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TECHNOLOGY OF STERLET REPRODUCTION BY MEANS OF CRYOPRESERVED SPERM

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The purpose of the work was to reproduce the sterlet of the Danube and Dnieper populations using cryopreserved sperm frozen in modified cryoprotective medium. Low-temperature cryopreservation of sterlet male reproductive cells was carried out in accordance with conventional procedures of cryobiology.

As a result of the performed work, it has been found that the applied cryoprotective environment, which includes methanol, enhances the cryoprotective effect and is technologically optimal for the process of the Danube and Dnieper populations of sterlet sperm storage and reproduction. Fish sperm cells retained viability after defrosting; their ability to fertilize the eggs was restored. The results of the conducted studies indicate that, despite a certain decrease in the quality properties of sperm after freezing/defrosting, its ability to fertilize did not significantly decrease. Revived in this way larvae from two populations of sterlet were used to reproduce repairing brood stock under conditions of fish farming.

Such a method of sterlet sperm cryopreservation could be recommended for use at commercial enterprises with sturgeon fish commodity cultivation.

Key words: sterlet, sperm, cryopreservation.

One of the most urgent problems in fish farming is the increase of the reproduction efficiency in populations of high-value aboriginal and disappearing economically valuable fish species in the reservoirs of Ukraine. These include the sterlet (*Acipenser ruthenus* L.), which lives in the rivers of Azov and Black Seas basins, and is included in the Red Data Book of Ukraine. The sterlet is characterized by relatively small body sizes and fast, compared with other sturgeons, terms of sexual maturity [1]. As an object of industrial and artificial reproduction, sterlet is widely used for the purpose of natural reproduction of populations and commodity cultivation [2]. Today, there is an urgent need for full-scale growing of sterlet in artificial conditions, using low-cost and efficient technologies. The most important direction in fish reproduction improving is the use of artificial insemination, one of the determining factors of its outcome is the improvement of methods for long-term storage of sperm at low temperatures (cryopreservation). We must also take into account the negative factors that are present at this and which cannot yet be completely avoided during the technological processing of sperm, its dilution with cryoprotective medium and storage in a cooled or frozen state. These manipulations to a certain extent lead to structural and biological defects in sperm cells, may cause violation of permeability of plasma membranes and the release of enzymes and other important metabolites of cell metabolism from spermatozoids, which significantly reduces the fertility of sperm [3, 4].

The technology of conservation and usage of frozen fish sperm is currently developed mainly for carp, silver carp, and some sturgeons [4, 5], but, in our opinion, for sterlet it is still imperfect. Therefore, the purpose of this work was to investigate the optimal cryoprotective medium for the reproduction of sterlet breeding nurseries of the Danube and Dnieper populations under the conditions of fish farming.

Materials and Methods

The research was carried out in the laboratory of biotechnology of the Institute of Fisheries and on the basis of the section of the "Odesa Sturgeon Complex" Ltd. (Vylkove town).

The object of the study was the sterlet ejaculate from the Danube and Dnieper populations, derived from native (control) and cryopreserved sperm. All manipulations with sterlet testicles (stimulation of maturation, obtaining of sexual products and assessment of their quality) were carried out in accordance with the technology of work in sturgeon farm [6, 7, 8].

The studies were conducted in several stages. At the first stage, specimens were sampled and the quality of native sperm was determined until freezing [9]. Sperm quality recognition was performed visually using a microscope. The condition of the sperm was evaluated in appearance (color, consistency), the concentration of spermatozoa was determined using a counting Goryaev chamber, while the sperm mobility was measured using a stopwatch.

At the second stage, the freezing/defrosting of sterlet sperm samples was used in accordance with generally accepted methods of cryobiology [6, 7]. Cryopreservation and defrostation of sperm were carried out according to the original method using methyl alcohol instead of traditional dimethyl sulfoxide (DMSO) [10, 11]. Technologically, this method was implemented by sperm dilution before freezing with a conservative protective solution containing sucrose, potassium chloride and methanol, followed by freezing in liquid nitrogen vapors. Freezing of diluted sperm was carried out in two stages: the first — from 5 °C to — 15 °C at a rate of 2–3 °C/min; the second one — from −15 °C to −70 °C at a rate of 15–20 °C/min, followed by a slow immersion into nitrogen up to −196 °C [7].

At the third stage, the fertilization of identical portions of spawn from two females was carried out by native and defrosted sperm. From each female, one portion of spawn was fertilized with native sperm from three males, the other — with frozen/defrosted sperm of the same males. For fertilization of each portion of spawn, defrosted sperm/protector (1: 1) mixtures from each male were used.

For eggs ungluing (the fourth stage), the frozen river silt was used. Incubation of the fertilized spawn was carried out in the apparatus "Osether-M". The embryonic period lasted about 6 days. During the incubation of the eggs, the temperature of the water was maintained within 13–14 °C. After free embryos hatching, the selection of dead individuals was carried out [12].

At the fifth stage, the larvae were kept in the apparatus "Osether-M" before the start of exogenous feeding. In this case, the temperature regime of the aqueous medium in the apparatus was gradually increased up to 18–19 °C.

The results were statistically processed by means of Student's t-test using the programs Statsoft Statistica 7.0 and Microsoft Excel.

Results and Discussion

The results of the sterlet sperm activity before and after its cryopreservation indicate that the mobility of defrosted sperm cells, in comparison with intact ones (almost 100%), in the Danube fish population is 53–55%, and in Dnieper fish population is 48–51% (Table 1).

It has been established that during defrosting of frozen sperm cells that retained viability after cryopreservation, their ability to fertilize the ovocyte was restored, despite the fact that the mobility of such sperm cells was slowed to some extent. Such changes are due to the fact that for sterlet sperm cells, which are characterized by the presence of acrosome, the methanol-containing cryopreservation medium creates conditions for increasing the cryoresistance of biomaterial [13, 14, 15].

The comparative characteristic of the sperm cells motility of the studied sterlet males from the Danube and Dnieper populations before and after cryopreservation indicates that the number of mobile gametes after defrost was decreased. These changes, in our opinion, are primarily due to certain changes in the filament fibrils and spermatozoa plasma membrane [16]. Of course, the most sensitive

Table 1. **Characteristics of sterlet sperm (*Acipenser ruthenus* L.) before and after its cryopreservation ($M \pm m$, $n = 10$)**

Sterlet populations	Mobility of native sperm cells, min (control)	Mobility of defrosted semen cells, min	The part of sperm cells that kept mobility, %	
			Native (control)	Defrosted
Danube	8.0–10.0	4.0–6.0 *	98–100	53–55 *
Dnieper	5.0–7.0	2.0–5.0 *	99–100	48–51 *

Notes: * — $P \leq 0.05$ (as compared to control).

to the cooling and freezing/thawing of sperm are the mechanisms of energy metabolism regulation. Experimental temperature manipulations with reproductive cells of animals lead to changes in the ordering and integrity of plasma and acrosomal membranes, the release of enzymes from cells, damage of mitochondrial membranes and impaired functioning of the respiratory chain [17, 18].

Intravital sampling of egg cells from 5-year-old females was performed by cutting the oviduct (Fig. 1, *a*), and the sperm was collected using a catheter (Fig. 1, *c*).

On average, 150–200 g of roe was obtained from each female, which is a characteristic value for fish that spawn for the first time. The average weight of the egg was volatile between 6.2 and 7.8 mg, the diameter was 2.2 mm. The use of proof sticks (Fig. 1, *b*) allowed determining the polarization coefficient of ovocytes, which was 8.0–8.3%, that indicates the beginning of ovocytes transition to the V stage of maturity, that is, to the stage of all-encompassing maturation of sex cells (Table 2).

In order to prevent the development of bacterial infection after the strip of eggs, the gentimicin antibiotic was administered to the females. Sterlet eggs, fertilized with both native and cryopreserved sperm, were laid for incubation into the apparatus "Osether-M" (Fig. 2, *c*).

The conducted researches showed that the proportion of fertilized eggs by native sperm was 90–95%, with cryopreserved sperm — 80–85%.

It should be noted that during the revision of biomaterial at the stage of embryogenesis, there were single deviations in the development of embryos in both variants. This is especially true for ovocytes with chaotic segmentation, in which the furrows were not laid simultaneously, and a significant number of ovocytes did not pass at all the stage of division. Such embryos did not enter the stage of gastrulation and slowly died. Probably, such deviations are due to parthenogenetic division [12].

In the process of eggs incubation, the observations on synchronicity and compliance

Fig. 1. **Sampling of sex products from sturgeon fish:**
female (*a*) and male (*c*); proof stick with withdrawn mature sterlet oocytes (*b*)

Table 2. **Fishery and biological indicators of sterlet females (*Acipenser ruthenus* L.) before spawning** ($M \pm m$, $n = 10$)

Sterlet population (females)	Length, cm	Mass, kg	Fish eggs size, mm		Polarization coefficient of fish ovocytes,%
			vertical	horizontal	
Danube	56 ± 0.05	1.350 ± 0.1	2.45 ± 0.005	2.19 ± 0.005	8.33 ± 0.002
Dnieper	62 ± 0.05	2.270 ± 0.1	2.43 ± 0.005	2.17 ± 0.005	8.00 ± 0.002

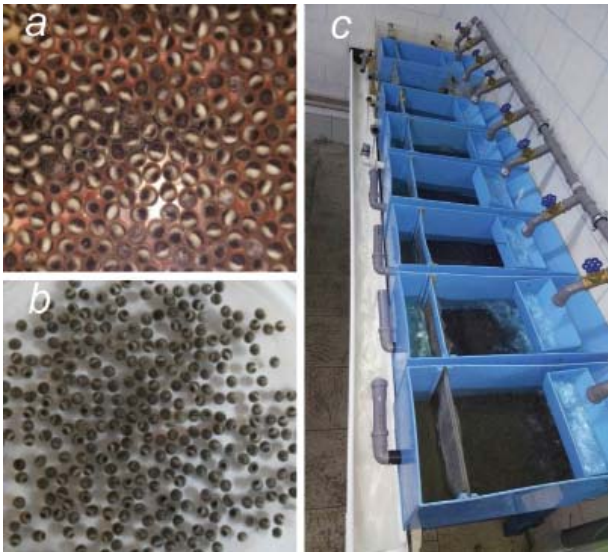

Fig. 2. **View of fish eggs fertilized with native (*a*) and cryopreserved (*b*) sterlet sperm; eggs incubation in the apparatus "Osether M" (*c*)**

with the norms of sterlet embryonic development were carried out (Fig. 3).

At the stage of the first division of Danube and Dnieper sterlet embryonic development, the degree of eggs fertilization was estimated. The experience of sterlet reproduction indicates that the number of fertilized cells is more rational and more reliable counting at the stage of the second division of blastomers (Fig. 3, *a*). Exactly at this stage, the identification of all fertilized eggs is securely ensured, whereas at the first stage of the division, those fish eggs, in which the division occurred with some delay, may be remain unaccounted.

The next control was carried out at the stage of lateral plates formation and the beginning of the tail segment of the embryo separation (Fig. 3, *c, d*). It is known that the number of developing embryos is proportional to the number of hatched pre-larvae.

The beginning of the hatching of sterlet free embryos of both populations took place on the sixth day, at water temperature of 14–15 °C. The average weight of pre-larvae was 8–9 mg. It was noted that the hatching of pre-larvae derived from cryopreserved sterlet sperm began an hour earlier than the hatching of pre-larvae derived from native sperm and was 65% of the eggs laid on the incubation, in contrast to the native sperm, which was 85%.

Peculiarities of the behavior of pre-larvae obtained from both the native and the cryopreserved sperm in the first days of life did not differ. They equally placed in the water column and formed the so-called "candles"

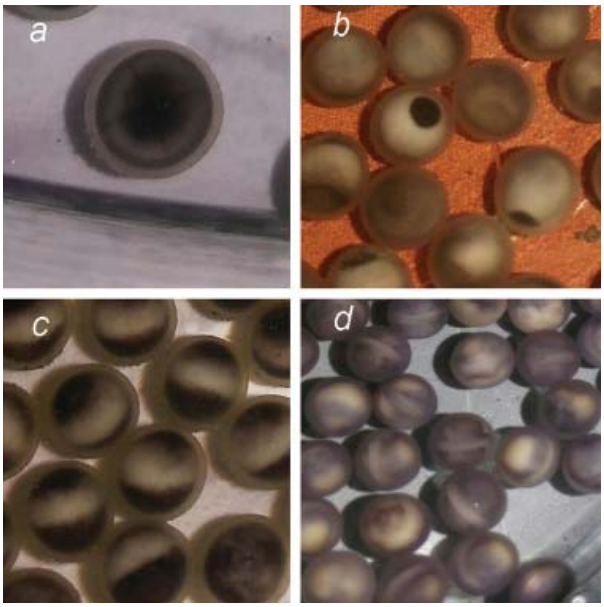

Fig. 3. **The appearance of fertilized sterlet eggs at various stages of embryonic development:**
a —of the second division of blastomers; *b* — of the "large and small yolk stopper"; *c* — of the slit-shaped blastopor; *d* — of lateral plates fusion and the beginning of the tail segment of the embryo separation

periodically rising to the surface of the water and descending to the bottom of the pool. This is explained by the fact that in the case of natural spawning, such behavior of sturgeon pre-larvae lets them, firstly, to avoid silting, and, secondly, passively moving with the flow, to reach faster the areas of the reservoir with a rich forage base.

In the transition to gill breath and with the onset of the stage of the digestive system formation (the so-called "swarming" period), pre-larvae obtained from the native and from the cryopreserved sperm migrated to the bottom of the basin and formed various agglomerations ("spots"). Behavior of individuals in both groups of fish did not differ from the general norms of development.

On the fifth day after hatching, the pre-larvae began to be transferred to mixed feed, and on the twelfth day — on active nutrition.

It has been established that the offspring obtained from fertilization with cryopreserved sperm proved to be viable. A small difference in the development of pre-larvae in both experimental groups may be due to differences in the cryostatability of the subpopulations of frozen cells [19]. The results of experimental studies have shown that methanol introduction into the composition of cryoconservant has increased the persistence of cryopreserved sperm from the Danube and Dnieper sterlet.

The methods used to evaluate the quality of cryopreserved sperm and to predict its fertilizing capacity can be used for successful and low-cost cultivation of young fishes and the formation of repairing brood stock of sterlet in regulated aquatic environment. Studies on adjusting the standard cryopreservation technique for sterlet sperm may be recommended both for use at commercial enterprises of sturgeon fish commodity cultivation and at plants for their reproduction.

A clear and objective assessment of the functional adequacy of the sperm of the Danube and Dnieper sterlet is important when creating the gene pool material bank.

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ТЕХНОЛОГІЯ ВІДТВОРЕННЯ СТЕРЛЯДІ З ВИКОРИСТАННЯМ КРІОКОНСЕРВОВАНОЇ СПЕРМИ

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Метою роботи було відтворення стерляді дунайської і дніпровської популяцій за допомогою кріоконсервованої сперми, замороженої в модифікованому кріозахисному середовищі.

Низькотемпературне консервування репродуктивних клітин самців стерляді проводили відповідно до загальноприйнятих у кріобіології методик.

В результаті проведених робіт встановлено, що застосоване кріозахисне середовище, до складу якого входить метанол, що сприяє кріозахисному ефекту, є технологічно оптимальним для процесу зберігання сперми і відтворення дунайської та дніпровської популяцій стерляді. Під час відтавання заморожених сперматозоїдів риб, які зберегли життєздатність після кріоконсервування, їхня здатність до запліднення яйцеклітин відновлювалася, незважаючи на те, що рухливість таких спермій до деякої міри сповільнювалася. Отримані таким способом личинки від двох популяцій стерляді використовували для відтворення ремонтно-маточних стад за умов рибного господарства.

Проведені дослідження оптимізації стандартної методики кріоконсервації сперми стерляді можуть бути рекомендовані для застосування на комерційних підприємствах з метою товарного вирощування осетрових риб.

Ключові слова: стерлядь, сперма, кріоконсервування.

ТЕХНОЛОГИЯ ВОСПРОИЗВОДСТВА СТЕРЛЯДИ С ИСПОЛЬЗОВАНИЕМ КРИОКОНСЕРВИРОВАННОЙ СПЕРМЫ

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Целью работы было воспроизводство стерляди дунайской и днепровской популяций с помощью криоконсервованной спермы, замороженной в модифицированной криозащитной среде.

Низкотемпературное консервирование репродуктивных клеток самцов стерляди проводили согласно общепринятым в криобиологии методикам.

В результате проведенных работ установлено, что использованная среда, в состав которой входит способствующий криозащитному эффекту метанол, является технологически оптимальной для процесса хранения спермы и воспроизводства дунайской и днепровской популяций стерляди. Во время оттаивания замороженных сперматозоидов рыб, которые сохранили жизнеспособность после криоконсервации, их способность к оплодотворению яйцеклеток восстанавливалась, несмотря на то, что подвижность таких спермиев в некоторой степени замедлялась. Полученные подобным способом личинки от двух популяций стерляди использовались для воспроизводства ремонтно-маточных стад в условиях рыбоводческого хозяйства.

Проведенные исследования оптимизации стандартной методики криоконсервации спермы стерляди могут быть рекомендованы для применения на коммерческих предприятиях с целью товарного выращивания осетровых рыб.

Ключевые слова: стерлядь, сперма, криоконсервация.