Quality evaluation of progeny of the sterlet Acipenser ruthenus (sterlet) obtained offspring

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Abstract. Preservation of biodiversity of rare and endangered fish species by deep freezing methods is relevant for modern science. For the first time defrosted reproductive cells of male *Acipenser ruthenus* frozen by a method of accoustic-mechanical impact with the use of piezoactuators were used for fertilisation of eggs of sterlet *Acipenser ruthenus*, which is included in the IUCN Red list The quality of defrosted semen was assessed to determine its fertilisation capacity, which was high, the activity of live active cells up to 79%, and the yield of free embryos up to 62%. The offspring obtained using cryopreserved semen had normal embryonic and postembryonic development, no significant differences from the offspring in the control batch were observed. In terms of reactivity of the central nervous system and receptor complex, the cryopreserved offspring sometimes surpassed the control batch in some parameters such as orienting and background activity, response to stimuli. Larvae after transfer to mixed feeding (10th day) did not significantly differ from the control in the character of the central nervous system reaction, except for the reaction to light stimulus. Thus, the use of frozen sperm using a new method allows us to obtain viable early juveniles adapted to life in natural water bodies for biodiversity conservation.

1 Introduction

Cryopreservation of reproductive cells of animals and fish has very broad prospects for the conservation of genetic resources, their rational use and application for obtaining high-quality juveniles for release into natural water bodies and marketable products in aquaculture to reduce the pressure on natural populations [13]. Progress in cryobiology, reproductive biology, population genetics and fish breeding, as well as in other areas of science, allows the development of new technologies for reproduction of rare and endangered fish species and innovative aquaculture, characterised by high economic efficiency and stability [45]. The most important direction of this activity is the formation and maintenance of gene pool collections, both living and preserved in the form of cryopreserved reproductive material (semen). Currently, cryotechnologies are strategically important for solving problems related

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to the conservation of the gene pool of rare and endangered fish species, which already include sturgeons [67].

In conditions of decreasing numbers of natural populations of economically valuable fish species, small in number and on the verge of extinction, as well as in connection with the violation of species and population balance, preservation of biological diversity is an important direction of modern science.

The *Acipenser ruthenus* Linnaeus, 1758 (further – sterlet) is listed in the IUCN Red list [8], and the Don population is listed in the Red Book of the Russian Federation (2021) [9] as endangered. The situation with sterlet stocks in the Lower Don basin is particularly tense. As a result of the construction of the Tsimlyanskaya HPP, the Don sterlet has been effectively split into two separate populations. The first one inhabits the upper and middle reaches of the Don and the upper part of the Tsimlyansk reservoir and is still in more or less satisfactory condition. The second population, living below the Tsimlyanskaya dam in the Lower Don, is currently in an extremely depressed.

The use of cryopreserved sperm in artificial reproduction plants and commercial aquaculture enterprises will allow the formation of genetically diverse and highly productive breeding stock of fish, which will reduce the area for producers, and the use of new cryotechnological methods to restore populations in natural water bodies. The use of cryosperm will eliminate the risks associated with maturation of producers and obtaining poor quality reproductive cells from them [1011].

In breeding, artificial insemination of farm animals using defrosted (cryopreserved) sperm has an industrial basis, in contrast to fish farming (aquaculture), and research in this area is mainly aimed at intensification of techniques. However, the previously established standard of motile defrosted sperm, which does not exceed 45-50 %, is still used in practice. Increasing the number of surviving sperm after deconservation is possible not only through the use of stabilising additives, but also through the influence of factors of acoustic or electromagnetic nature [12].

Previous studies have shown that the application of ultrasound, electric and magnetic fields to assist or accelerate the freezing process have revealed great potential to improve the quality of frozen foods using small and uniformly distributed ice crystals [1314]. Exposure to electrical stimulation before equilibration and treadmill withdrawal using NaCl showed that exposure of sturgeon fish reproductive cells to electrical signal promoted an increase in the permeability of sperm membranes for cryoprotectants, ensuring the preservation of cell integrity in the process of low-temperature preservation.

The use of accoustic-mechanical impact with the use of piezoactuators in the technology of low-temperature preservation of fish reproductive cells has shown good results with optimal parameters of impact on sturgeon sperm for one minute with a signal frequency of 500Hz, which has the best effect on the reproductive qualities of defrosted sterlet sperm. The method was first used in vitro in 2021 on sperm of sturgeon fish species, which showed that cells frozen by this method after defrosting had high survival rate and activity [15–17].

However, one of the most important indicators after deep freezing of semen is its fertile potential, which is manifested in its ability to be activated, yielding a certain concentration of motile spermatozoa for oocyte fertilisation, as well as the retention or motility time. These indices are used to test cryopreservation techniques and protocols developed and more adequately assess the fertile potential of sperm [18–19]. Testing the quality of reproductive cells (sperm) after deep freezing is an integral part of cryopreservation technology. According to literature data [20], one of the main reasons for the death of early juvenile fish reared in artificial conditions is the poor development of defensive behaviour and the ability to respond quickly to external stimuli.

In connection with the above, the main goal of our research is to assess the fertile potential of cryopreserved sterlet sperm and the offspring obtained from it.

2 Material and research methods

The studies were conducted from 2020 to 2023. The material for the research was reproductive native and cryopreserved cells (spermatozoa), embryos, larvae and early juveniles of sterlet. Eggs (oocytes) from female Don sterlet was obtained during their lifetime by cutting the oviduct [21].

Sperm was collected from male sterlet grown in fish farms in Rostov and Volgograd regions. The males were hormonally stimulated by injection of carp pituitary gland at a dose of 5 mg per kg of body weight. Sperm was obtained by straining. A total of 6 males were used

Sperm activity was determined under a microscope using the 5-point scale of G.M. Persov [22], recording the percentage of sperm with progressive and oscillatory movement from the total number of living spermatozoa. To estimate the quality of reproductive cells we used the indicator - sperm life time after their activation. The quality of biological material was assessed by the time from the moment of activation to the complete stop of the last sperm in the field of view of the microscope. The sperm used in the cryopreservation studies had an activity score of 4 and 5. The motility time was determined using a stopwatch. Semen of male sterlet was frozen using acoustic-mechanical action [15-16, 23]. During the equilibration period, the interaction between cryoprotectant and sperm, acoustic-mechanical influence was performed with a signal frequency of 500 Hz for 1 minute. The acoustic medium was cryoprotector (modernised Stein medium) mixed with sperm, having the following composition: NaCl; KCl; $CaCl_2$; $NaHCO_3$; HCl; sucrose; mannitol; egg yolk - 10%; dimethyl sulfoxide - 10%.

Before low-temperature preservation the sperm from each male was mixed at a concentration of 1:1 with a protectant. The solution was processed by a piezo actuator. After balancing, the samples were gradually frozen in a Planer programmable freezer to a temperature of -70 °C, followed by immersion of the samples in liquid nitrogen (-196 °C). The defrosting of the sperm was performed on a water bath at a temperature of 38–40 °C . The defrost time averaged no more than 35–45 s. Ten sperm samples from five males (two samples from each male) were removed from the cryocontainer and thawed, with each sample volume being 1.5 ml.

One batch of eggs taken from a female sterlet was fertilized with native sperm (control), the second batch of eggs was divided into 5 parts and fertilized with thawed sperm from 5 males (experiment). Before fertilisation, sperm quality was determined: percentage of sperm motility and time of progressive sperm motion. The sperm activity was determined by microscopy using an HB-200 video eyepiece on a personal computer monitor.

The eggs were fertilised by semi-dry fertilisation according to standard methods [24]. The volume of used sperm was determined in accordance with fish breeding regulations at the rate of 10 ml of sperm per 1 kg of eggs. After fertilisation, eggs were decellularised with river silt. Decellularised eggs were placed in Weiss apparatuses for incubation. The incubation process was continued for 4 days at the water temperature of 15.5–17.2°C. During incubation (embryogenesis), the percentage of fertilisation of eggs at the fragmentation stage (first fragmentation furrow) was assessed. Percentage of fertilisation of eggs was determined during embryogenesis when the first crushing furrow was laid. After incubation, the % of surviving free embryos was determined.

After 10 days of active feeding, the control and experimental batches of juvenile sterlet were tested using the "open field" methodology [25]. The "open field" method is used to estimate behavioural reactions of fish in a minimally structured environment. The test is intended to characterise the reactivity of the central nervous system of fish. Young fish are estimated by reaction to external stimuli. The magnitude of motor activity is determined by the average number of fish crossing the lines of a coordinate grid drawn on the bottom of the

experimental setup. In the study, a chamber with an area of 50x80 cm, a water layer of approximately 2–3 cm, and the size of the grid squares 5 × 5 cm was used.

The average number of crossings of coordinate lines during the first 30 s after placing a sample of fish in a new location characterises their orientation activity (OA, units/minute). Adaptation of juvenile sturgeon fish to a new location takes no more than 3 min, after which their motor activity reaches a relative plateau. According to this, the average number of crossings of coordinate lines of each fish during the period from the 4th to the beginning of the 7th minute of the experiment was considered as background motion activity (FA, units/minute). After 6 minutes from the beginning of the experiment, the stimulus imitating a low-frequency signal, the average frequencies of which lie in the range of 20–150 Hz, which is perceived by the lateral line of fish, was applied. The average number of crossings of grid lines per 1 individual for a period of 30 s was recorded. After that, the fish reactivity to this stimulus was determined (P1, units/minute). Through 7.5 min (at the 8th minute) after the beginning of the experiment, the second stimulus (P2, units/min) was applied with a high-frequency acoustic stimulus - a shock, the average frequencies of which ranged from 800 to 2000 Hz. This type of signals is perceived by fish by the inner ear. The average motor activity of the tested planting material during 30 s after exposure to this stimulus is considered as reactivity to the high-frequency signal (P2, units/minute). Then, at the 10th minute, a light with a power of 250–300 lux was lit above the experimental chamber and the reaction of fish to the light stimulus was evaluated (P3, units/minute). Illumination was measured using a luxmeter. The last stimulus affects the visual lobes of the brain. 10 individuals each from experimental and control groups were tested at the developmental stages after transition to active feeding at the age of 10 days.

The indicators of activation of motor activity and reactivity to the stimulus were calculated according to the formula [24]:

$$AI\% = \frac{A}{BA} \times 100\% \quad (1)$$

$$RI\% = \frac{RA}{BA} \times 100\% \quad (2)$$

where AI% – activation index; IA (units/min) – indicative motor activity; BA (units/min) – background motor activity; PI – reactivity index; RA (units/min) – reactivity.

The experiments were carried out in threefold repetition, the data were subjected to statistical processing in MS Excel and STATISTICA 10 programmes. Statistical significance of differences was established by Student's t-criteria.

3 Results and discussion

The preservation of cellular structure and functionality depends on the cryopreservation protocol. For oocyte fertilisation, spermatozoa must retain the integrity of the plasma membrane and their motility when exposed to water to reach the oocyte. Sperm motility is crucial and depends on various aspects of the cell such as mitochondrial state, ATP production, plasma membrane channel activity and flagellar structure.

One of the most important characteristics of sperm quality is fertilisation capacity (assuming quality female gametes), and variability in fertilisation rate is positively correlated with sperm velocity, and sperm velocity affects fertilisation capacity. The fertilisation ability of sperm is influenced by the duration of their activity after activation [26]. After deep freezing, which lasted 30 days, defrosted sperm started oscillatory movements after activation after 30–40 seconds, while native sperm started movements immediately after entering water. Previously, many authors have noted that cryospermatozoa differ from native sperm in that they do not start to move immediately after deconservation and activation with

water. Data on thawed sperm are presented in Table 1. For each of the five males, the average values for two samples are given.

Table 1. Comparative indicators of fresh sperm and cryosperm of sterlet from males kept in industrial farms of the Rostov and Volgograd regions in the period from 2020 to 2023

Experience Variant	Motility before cryopreservation, %	Time of life before cryopreservation, s	Mobility after cryopreservation, %	Time of life after cryopreservation, s
1	75	402	61	320
2	77	428	67	375
3	72	352	59	293
4	72	383	68	310
5	89	475	77	405
Control (fresh sperm)	90	460	-	

The comparative evaluation of native and defrosted semen revealed some differences in such parameters as the proportion of live sperm cells, time of progressive movement. The proportion of live cells in native semen was 90% and the time of movement was 460 sec, which corresponded to semen of high quality. In defrosted semen, the proportion of live cells in the samples ranged from 58 to 79%, and the time of translational movement from the beginning and full stop ranged from 290 to 420 sec. However, it should be noted that in some samples the defrosted semen was of high quality: the semen of male No. 2 sample 2 had a percentage of live cells 69, time of motion 400 sec, the same as in the control (fresh semen), in sample 1 and 2 of male No. 5 the survival rate of sperm was 75 and 79%, in the time of forward motion 420 and 390 sec, respectively. Such semen is considered to be of high quality and recommended for artificial reproduction, therefore, it can be said that these samples were in compliance with the requirements of the control (fresh semen). The mean values of all selected samples of male sperm were 66.4% for survival rate and 340.6 s for activity. These are high indicators for defrosted semen [20].

We performed fertilisation of eggs (sterlet eggs) with native and defrosted sperm. It should be noted that the high fertilisation rate was observed in the control (native sperm), it was 82%, in the experiment variants the fertilisation rate ranged from 52 to 69%, for cryopreserved sperm the rate above 40 is normal [27]. We noted the correlation of fertilisation percentage and sperm movement time, in the variants of the experiment with defrosted semen, the fertilisation percentage was higher where the activity was between 350-400 sec (male №2) and 390-420 sec (male №5).

The result of fertilization of sterlet eggs is shown in the graph (fig. 1).

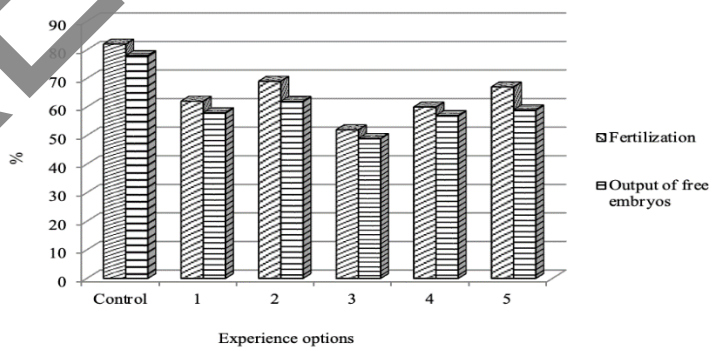
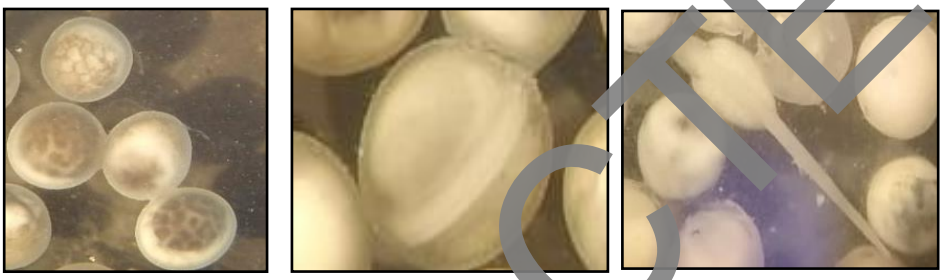


Fig. 1. Percentage of fertilization of sterlet eggs and hatching rate of free embryos in experimental variants and control

Incubation of eggs (embryogenesis process) is one of the most important processes in aquaculture and artificial reproduction technologies. The results of sterlet eggs incubation showed that the embryogenesis process took place under optimal conditions of hydrochemical regime, with water temperature within 15,5-17,2 ºC, oxygen content of 8-9 mg/l and pH 7.5. The yield of free embryos in the control from eggs laid for incubation, fertilised with native sperm was 78%, in the experimental variants the yield ranged from 49 to 62%.

The beginning of hatching of larvae in the experiment was observed after 110–114 hours from the moment of egg laying for incubation, in the control after 118 hours. Also the results of embryonic development of fertilised sterlet eggs showed that they developed normally and no deviations in the development of embryos during embryogenesis in the experimental batch relative to the control (fig. 2).



a b c

Fig. 2. Embryonic development of sterlet at different stages: a – fragmentation (multicellular morula); b – gastrulation, c – beginning of embryo emergence.

It was noted that free embryos in some variants of the experiment exceeded the individuals in the control in terms of weight and length (experiment 1, 4), in other variants they had no significant differences from the control in terms of weight and length. At comparative analysis of weight and length of larvae that switched to active feeding it was noted that they were ahead of larvae from the control group in growth, and in four variants reliable differences were noted (at $p < 0.05$). The study of the behaviour of larvae and juveniles in the studied groups revealed no differences, all early juveniles were active and responded to live feeds during the transition to active feeding. The growth of free embryos and sterlet larvae was further studied. It was observed that free embryos after hatching and larvae after transition to active feeding obtained from preserved semen did not differ in development from offspring obtained from native semen. They actively switched to feeding on external food and did not differ externally from the control. Size and mass characteristics are presented in Table 2.

Table 2. Length and weight parameters of sterlet embryos and larvae, obtained from cryosperm

Indicators	Free embryos (larvae)					
	control	Experience options				
		1	2	3	4	5
Weight, mg	10.78±0.88	11.37±1.07	10.60±0.72	10.15±0.66	11.14±0.92	10.97±0.55
Length, mm	8.61±0.50	9.17±0.47	9.13±0.51	8.53±0.36	9.32±0.38	9.67±0.15
	Transition to active feeding (age 10 days)					
Weight, mg	21.28±1.01*	23.02±0.37*	23.15±0,85*	21.35±0.89	23.25±0.72 *	24.92±0.49*
Length, mm	14.30±0.40	15.03±0.42	15.5±0.42	14.91±0.55	15.6±0.46	15.8±0.49

* differences are reliable at $p<0.05$

For a more complete evaluation of the offspring obtained from cryopreserved and defrosted sperm, we compared changes in the locomotor activity of juvenile sterlet in response to various stimuli such as shock, sound and light. Comparative analysis of experimental and control variants carried out using the "open field" method revealed changes in the behaviour of juvenile sterlet after the transition of juveniles to active feeding at the age of 10 days. We chose the time of testing not accidentally, as at this time the hatched embryos, prelarvae and larvae transferred to active feeding have passed all critical periods, which are the most sensitive for fish.

Earlier studies on the behaviour of juvenile sturgeon fish by determining the orientation and background activity in the "open field" test [20, 24] have shown that juvenile fish upon entering an unfamiliar habitat react instantly by increasing their motor activity during the first 3 minutes, then their movements slow down and come to a state of calm swimming and uniform distribution in the tank. It is noted that when releasing juveniles into the natural habitat, initially the fish are characterised by chaotic movements, which disappear after a certain time when adapting to the new environment [28-29].

Further on the basis of the obtained absolute characteristics we calculated relative indices (OA and RA), which allowed us to estimate the degree of motor activity of young sterlet under the influence of various stimuli. In the experimental groups, no significant differences between the variants of the obtained mean values of OA and BA were observed. Differences in some variants of experimental batches and control were significant, so experimental batch of fish in variant 2 significantly differed in OA and BA from control at $p < 0.05$. The decrease in motor activity indicates the initial adaptation of the object to the environmental conditions after 3 minutes of stay in an unfamiliar environment, which is typical for early juvenile sturgeon fish species [20]. The study of a low-frequency stimulus conducted 6 min after the start of the experiment or exposure to a low frequency sound of 20 Hz (S1) revealed changes in the response of the studied groups of fish with exposure time over the first 30 sec. It was noted that in the control group, during this time interval, the reactivity of swimming movements was lower by 11.3-19.1% compared to the experimental groups. All parameters were higher in the experimental groups, in variants 1, 4 and 5 had a significant difference from the control group (at $p < 0.05$) (Table 3).

Table 3. Behavioural changes in juvenile sterlet in the studied groups

Indicators	Control	Experience options				
		1	2	3	4	5
Orientation activity (OA)	18.11±3.1*	19.11±2.51	20.70±2.67*	19.12±2.33	19.30±1.34	19.41±3.23
Background activity (BA)	15.60±3.5*	18.50±1.84	18.63±2.39*	17.85±1.54	18.73±1.49	18.51±2.30
Low-frequency stimulus (S1)	16.51±3.3*	20.10±1.20*	19.91±1.37	18.61±1.43	20.10±1.05*	20.40±1.43*
High-frequency stimulus (S2)	15.75±2.6*	18.76±4.1*	18.16±3.2*	16.11±3.1	18.73±3.1*	17.87±2.89
Light stimulus (S3)	11.75±3.9*	13.49±8.2*	13.50±1.57*	12.90±1.52	13.10±2.47	13.45±2.68*

* differences are reliable at $p<0.05$

Young sterlet in both experimental and control groups began to move rapidly after the signal action, followed by a hiding reaction, but in the control group the halt of movement was faster. It should be noted that low-frequency signals are picked up by the lateral line of fish, and the behavioural response to this stimulus is manifested in rapid escape and hushing

of fish. It is manifested just at the beginning of the larval period after the transition to active feeding [28-29].

Exposure to high-frequency acoustic stimuli with an average frequency from 800 to 2000 Hz (S2), after 8, 5 minutes after the beginning of the experiment revealed a change in the motor response of the studied objects. The locomotor activity of fish was higher in all variants of the experiment by 2,2-16,0%, variants 1, 2 and 4 had significant differences.

Frequencies from 200 to 2000 Hz are perceived by the inner ear, fish react to such influence by a sharp increase in motor activity fading after 30–35 sec. Investigation of the effect of light of 250–300 lux (S3) was carried out at the 9th minute of the total experiment. The reaction to light in the first seconds in fish revealed the reaction of quiescence (stopping movement), but after a few seconds between 20 and 30 sec fish in the control group started to move actively. The reaction of slowing down movement was longer in the control. Based on the obtained factual data, relative indicators were calculated to determine the degree of motor activity; in the control, it was 11.75±3.9 units/min, which is 8.9-13.0% lower than in the experimental variants. Significant differences with control were in variants 1, 2 and 5.

It was noted that the activation index was higher in the control and was 116.1% in the groups of experimental batches it varied from 103.0 to 111.7%, but no significant differences were obtained on this indicator (Table 4).

Table 4. Relative indices of motor activity estimation

Indicators	Control	Experience options				
		1	2	3	4	5
Activation rate, %	116.1	103.2	111.1	107.1	103.0	105.0
Reactivity index for S1	105.8	108.6	106.8	104.2	107.3	110.2
Reactivity index for S2	100.1	101.4	97.5	90.2	100.0	96.5
Reactivity index for S3	75.3	72.9	72.5	72.3	69.9	72.6

Further, the dynamics of changes in response parameters of larvae and early juvenile sterlet to various stimuli was traced (fig. 3).

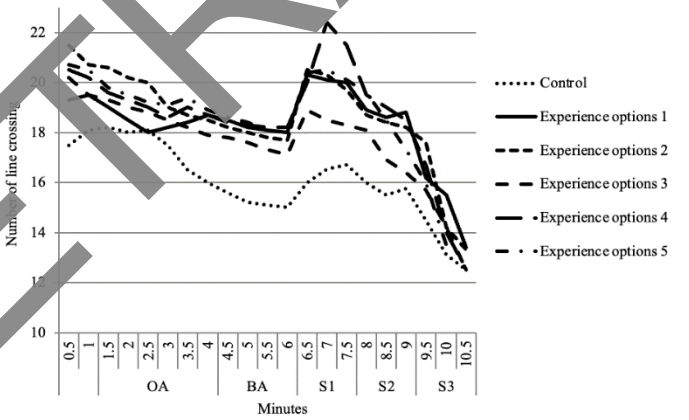


Fig. 3. Dynamics of changes in locomotor activity of juvenile sterlet in experimental and control groups. Designations: OA – orientation activity, BA – background activity, S1 – low-frequency stimulus, S2 –high-frequency stimulus, S3 – light.

It should be noted that the points of increased activity in the groups of fish in the experiment and control coincide, the early juvenile sterlet responds in the same way, changing motor activity to stimuli.

According to the reactivity indicators, also no reliable differences were noted. According to the indicators of reactivity, there were differences between the control and the experiment

in the action of low-frequency stimulus, the reactivity was slightly higher in the experimental groups. The reactivity index at action by high-frequency stimulus was the same in the control and in the 1st and 4th variants of the experiment, in other variants the differences were unreliable. Analysis of changes in locomotor activity of juvenile sterlet obtained from defrosted and native semen as a response to different stimuli was an indicator of the evaluation of the method of cryopreservation under accustic-mechanical influence for use in aquaculture.

4 Conclusion

As a result of the conducted research, it should be noted that cryopreservation of sterlet germ cells allows obtaining high-quality sperm after deconservation. It is known that sturgeon germ cells have low cryoresistance, which is explained by the peculiarities of their structure.

The cryopreservation method allowed preserving the structure of spermatozoa and their functionality. A comparative analysis of the studied native and defrosted sperm showed that the proportion of living active cells in native sperm was 90%, and the time of progressive movement was 460 sec, which corresponded to 5 points. Defrosted sperm was also of high quality, the number of living active cells was from 58 to 79%, and the time of movement was from 290 to 420 sec. In two variants of the experiment, the number of spermatozoa corresponded to 4 and 5 points, which characterized it as high-quality for fertilization.

When sterlet eggs were fertilised with native sperm, the fertilisation rate was high at 82%, while defrosted sperm was between 52 and 69%, confirming the fertilisation ability of cryopreserved sperm. The correlation of fertilisation percentage and sperm movement time was observed in the experiment variants with defrosted semen. The yield of free embryos in the control from eggs laid for incubation, fertilised with native sperm was 78%, in the experimental variants, when fertilised with defrosted sperm, the yield ranged from 49 to 62%.

The offspring obtained using cryopreserved semen, which was frozen under accoustic-mechanical influence and using piezoactuators had normal embryonic and postembryonic development, no significant differences from the offspring in the control batch were observed. According to their morphological characteristics, free embryos had no abnormalities, and larvae after 10 days switched to active feeding and did not differ from the offspring obtained from native semen.

The "open field" test showed that the CNS reactivity of early juvenile sterlet obtained from defrosted semen did not differ from the control group, but the indices of orientation and background activity were higher and the juveniles adapted to new conditions faster. The juveniles in the experimental groups reacted faster to different stimuli and in some groups had significant differences from the control. The dynamics of locomotor activity demonstrated a more intensive reaction of juveniles in the experiment to stimuli, which is of great importance in the adaptation of juveniles to different aquatic conditions.

Thus, the use of sperm from the cryobank allows the production of viable juvenile fish and can be recommended for use in state plants for artificial reproduction and in private aquaculture enterprises. Calculation of relative indices (OA and RA) allowed us to evaluate the degree of motor activity of juvenile sterlet under the influence of various stimuli. Relative values of activation and reactivity to different stimuli had no significant differences in juvenile sterlet in the experimental and control variants. Thus, the use of sperm cryopreserved by a method with the use of accoustic-mechanical influence during the period of equilibration allows after defrosting to obtain viable young fish and can be recommended for use in artificial reproduction enterprises for the restoration of populations in the natural habitat.

Compliance with ethical standards. This article does not describe any studies performed by the authors involving humans or using animals as subjects.

Conflict of interest. The authors declare no conflict of interest.

5 Acknowledgements

The publication was prepared as part of the implementation of the SA of the SSC RAS, No. 122020100328-1. using the Bioresource collection of rare and endangered species of fish SSC RAS No. 73602.

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