during cryopreservation of pikeperch sperm on sperm quality indicators.

MATERIALS AND METHODS

Research materials

Scientific research on the study of sperm cryopreservation was carried out from wild male pikeperch, *Sander lucioperca*, obtained in March 2022 from the reservoir of the city of Astana and delivered to the research center "Fishery" of the Kazakh Agrotechnical University named after S. Seifullin, Astana, (Kazakhstan). The males were placed in pools with a recirculating water supply. A morphobiological analysis of fish was carried out (Syzdykov *et al*. 2019; Table 1). According to the results of the analysis, the males were divided into two groups. The first group included males with additional exposure and hormonal stimulation, while the second group included males with fluid. For stimulation, HCG was used at a dose of 500 IU kg^{-1} at a temperature of 13 ℃. The period between injection and collection of semen was 12 hours, which is optimal for the collection of high quality pikeperch semen. Sperm was collected by pumping into a dry plastic container.

Assessment of the quality of fresh semen

The quality of freshly obtained sperm of pikeperch males was assessed by determining the motility of spermatozoa using a microscope. The sperm quality was determined according to the Persov scale. Samples were taken with an activity of 4 and 5 points (Matrossova 2020). Sperm was assessed by appearance for color and consistency. The sperm activity time was set using a stopwatch. The concentration of native sperm was determined by conventional methods visually and using a Goryaev camera (Polyantsev 2022)

Cryopreservation protocol

Cryopreservation was carried out according to the following scheme: semen dilution with a cryoprotective medium, equilibration, freezing, defrosting. In the process of cryopreservation, cryoprotectants containing the following components were used: 0.1% sucrose, 0.35% NaCl, 10-20% dimethyl sulfoxide (DMSO), 7% ethylene glycol. The semen selected after evaluation from 2 groups of 5 males in separate plastic containers was placed in a refrigerator for exposure to a temperature of 10-15 ℃ for 15-20 minutes. After equalizing the temperature, the semen was diluted with a cryoprotectant in a ratio of 1:1. The medium was added slowly, drop by drop, with continuous stirring of the container. The resulting suspension of sperm-cryoprotective medium was poured into

cryovials with a volume of 0.9-1.5 mL for several minutes. Cryotubes with the solution were placed in a refrigerator for equilibration during 15 minutes. Afterward, the cryomaterial were placed on a raft formed from foam with dimensions of 10 × 10 × 2 cm. Next, the raft was immersed in a foam plastic box with a wall thickness of 4 cm and filled with liquid nitrogen to a level of 7 cm. The raft with cryovials was kept until the temperature dropped to 70 °C. To control the temperature, a wire sensor from a low-temperature thermometer was placed in one of the test tubes. After reaching the required temperature, the raft with test tubes was turned over into liquid nitrogen. After processing and freezing of all samples, the tubes were transferred from the box to the Dewar vessel for further transportation and storage. Cryotubes were thawed in distilled water at a temperature of 38 °C for 1 minute using a water bath.

Motility analysis of defrosted semen

The parameters of defrosted sperm were studied in the laboratory of JSC "Republican Center for Breeding in Animal Husbandry "Asyl Tulik". Sperm motility and activity time were recorded on a personal computer monitor using a video attachment under a trinocular microscope with a camera and software CEROS computer technology system CASA (IMV-technologies, France). Sperm was activated with water at a ratio of 1:300.

Statistical processing of data

Statistical processing was carried out according to the guidance of G.F. Lakin (Lakin1990) and on a PC using the Excel program (Korossov *et al*. 2007). Results were presented as mean (M) ± standard deviation (m). To determine the significance of the recorded differences in the values of sperm motility and the time of motor activity between the studied objects, Student's t-test was used.

RESULTS

According to the results of the assessment, the males were divided into two groups and placed in the RWI pools. Males were evaluated according to the following parameters: body weight, fatness, size and appearance (Table 1). A total of 15 males were selected. The average body length was: 54.8 cm, body length without caudal fin 48.27 cm, body weight 1707.2 g, Fulton fatness 1.56. Head length averaged 28.26%. Male pikeperch sperm from the first group was obtained without stimulation, while from the second group after hormonal stimulation by pumping. A visual assessment of the quality of sperm by color and consistency was carried out during straining of the genital products. Assessment of the quality of pikeperch spermatozoa by mobility was carried out under a microscope by the percentage of spermatozoa with rectilinear-progressive movement to the total number of spermatozoa in the field of view. The sperm concentration was determined visually and using a Goryaev camera. During the research, 15 samples collected from 15 males were divided into two groups, of which, after being evaluated on the Persov scale, 10 samples had mobility indices of 4 and 5 points. The average sperm concentration with 4 and 5 points was 17.66 ± 0.27 billion mL^{-1} in the first group, while 17.3 ± 0.20 billion mL^{-1} in the second group (range: 16.9-18.1 billion mL^{-1}). When comparing the two studied groups of males, the motility of spermatozoa of fresh sperm in group 1 showed the highest rates, which confirms the positive effect of hormonal stimulation on sperm quality.

Table 1. Biological indicators of pikeperch (n = 15).

Characteristics	min-max	M ± m
L (cm)	45-62	54.8 ± 4.61
l (cm)	39-55	48.27 ± 4.28
Q (g)	1124-2154	1707.2 ± 360.8
Fulton	1.1-2.64	1.56 ± 0.36
	B (%)	
lc (cm)	25.64-30.23	28.26 ± 0.92

Note: * L: total length of the fish; l: body length without caudal fin; Q: total weight, Fulton: the Fulton body condition coefficient, lc: length of the head.

Impact on sperm motility when using various cryoprotectants

In our studies, two cryoprotectants, i.e., DMSO 10% and DMSO 20% were used, in which the best results were obtained using DMSO 10% ($p < 0.01$), where the motility of spermatozoa after equilibration in the first group was 70.2 ± 1.76% while in the second group 60.2 ± 1.84%, after freezing 68.2 ± 2.24% and 58.8 ± 2.16%, respectively. When using DMSO, 20% spermatozoa motility after equilibration was 11.8 ± 1.36% in the first group, 8.8 ± 0.72% in the second group, after freezing 10.6 ± 0.88% and 7.2 ± 1 .04% respectively (Fig. 1).

Table 2. Characteristics of fresh semen.

Sperm quality indicators	Sperm concentration (billion mL^{-1})	Fresh sperm motility (%)
Group 1 with hormonal stimulation (n = 5)	17.66 ± 0.27	90.2 ± 1.84
Group 2 without hormonal stimulation (n = 5)	17.3 ± 0.20	84.4 ± 2.72

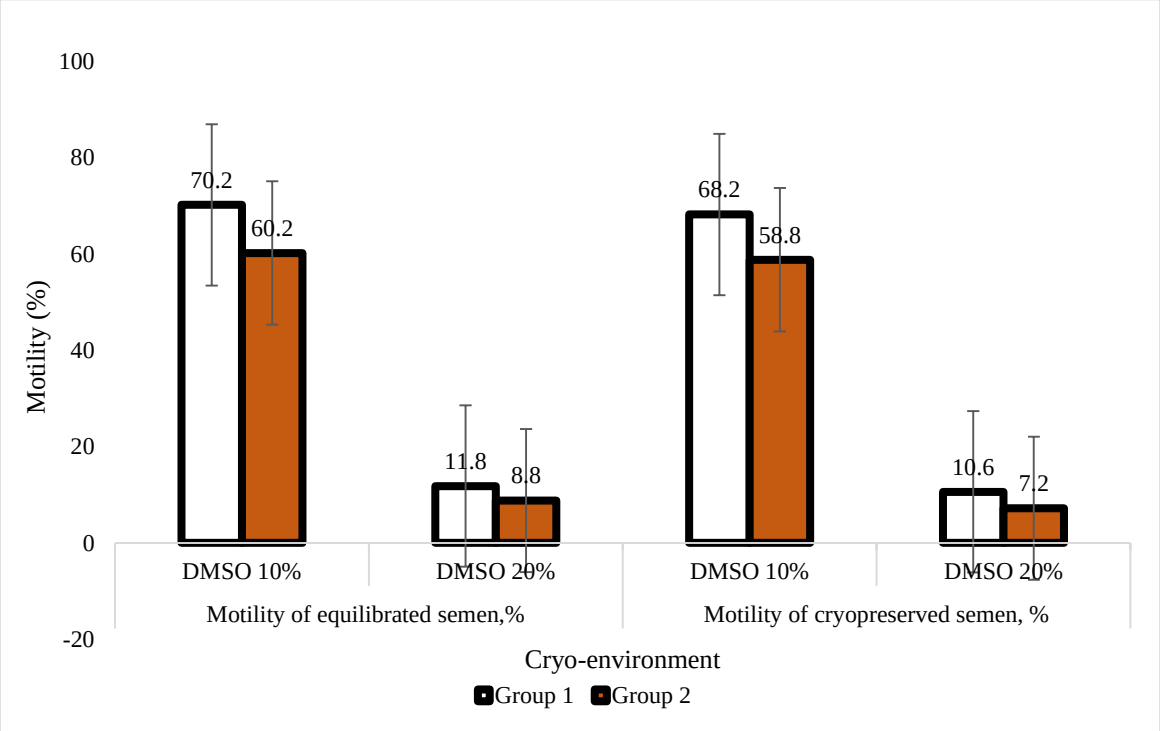

Fig. 1. Motility of pikeperch spermatozoa after equilibration and freezing.

In addition, 10% DMSO was much more effective than 20% DMSO. The highest rate (%) of sperm motility after equilibration and freezing were recorded in group 1. The rates (%) of sperm motility of group 1 after equilibration and freezing were slightly lower compared to fresh sperm ($p < 0.01$). In the course of the research, experiments were carried out to identify the effect of equilibration on the motility of frozen sperm. Equilibration was an exposure of diluted sperm with a cryoprotective medium in a refrigerator for 15 minutes. As a result of these studies, it was found that in frozen sperm with 10% DMSO cryoprotectant, the motility of spermatozoa with an equilibration of 3.6% in the first group (68.2±2.24%) and 2% in the second group were higher (58.8 ± 2.16%; $p < 0.01$) than without equilibration (group 1, 64.8 ± 2.56%; group 2, 56.8 ± 2.2), and with cryoprotectant DMSO 20% by 2.4% in the first group (10.6 ± 0.88%) and by 0.6% (7.2 ± 1.04%) in the second group higher than in the studied groups without equilibration (group 1, 8.2 ± 0.64 %; group 2, 6.6 ± 0.72) (Fig. 2). The process of equilibrium exhibited a positive effect on sperm motility. The males of the first group also showed high results of sperm motility compared to the second group ($p < 0.01$). The activity time of defrosted sperm was studied using various cryoprotectants. The highest activity time was found in the first group using 10% DMSO by 883.4 ± 15.12 s, which was 84 s higher ($p < 0.05$) than in the second group (799.4 ± 10.32 s). The activity time of frozen sperm by 20% DMSO showed much lower results, which amounted to 188.2 ± 11.84 s in the first and 157 ± 12.8 s in the second group (Fig. 3).

Dependence of motility and activity time of frozen pikeperch semen

In the course of the study, a direct relationship was observed between the motility and activity time of pikeperch spermatozoa (Fig. 4).

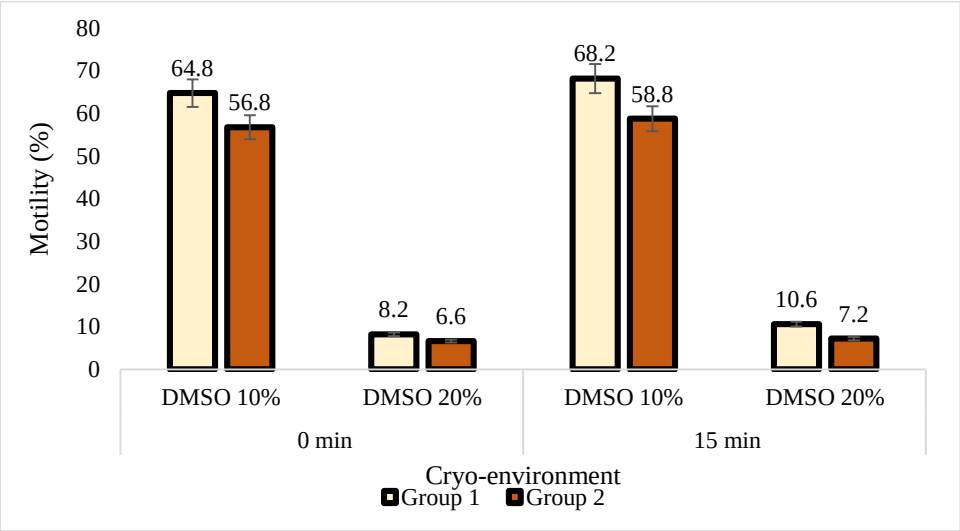

Fig. 2. The effect of equilibration on the motility of pikeperch spermatozoa.

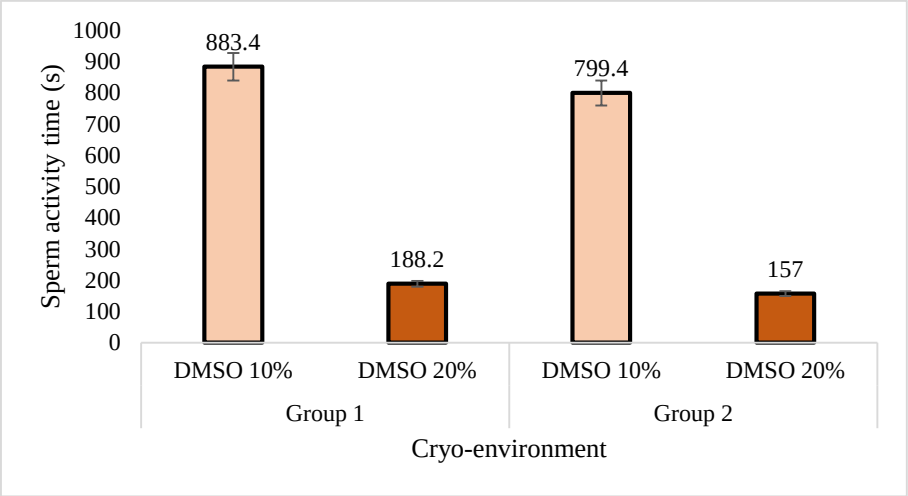

Fig. 3. The time of sperm activity in different groups.

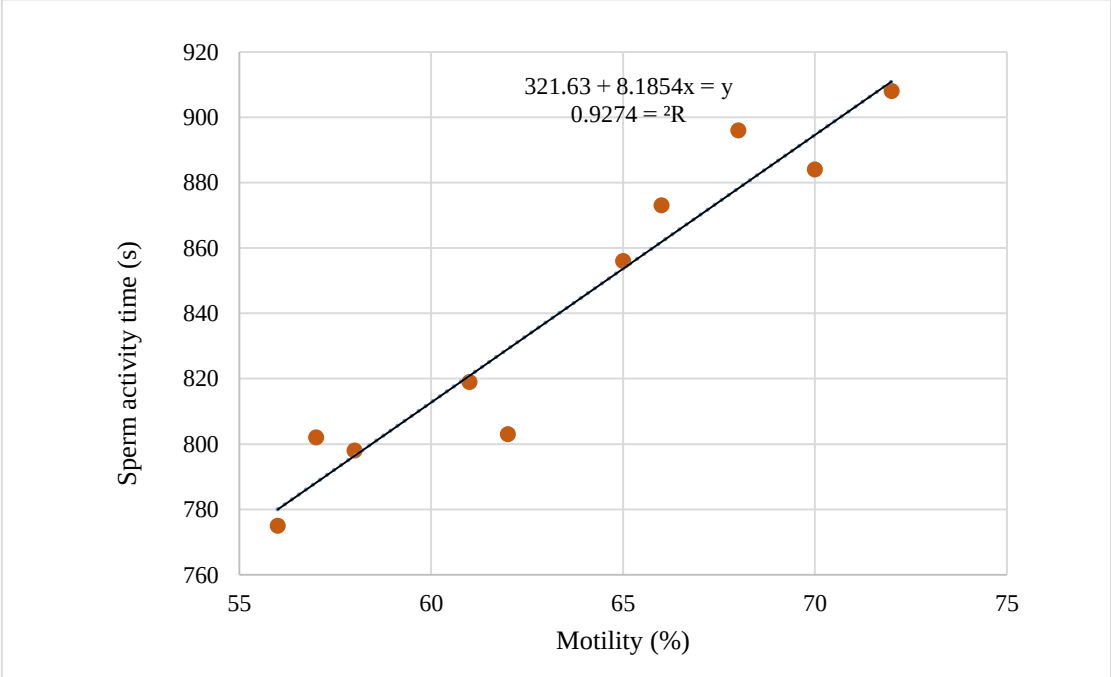

Fig. 4. Relationship between motility and activity time of pikeperch spermatozoa (n = 10).

DISCUSSION

In our research, we studied the cryopreservation of pikeperch sperm with the determination of the optimal environment, which makes it possible for long-term storage in liquid nitrogen for many years. The studies were carried out on the basis of the developed technologies for cryopreservation of cyprinid fish sperm (Akos Horvath *et al*. 2003). We have established the optimal environment for cryopreservation of pikeperch sperm. However, according to scientists, the effect of the cryoprotective environment does not depend on the family of fish (Labb'e & Maisse 1996). To date, the results of several studies on the cryopreservation of pikeperch sperm are known (Bokor *et al*. 2007, 2008; Judycka *et al*. 2021). Bokor *et al*. (2007) in their studies reported a cryopreservation procedure based on a dilution ratio of semen and diluent of 1:9 or 1:1, which results in defrosted sperm motility of 40 or 53%, respectively. Judycka *et al*. as part of their research, examined the final concentrations of pikeperch sperm for the success of cryopreservation, and exhibited sperm motility after freezing / thawing using 7.5% methanol with maximum values at 0.13 M (44 ± 12%). In the present study, we examined cryopreservation of pikeperch sperm with DMSO cryoprotectant at different concentrations of 10% and 20%. The results exhibited that the cryoprotective effect was enhanced at a concentration of 10% DMSO with a motility of pikeperch spermatozoa in the studied groups as 68.2% and 58.8%, and at a concentration of cryoprotectant DMSO of 20%, the motility of pikeperch spermatozoa was low (10.6% and 7.2% respectively). Cryoprotector, i.e., DMSO is successfully used in the cryopreservation of sperm of valuable fish species (carp, salmon and sturgeon) in various concentrations. In the studies of Ponomarev E.N. *et al.* on the cryopreservation of sturgeon male reproductive cells according to the developed methodology using DMSO, however, the content of the cryoprotective medium included a multicomponent composition, so that, the content of DMSO was adjusted for each object under study. They decreased the volume of the protector, which reduced the toxic effect, leading to an elevation in the activity time of defrosted sperm in beluga by 20% and in Russian sturgeon by 47% (Ponomarova *et al*. 2016). Our study exhibited similar results displaying that sperm motility is higher by 10% DMSO compared to 20% DMSO.

The composition of the cryoprotective medium included 0.1% sucrose, which is of no small importance in the success of cryopreservation of pikeperch sperm. Judycka *et al*. (2021) used glucose at different concentrations, where the final concentration of 0.13 M favorably affected the success of cryopreservation of pikeperch sperm (Judycka *et al*. 2021). In our study, a positive effect of equilibration was observed on spermatozoa motility, where the sperm by the cryo-environment was kept for 15 minutes in a refrigerator at a temperature of 4 ℃, then it was frozen according to the abovementioned method.

The highest results were observed by males of the first group (68.2%) and the second group (58.8%) when they were frozen with 10% DMSO, while without equilibration it was 64.8% and 56.8%, respectively. However, recent studies reported the opposite results, where a drop was found in the proportion of sperm motility compared to fresh sperm (from 73-79 to 46-64%), to which the authors claimed unexpected results, where in previous studies of cryopreservation, the percentage of sperm motility during equilibration either remained or increased (Judycka *et al*. 2017; Judycka *et al*. 2019; Judycka *et al*. 2021) compared to fresh semen.

CONCLUSION

According to the results of the research, we found that the optimal cryoprotectant is DMSO with a 10% concentration, which favorably affected the motility of freezing/thawing sperm (in the two study groups were 68.2 ± 2.24% and 58.8 ± 2.16%) compared to 20% DMSO, at which low results of sperm motility (10.6 ± 0.88% and 7.2 ± 1.04%) were obtained respectively. The effect of equilibration on the motility of spermatozoa of defrosted sperm was studied, during which positive results were obtained compared to sperm frozen without equilibration amounting to 68.2 ± 2.24% in the first and 58.8 ± 2.16% in the second group after freezing by 10% DMSO while 10.6 ± 0.88% and 7.2 ± 1.04% when frozen with 20% DMSO, respectively.

When studying the activity time of defrosted sperm, the highest results were also obtained when freezing sperm using 10% DMSO. So that, in the studied groups, we recorded 883.4 ± 15.12s and 799.4 ± 10.32s, while using 20% DMSO 188.2 ±11.84 s and 157±12.8 s respectively. There is a direct relationship between the motility and activity time of pikeperch spermatozoa. In the present study, two groups of males with and without hormonal stimulation were examined, the best results in terms of the quality of defrosted sperm were observed by the males of the first group. Thus, according to the results, we found that in the case of the cryopreservation of pikeperch sperm, it is more efficient to use DMSO cryoprotectant with a concentration of 10%.

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Not applicable.

Data Availability Statement
Not applicable.

Conflict Of Interest
The authors declare no conflict of interest. This manuscript has not been published or presented elsewhere in part or in its entirety and is not under consideration by another journal. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these.

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