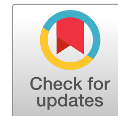

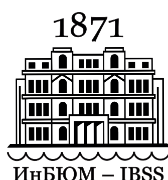

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PRESERVATION OF BIOLOGICAL DIVERSITY BY CRYOPRESERVATION METHODS: EXPERIENCE OF THE SOUTHERN SCIENTIFIC CENTER OF THE RAS

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One of the promising directions for increasing animal genetic diversity is the formation of cryobanks and long-term storage of reproductive cells in liquid nitrogen. Methods of sperm cryopreservation are known for more than 200 fish species. The resistance to sperm cryodamage in different fish species varies dramatically. There is no unified cryopreservation technique for fish since the habitats vary greatly for different species. In Russia, cryopreserved sperm is currently used extremely insufficiently in aquaculture, but the practice dictates the need for widespread use of cryosperm to solve the problems of producing high-quality fish seed material and for breeding work. The formation of cryobanks is very relevant due to extensive development of aquaculture. Providing commercial and farm enterprises with elite genetic material capable of reproduction at any time of the year will allow not only to set up a biotechnological process, but also to eliminate inbreeding.

Keywords: cryobank, cryopreservation, quality assessment, mobility

Currently, marine fish resources are depleted as they are affected by anthropogenic factors, many of which have an irreversible impact on inland waters (Balykin & Khodorevskaya, 2021). At the same time, the number of commercially important fish species has decreased so much that the question arises of forming broodstocks: those are capable of restoring the normal functioning of natural populations of these fish, maintaining their genetic diversity, and intensifying commercial aquaculture. It will reduce the pressure on wild populations which are significantly undermined by fishing. This is possible only when the formation of artificial populations and commercial aquaculture are based on genetic principles allowing to reduce the risk of a significant depletion of the gene pool for restored populations and to grow fish with high values of commercially important traits. However, formation of broodstocks on fish farms and their management should involve the same principles as the well-being of natural populations: their basis is maintenance of the optimal level of genetic diversity. Cryopreservation is one of the methods of reproductive biology directly related to the preservation of bioresources with the possibility of subsequent restoration of their reproductive functions. In the literature, the term “cryopreservation” usually refers to the storage of biological objects at liquid nitrogen temperature (−196 °C), and the process is considered effective only if the cells or tissues are completely viable after thawing (Amstislavsky et al., 2014).

History of cryopreservation. The first to put forward the idea of freezing reproductive cells was the Italian physician P. Mantegazza. In 1866, he published a monograph on the preservation of the ability of bull and stallion ejaculate to fertilize after its freezing down to −15 °C and subsequent thawing. At the late XIX century, the scientific foundations of cryobiology were laid by the Russian scientist P. Bakhmetyev who studied peculiarities of hypothermia in insects and anabiosis in bats. The French biologist P. Becquerel (1904–1936) and the Austrian scientist P. Rahm (1919–1924) revealed the ability of various organisms (microorganisms and invertebrates), as well as seeds and spores, to tolerate deep freezing (down to −269 and −271 °C, *i. e.*, to temperatures close to absolute zero) in the dried state. It was proved later that some animals and plants survive when the water they contain is frozen. In our country, the first experiments on freezing farm animal spermatozoa were carried out by the prominent Russian biologist I. Ivanov. In 1907, he showed that stallion sperm restored its fertility after freezing down to −15 °C and subsequent thawing. In 1947, I. Sokolovskaya, V. Milovanov, and I. Smirnov obtained offspring from insemination of females with thawed rabbit spermatozoa previously stored at −78 °C. Studies of A. Smith and Ch. Polge were of great importance as well: in 1949, these researchers were the first to propose to use glycerol for cryopreservation. Preservation of sperm fertility after freezing–thawing was shown for 16 mammalian species, 2 mollusc species, 5 bird species, 6 echinoderm species, and 1 amphibian species (Ponomareva et al., 2017a).

The first successful reproducible results of fish spermatozoa cryopreservation were obtained for the herring (Blaxter, 1953). The results of sperm cryopreservation for several sturgeon species – the Beluga sturgeon *Huso huso* Linnaeus, 1758, the sterlet *Acipenser ruthenus* Linnaeus, 1758, the kaluga *Huso dauricus* (Georgi, 1775), and the hybrid *H. huso* × *A. ruthenus* – were obtained for the first time by I. Burtsev and E. Serebryakova (1969). The first possibility of salmon sperm preservation was demonstrated on the example of the chinook salmon *Oncorhynchus tshawytscha* (Walbaum, 1792); its sperm previously stored in liquid nitrogen for seven days showed a fertilization rate of 77.7 % (Ott & Horton, 1971). The first to obtain good results of using cryopreserved sperm to fertilize bighead and silver carp eggs was A. Sin (1974). In 1976, when using cryosperm of the common carp *Cyprinus carpio* Linnaeus, 1758, the rate of fertilized eggs was 11 % (Pavlovici & Vlad, 1976). There is a positive experience of using cryopreserved sperm to restore and maintain the population structure of the salmon in Iceland, Norway, and Canada. Commercial cryobanks operate in the USA, Norway, Japan, and France.

Prospects for creating a cryobank. Methods of sperm cryopreservation are known for more than 200 fish species. In different species, the resistance to sperm cryodamage varies dramatically. There is no unified cryopreservation method for fish since the habitats of different species (marine, freshwater, anadromous, sedentary, and non-migratory ones) vary greatly. For marine fish resistant to high osmotic water pressure, it is easy to obtain good rates of spermatozoa survival after cryopreservation; for freshwater and anadromous species, it is necessary to search for cryoprotective media (Asturiano et al., 2017 ; Maisse, 1996 ; Martínez-Páramo et al., 2017). So far, experiments on cryopreservation of spermatozoa and somatic cells have been carried out on more than 30 species of marine fish (Cabrita et al., 2010 ; Mauger et al., 2006 ; Suquet et al., 2000). The rate of spermatozoa that survive cryopreservation and are active after it is much higher in marine fish species (80–90 %) than in freshwater ones (40–50 %) (Scott & Baynes, 1980).

In Russia, cryopreserved sperm is currently used extremely insufficiently in aquaculture. However, practical possibilities allow to widely apply cryotechnologies for reproduction of high-quality fish seed material and for breeding work. Considering extensive development of aquaculture, the creation of a cryobank is very promising and relevant. Commercial and farm enterprises will be provided with elite genetic material capable of reproducing regardless of presence of males; the farms will be able both to set up a biotechnological process and to eliminate inbreeding (Savushkina, 1999 ; Cabrita et al., 2015 ; Zhang, 2018).

Creation of a cryobank allows:

1. To preserve the genetic information of rare, endangered, and commercially important animal species in liquid nitrogen for decades. Storage of frozen cells at −196 °C is possible up to 50 years or even longer without the formation of much abnormal DNA sections.
2. To transport genetic material to an area of population reduction or extinction in order to restore the species.
3. To provide opportunities for breeding and genetic work.
4. To form and maintain a genetic collection of various hydrobiont species.

When designing and constructing fish farms, the presence of a regional cryobank should be provided: this will greatly facilitate the work of enterprises in the future. The annual renewal of broodstocks will contribute to "infusion of fresh blood" and rejuvenation of the herd. Moreover, this will allow minimizing the number of males on farms. The cost of sampling and storing genetic material is five times lower than the cost of fish food. The operation of a cryobank can contribute to development of aquaculture in the regions.

Differences of the cryobank-reproducer from existing analogues are as follows:

1. The cryobank-reproducer allows both to store genetic material and to provide fish farms with the required amount of sperm at a convenient time.
2. The exchange of cryopreserved sperm between fish farms will result in an increase in genetic diversity and, consequently, a rise in the quality of juveniles. The exchange of native sperm is not always possible since the timing of spawning activities varies on different enterprises. Moreover, with a significant distance between the farms, there is a problem of quality loss during transportation.
3. Sperm left on fish farms after fertilization can be stored frozen in the cryobank-reproducer and used later.
4. Sperm samples in the cryobank allow farms to reduce the number of males in the broodstocks. This results in reducing the cost of maintaining fish or replacing some males by females aimed at obtaining more juveniles or food caviar.
5. The applying of cryopreservation methods for fish sperm with a high survival rate after freezing–thawing makes it possible to obtain physiologically high-grade offspring.

To ensure the operation of a cryobank, legal regulation is required: this enables the purchase of material from fish farms and the use of cryosperm there.

When forming cryobanks of male reproductive cells, it is important to store high-quality material. Knowledge of specific morphophysiological peculiarities of fish reproductive cells will help in developing more effective cryopreservation methods. Those will consider the need to combine penetrating and non-penetrating cryoprotectants, osmotically active compounds, and antifreezes. Moreover, those will consider the inclusion of cell membrane stabilizers and antioxidants in the media. All this will provide reliable protection of fish sperm from cryodamage during freezing–thawing and optimize

all stages of cryopreservation. The scale of the described problem is determined by the coverage by studies of a large group of commercially important, native, unique, and endangered fish species; those can be used to save endangered fish. In breeding, cryopreserved sperm can serve as a source of the gene pool.

The cryobank of the Southern Scientific Center of the RAS. Researchers of the SSC RAS work on cryopreservation of reproductive cells of rare and endangered fish species of the southern seas of Russia since 2004. The key aim of our investigation is to optimize the process of fish sperm cryopreservation by selecting optimal cryoprotectors and reducing their negative effect on cells. During cryopreservation, crystallization of intracellular and extracellular water occurs, and membranes of germ cells are destroyed; this leads to their death. To prevent cell damage, cryoprotectors and membrane stabilizers are used. Various stimulation methods (chemical, mechanical, magnetic, *etc.*) contribute to a better penetration of protectors into cells. Electrical stimulation is one of the promising directions in cryopreservation work.

The first experiments were carried out with sperm of the Russian sturgeon *Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833 obtained from the Bertyulsky sturgeon hatchery (the Astrakhan Region). In research on cryopreservation, sperm with activity ranks 4 and 5 according to the G. Persov scale was used (Persov, 1953). For cryoprotection, we used Stein medium (NaCl, KCl, $NaHCO_3$, glucose, 12.5 % egg yolk, and 12.5 % DMSO) and the cryomedium developed by us (NaCl, KCl, $NaHCO_3$, $CaCl_2$, mannitol, sucrose, 10 % egg yolk, and 10 % DMSO). Freezing was carried out according to the method of L. Tsvetkova and S. Savushkina (1997). A high survival rate was registered – up to 85 %; this value is higher than that of sperm frozen in the developed cryomedium and then thawed. In the experiments, the optimal parameters of the electrical signal were established, at which the survival rate and time of spermatozoa activity increase. Those are frequency of 20 Hz and amplitude of 150 mV. When exposed to an electrical signal for 1 min, thawed sperm of better quality was obtained according to both parameters. Specifically, sperm survival of the Russian surgeon accounted for 50 %, and lifetime was of 290 s; the values for the stellate sturgeon were 56 % and 693 s, respectively.

Since 2007, the researchers work with the white salmon (inconnu) *Stenodus leucichthys* (Güldenstädt, 1772). Its eggs were fertilized with sperm stored for two years in liquid nitrogen. Exposure to electric current during the equilibration and removal of the protector during cell thawing increase the survival rate of germ cells of the sturgeon by 1.4–1.6 times. When using electrical stimulation at the equilibration stage, membrane permeability rises; cryoprotectors penetrate into cells and prevent cryodamage. The survival of spermatozoa with the use of electrical stimulation after thawing increases compared to the survival of sperm frozen by the traditional method (90 % and 60 %, respectively). Sperm of such a high quality can be recommended for artificial insemination of eggs. When carrying out experiments on the insemination of eggs with thawed sperm, the success of fertilization was 80–96 % for the Russian sturgeon and 64–84 % for the stellate sturgeon. The fertilization of the same batches of eggs at a sturgeon hatchery reached 75–80 %. The obtained results indicated high quality of cryopreserved sperm (Bogatyreva, 2010 ; Krasilnikova, 2015 ; Krasilnikova & Tikhomirov, 2018 ; Ponomareva et al., 2017b).

Thus, it was established that deep freezing of the Russian sturgeon sperm and its storage in liquid nitrogen at −196 °C for two years do not adversely affect the quality of thawed sperm, embryonic development of fish larvae and juveniles, and their morphometric parameters. Therefore, the use of thawed germ cells for artificial insemination of eggs is advisable in the lack of producers at sturgeon hatcheries.

Together with the colleagues from the Institute of Cell Biophysics, Russian Academy of Sciences, we developed a method for reducing the low-temperature jump during crystallization of cryoprotective solutions in order to increase the thawed cell integrity after cryopreservation. The core is as follows: in the method which involves freezing of the cryosolution with biomaterial in liquid nitrogen, *prior* to the operation of the freezing of the cryosolution with cells of living organisms, the solution is remotely affected by ultrasonic radiation with a frequency of 0.50–10 MHz (Patent 2540598 RF, 2015). The dependence between the volume of frozen material and survival after thawing was recorded (Krasilnikova & Tikhomirov, 2014a); the possibility of freezing seminal fluid on grids in the form of a thin film was described (Krasilnikova & Tikhomirov, 2014b). Moreover, the effectiveness was established of reducing the volume of toxic substances in the composition of the cryoprotective medium for spermatozoa of sturgeon species; this, in turn, reduced the toxic effect of the latter on the object and led to an increase in the lifetime of thawed cells (Krasilnikova & Tikhomirov, 2015). The obtained results make it possible to recommend the adjustment of the concentration of penetrating protectors in the cryoprotective solution depending on the amount of intracellular water to increase the survival of male reproductive cells after a double temperature shock.

The sperm bank of sturgeon and other fish species has been replenished in the cryobank of the SSC RAS since 2006. All reproductive cells are frozen according to technological methods developed by the researchers of the center. The material is sampled on fish farms of Astrakhan, Volgograd, and Rostov regions; this enables the exchange of genetic material within the Southern Federal District of Russia (Table 1).

Table 1. Collection of fish reproductive cells in the cryobank of the Southern Scientific Center of the RAS

Species	Number of samples
Russian sturgeon *Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833	398
Siberian sturgeon *Acipenser baerii* Brandt, 1869 (the Lena River population)	224
Stellate sturgeon *Acipenser stellatus* Pallas, 1771	38
Ship sturgeon *Acipenser nudiventris* Lovetsky, 1828	196
Hybrid *Huso huso* Linnaeus, 1758 × *Acipenser ruthenus* Linnaeus, 1758	125
Beluga sturgeon *Huso huso* Linnaeus, 1758	105
Sterlet *Acipenser ruthenus* Linnaeus, 1758	337
Paddlefish *Polyodon spathula* (Walbaum, 1792)	20
Amur sturgeon *Acipenser schrenckii* Brandt, 1869	50
White salmon (inconnu) *Stenodus leucichthys leucichthys* (Güldenstädt, 1772)	140

The preserved genetic material can be used to fill the shortage of producers and to adjust existing technologies for the artificial reproduction of rare and endangered fish species. Thus, cryopreservation of male reproductive cells is a key direction in the strategy for the preservation of genetic biodiversity and in the development of fisheries and aquaculture.

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СОХРАНЕНИЕ БИОЛОГИЧЕСКОГО РАЗНООБРАЗИЯ МЕТОДАМИ КРИОКОНСЕРВАЦИИ: ОПЫТ ЮЖНОГО НАУЧНОГО ЦЕНТРА РАН

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Одним из перспективных направлений увеличения генетического разнообразия животных является формирование криобанков и долгосрочное хранение репродуктивных клеток в жидком азоте. Известны методы криоконсервации спермы более чем 200 видов рыб. Устойчивость к криоповреждениям спермы у разных видов рыб различается кардинально. Единой методики криоконсервации для рыб нет, так как среда обитания имеет значительные различия для разных видов. В аквакультуре России криоконсервированная сперма в настоящее время используется недостаточно, однако практика диктует необходимость широкого применения криоспермы для решения проблем производства качественного рыбопосадочного материала и для селекционно-племенной работы. В связи с широким развитием аквакультуры создание криобанка является весьма актуальным. Обеспечение товарных и фермерских хозяйств элитным генетическим материалом, способным к воспроизводству в любое время года, позволит не только наладить биотехнологический процесс, но и исключить инбридинг.

Ключевые слова: криобанк, криоконсервация, оценка качества, подвижность