Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Applied aspects of fish sperm cryopreservation

Aplikované aspekty kryokonzervace rybích spermií

Yevhen Horokhovatskyi

Czech Republic, Vodňany, 2018

I, Yevhen Horokhovatskyi, hereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

I hereby declare that, in accordance with the § 47b Act No. 111/1998 Coll., as amended, I agree with publicizing of my Ph.D thesis in full version electronically in a publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice on its web sites, with keeping my copyright to the submitted text of this Ph.D. thesis. I also agree so that the same electronic way, in accordance with above mentioned provision of the Act No. 111/1998 Coll., was used for publicizing reviews of supervisor and reviewers of the thesis as well as record about the progress and result of the thesis defence. I also agree with compering the text of my Ph.D. thesis with a database of theses "Theses.cz" operated by National Register of university theses and system for detecting of plagiarisms.

In Vodňany 3rd May, 2018

Supervisor:
Assoc. Prof. Borys Dzyuba
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Laboratory of Reproductive Physiology
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Consultants:
Marek Rodina, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Laboratory of Reproductive Physiology
Zatisi 728/II, 389 25 Vodnany, Czech Republic

David Gela, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Genetic Fisheries Center
Nad Velkou podvinicí 1153, 389 01 Vodnany, Czech Republic

Head of Laboratory of Reproductive Physiology:
Sergii Boryshpolets, Ph.D.

Dean of Faculty of Fisheries and Protection of Waters:
Prof. Pavel Kozák

Board of doctorate study defence with reviewers:
Assoc. Prof. Josef Matěna – head of the board
Prof. Petr Ráb – board member
Assoc. Prof. Tomáš Policar – board member
Prof. Lukáš Kalous – board member
Assoc. Prof. Ondřej Slavík – board member
Assoc. Prof. Zdeněk Adámek – board member

Dr. Ian Anthony Ernest Butts – Technical University of Denmark, DTU Aqua, Denmark – thesis reviewer
Dr. Akos Horvath - Szent Istvan University, Department of Aquaculture, Hungary – thesis reviewer

Date, hour and place of Ph.D. defence:
20th September 2018 in USB, FFPW, RIFCH, Vodňany, Czech Republic, 11 a.m.

Name: Yevhen Horokhovatskyi

Title of thesis:
Applied aspects of fish sperm cryopreservation
Aplikované aspekty kryokonzervace rybích spermií

Ph.D. thesis, USB FFPW, RIFCH, Vodňany, Czech Republic, 2018, 82 pages, with the summary in English and Czech.
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz
ISBN 978-80-7514-074-6

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

THE BACKGROUND OF LOW TEMPERATURE USE FOR PRESERVATION OF BIOLOGICAL OBJECTS

Cryopreservation of biological objects at the temperature of liquid nitrogen (-196 °C) is considered as one of the effective techniques for achieving long-term preservation, and it has been applied to a broad range of biological materials (Watson and Holt, 2001). However, to be able to successfully put the cellular system, tissue or organism, into a deep freeze condition and then bring back to a functional state, we need to understand the nature of complex phenomena which happen during freezing and thawing processes.

During freezing and thawing, cells are exposed to the influence of numerous physical and chemical factors which occur in response to changes in the physical state and chemical properties of the surrounding environment (Mazur, 2004). The harmful effect of these factors mostly depend on a freezing rate at which cells are cooled. According to 'Two factor hypothesis of freezing injury', there are different causes of injury during rapid freezing as well as during slow freezing. It has been suggested that slow cooling results in osmotic effects caused by enormous concentrations of solutes, while rapid freezing leads to intracellular ice formation (Mazur et al., 1972). Moreover, water can recrystallize into larger lethal-size ice crystals during warming, as a result of differences in surface free energy, and damage the cell (Seki and Mazur, 2008).

Nevertheless, for a better understanding of the specific mechanism of these events related to freezing rate changes, we should examine it more closely. In fact, most of the harmful events during the cell cryopreservation process are associated with osmotic water movement via the cell membrane (Richard et al., 2011). Depending upon the cell type, it contains from 60% to 80% of the total volume as liquid water and some of this water is bound to intracellular solutes or molecules (Mazur, 2004). All of these constituents are surrounded by a semipermeable membrane which maintains an equilibrium between the concentration of solutes and water inside and outside the cell (Denniston et al., 2011). The membrane permits water penetration relatively freely and acts as a barrier to large molecules of dissolved components. When the cellular system is exposed to fast freezing, dehydration of the cell does not occur and water freezes internally with a significant increase in volume (lethal-size ice crystals), which can lead to mechanical damage to the cell organelles and membrane layers with lethal consequences. However, if the freezing rate is too slow, intracellular formation of ice is minimized, but water will move out of the cell over an extended time and this will lead to dehydration and shrinking of the cell with a high probability of damage. Moreover, in this case the cell becomes exposed to enormous concentrations of solutes which lead to a toxic effect with subsequent damage to the cell (Mazur, 1996).

The dynamics of these two factors related to ice formation during cooling to very low temperatures can be minimized by using additives such as alcohols, polyols, and sugars; these are called cryoprotectants. Cryopreservation nearly always entails the use of different cryoprotectants that ensure protection of the cell at these temperatures. Nevertheless, they have both positive and negative influence on the cell, depending on exposure time and concentration (Richard et al., 2011). On the one hand, the presence of a cryoprotectan in the solution permits adequate dehydration of the cell and but prevents lethal-size intracellular ice formation. This phenomenon is closely related to the fact that most of the cryoprotectants can easily penetrate cell membrane to replace the water, and ensure equilibration of the solute concentration (Fahning and Garcia, 1992). Moreover, the interaction of cryoprotectant with cell membrane lipids increase pliability, reducint the likelihood of damage during shrinking due to the freezing process (Richard et al., 2011). On the other hand, many cryoprotectants become increasingly toxic for the cell as the exposure time or concentration increase (Best, 2015). Thus,

the cell exposure time during equilibration with cryoprotectant solution should be minimised. In addition, the balance between the minimum toxic influence of cryoprotectant to the cell and maximum degree of protection should be kept when freezing to very low temperatures. Although, most of the damage can be greatly reduced by using different cryoprotectants, nevertheless, this doen not totally prevent the damage, only decreases it.

A current theory considers that to survive cryogenic temperatures, which are required for stable and long-term preservation, the cell should be vitrified (Katkov et al., 2012). Water transition from a liquid to a glass-like solid state is called vitrification. If the cell is vitrified, damage due to intracellular ice formation is prevented by converting water into non-crystalline amorphous solid state. The success of water vitrification depends on an increase in viscosity within the cell, which occurs as cells solutes become concentrated. The increased viscosity of the intracellular and extracellular components prevents water molecules from coming together to form ice and thus avoids damage to the plasma membrane and other constituents of the cell. The successfully cryopreserved cells are vitrified while extracellular water is partially crystallized. However, vitrification of both intracellular and extracellular liquids is the challenge which requires highly permeable cryoprotectant concentrations. As the concentration increases toxicity increases for the cell. In view of the currently known biological and chemical effects of cryoprotective agents, the toxic impact appears to be the main limiting factor in cryobiology (Fahy and Wowk, 2015). Thus, the balance between low toxicity and high survival is vital for successful vitrification of any biological system.

In summary, better understanding of biological and physicochemical effects on the cell during cryopreservation will help to improve present results while pointing the way to new strategies that may be yet more successful in the future.

STATE OF THE ART OF FISH SPERM CRYOPRESERVATION: IMPORTANCE AND LIMITATIONS

Fish have traditionally been, and continue to be one of the most important parts of human nutrition. Fish are a source of high-quality animal proteins, minerals, amino acids and the essential healthy fats for billions of people throughout the world (Rice and Garcia, 2011). Global fish consumption has increased by more than 80 times since 1950 (FAO, 2006). Nowadays, fish account for more than 16 percent of the global population's intake of animal protein which continues to grow in relation to expansion in the world's human population, economic development, and global urbanization (FAO, 2014).

The constantly growing demand for the fish products has caused widespread overfishing of wild stocks and the populations of many fish species have decreased. In addition, many fish species have been negatively impacted through other human activities such as construction dams with consequent blockage of spawning migrations, water pollution with pernicious effectson water quality, etc. Because of these habitat degradations, numerous species have been at risk of extinction (FAO, 2014). As a result, many fish species are included in biodiversity conservation programs for population restoration (Freyhof and Brooks, 2011). Fish farming has developed as an alternative to supplement the growing commercial demand and reduce reliance on wild fisheries (Duarte et al., 2007).

One of the useful tools for both fish biodiversity conservation programs and controlled reproductive processes on farms is cryopreservation of fish gametes. Artificial breeding using frozen gametes has been practiced in the agricultural sector for more than fifty years (Watson and Holt, 2001). Both male and female gametes are needed, however, compared to spermatozoon, fish eggs and embryos are large because of the large amount of yolk and investing chorion. Therefore, uniform penetration of the cryoprotectant and cooling during

cryopreservation is challenging in these large and dense specimens (Chao and Liao, 2001). At the same time, the much smaller fish spermatozoa have a greater surface - volume ratio which allows cooling more uniformly and quickly. Moreover, cryoprotectants can penetrate spermatozoon membranes more easily and evenly to replace water. Spermatozoon dehydration can occur rapidly and water-salt balance is easlly restored after thawing (Kopeika, 2007).

The inability to cryopreserve fish female gametes could be circumvented by using androgenesis in species restoration where only cryopreserved sperm is used with the eggs of a related species (Martínez-Páramo et al., 2017). In practice, this approach involves inactivation of the oocyte genome through UV or gamma irradiation, and developmental activation of these eggs with the cryopreserved sperm of the donor with subsequent diploidization. The diploid or so called double haploid state of the embryo, in this case, is achieved by influence of cold, heat or pressure shock applied during the first mitotic division (Komen and Thorgaard, 2007). Therefore, the double haploid individual would carry only the duplicated set of chromosomes inherited from the cryopreserved spermatozoa of the given species. Cryopreserved sperm has been used successfully in androgenesis on many fish species, including some most economically important freshwater fish as sturgeon (Grunina et al., 2006) and carp (Bongers, et al., 1994), showing the big potential of this approach.

However, androgenesis is complicated by problems which have prevented its use except on experimentally. A major problem is the high level of homozygosity of the progeny which results in mass mortalities in embryonic and post-embryonic age, showing a very low efficiency of androgenesis.

Thus, it is clear that long-term cryopreservation of fish sperm will not satisfy all needs for successful population restoration; nevertheless, it can serve as a supplementary management tool for efficient breeding management of farmed fish, hybridization and selective breeding, biodiversity programs and for the conservation of endangered species (Kopeika et al., 2007). Moreover, it has many advantages for the farm sector as it affords an opportunity to synchronize gamete availability of both males and females, rational use of sperm, the decrease of the male number needed at a hatchery, simplification of broodstock maintenance and gamete transport between farms (Cabrita et al., 2010).

Therefore, long-term cryopreservation of fish sperm is becoming a supplementary management tool for efficient breeding management of farmed fish, hybridization and selective breeding, biodiversity programs and for conservation of endangered species (Kopeika et al., 2007).

Nowadays, there is a high interest in elaboration of the most effective cryopreservation protocols for fish sperm cryopreservation. Detailed literature analysis in the “Web of Science” database (www.webofknowledge.com) using “fish sperm cryopreservation” as key word showed an increasing trend in research related to this topic over the last 20 years (Fig. 1).

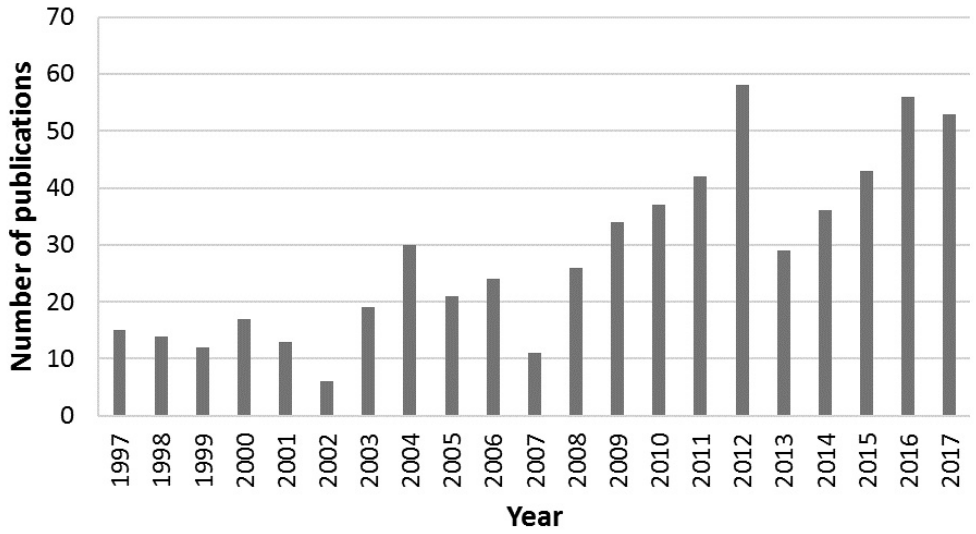


Figure 1. *Number of scientific publications on topic fish sperm cryopreservation, which can be found through a Web of Science. Search entry parameters: TOPIC: (fish sperm cryopreservation) and YEAR PUBLISHED: (1997–2017).*

However, fish sperm cryopreservation status nowadays is highly contentious compared to mammals, where cryopreservation of both cattle and human gametes is a routine tool and widely applied in agriculture and medicine. In contrast, for fish the absence of specific techniques and standardization in the developed cryopreservation protocols still presents challenges, which to date influence the efficiency of this approach (Asturiano et al., 2016). Among these challenges are: intra-species differences of sperm cryoresistance (Horokhovatskyi et al., 2016), a lack of tools to characterize cryodamages and correct assay of seminal quality (Cabrita et al., 2014). Moreover, the published methods have limited the use of cryopreserved sperm in aquaculture due to difficulties in obtaining reproducible results. For this reason, AQUAEXEL 2020 a Horizon 2020 research infrastructure project was developed to support the sustainable growth of the aquaculture sector in Europe. In particular, one of the task of this project was to establish common procedures to secure the cryobanks already existing in Europe, standardize the procedures for cryopreservation, traceability, and quality assessment and to disseminate the cryobanking strategy to facilities in need of genetic resources management and preservation. The present PhD study was performed partially in the frame of AQUAEXEL 2020 project, aiming to standardization of fish sperm cryopreservation approaches in Europe.

As an example, Czech Republic is one of the largest carp producers and exporters in Europe, constituting nearly 97% of the total national freshwater fish production (European Commission, 2016). Carp breeding programs involve many different strains typically used in aquaculture production and most of them are included in the National Program on Conservation of Farm Animal Genetic Resource. One part of this programme is the establishment of fish sperm cryobank at the Faculty of Fisheries and Protection of Waters of the University of South Bohemia in Ceske Budejovice. However, over the nearly 20 years of its activity, cryopreservation of carp sperm from some fish individuals still can present challenges and prevent achieving of satisfactory results on a regular basis. It has been suggested that these challenges can

be related to differences in the cryoresistance of carp sperm while causes of this still remain unclear.

Another example regards sturgeons, which are valuable for caviar and boneless meat all around the world (Bronzi et al., 2011). The demand for sturgeon aquaculture has increased, due to continuous overfishing of a wild stock, sturgeon population have decreased and many species have been included to biodiversity conservation programs for population restoration (Mims, 2011). Many cryobiological studies have been conducted on sturgeon sperm and broad range of successful protocols for cryopreservation were elaborated over the past decades (Kopeika, 2000; Glogowski et al., 2002; Boryshpolets et al., 2011; Judycka et al., 2015). Nevertheless, such factors as initial sperm quality, extender and cryoprotectant type, equilibration conditions and, freezing/thawing regimes still can influence cryopreservation success (Kopeika et al., 2007; Horokhovatskyi et al., 2017).

Initial sperm quality is considered as one of the most important parameters for sample keeping-discarding for cryopreservation. After collection, sperm quality of many fish species decrease rapidly, however, sturgeon sperm can be stored at ice temperatures (0–4 °C) for several days and retaining high motility percentage (Aramli et al., 2013). Moreover, for sturgeon sperm, this period can be greatly prolonged by mixing with extenders of various composition, antibiotics and/or storage under oxygen conditions (Billard et al., 2004). However, the quality of sperm gradually decreases over time, depending on the storage conditions and individual male variability. It has been suggested that oxidative stress and decrease in ATP content might be the main causes of the decline in sperm quality during storage (Perchec et al., 1995; Aitken and Baker, 2006).

The fish spermatozoon membrane is particularly receptive to lipid peroxidation as it contains great amounts of polyunsaturated fatty acids (Martínez-Páramo et al., 2012). An imbalance between reactive oxygen species and spermatozoa antioxidants can cause lipid peroxidation and alter membrane structure (Li et al., 2010b). This in turn, can influences membrane permeability to water and other molecules, membrane fluidity and lipid phase transitions in the membrane bilayer (Holt, 2000). As a result of these changes in the spermatozoa plasma membrane, sperm cryoresistance can decrease. Moreover, this also can be considered as a main reason of sperm cryoresistance heterogeneity.

During freezing and thawing, spermatozoa are exposed to numerous biophysical and chemical factors, including osmotic changes, dehydration and rehydration with subsequent changes in cell volume, ice crystals formation, cryoprotectants toxicity and etc. (Asturiano et al., 2016). Being specifically sensitive to environment tonicity and chemical composition, spermatozoa can be injured during cryopreservation with consequent decrease in their viability and fertilization capacity. The formation of ice crystals during freezing and thawing is considered as a major source of dramatic changes of extracellular osmolality, which in turn leads to spermatozoa cryoinjuries (Billard et al., 2004; Zilli et al., 2008). According to the 'Two factor hypothesis of freezing injury', there are different causes of injury during rapid and slow cooling of cells. It has been suggested that slow cooling results in osmotic effects from high solute concentration, while rapid freezing leads to intracellular ice formation and its subsequent recrystallization during warming (Mazur et al., 1972). The demonstration of optimal freezing rates would provide the possibility of reducing the effect of these damaging factors, but precise ranges of optimal rates still remain to be identified.

Therefore, spermatozoa cryoresistance is strongly dependent on the capacity of the cell to resist osmotic changes which is related to spermatozoa plasma membrane properties. The cryopreservation process could also modify membrane lipid profile, shedding phospholipids, saturated fatty acids and cholesterol (Labbe et al., 1997; Chakrabarty et al., 2007). Such modifications in the sperm membrane profiles have detrimental effects on spermatozoa,

resulting in subsequent leakage of the intracellular organelles, including cytoplasmic and membrane-bound proteins and enzymes as well as other components, which co-elute from spermatozoa (Li et al., 2010a). Moreover, the freeze-thaw process can also provoke alteration in DNA integrity and protein profile (Zilli et al., 2003; Cabrita et al., 2005). Defects in sperm proteins may in turn have a pernicious effect on sperm motility parameters, fertilization capacity, and early events after fertilization (Lessard et al., 2000; Li et al., 2010). Different studies conducted on this topic have shown alteration in fish sperm protein profile after cryopreservation (Zilli et al., 2005; Li et al., 2010; Nynca et al., 2014). It has been shown that some proteins decrease in their abundance after cryopreservation and, these changes can be related to leakage of proteins from inside the spermatozoa to the extracellular medium (Li et al., 2010). However, it has been also observed that cryopreservation can increase some protein content in spermatozoa through their modification (Ardon and Suarez, 2013). All these observations indicate that cryopreservation has a complex effect on fish sperm proteins.

Nevertheless, many published results are unclear as they are based on analysis of frozen/thawed sperm suspension which contains not only the fraction of interest presenting viable spermatozoa but also lethal and sublethal damaged spermatozoa which, are not actually involved into fertilization process. The analysis of such suspensions could obscure real cryopreservation effect on survived spermatozoa showing suitable motility parameters and involved into fertilization process. Moreover, the presence of damaged spermatozoa, could negatively influence fertilization process if post-thaw mixture of damaged and viable spermatozoa is used (Ahmadi, 1999).

In mammals, there have been several widely used different techniques for selecting sperm subpopulation with high parameters in assisted reproductive techniques. Among them are swim-up, swim-down, fluorescence cell sorting, electrophoresis, migration-sedimentation, glass wool filtration, density gradient centrifugation which considered as effective techniques for sperm separation (Lan, 2000; Beydola and Agarwal, 2014). In principle, all these techniques provide an opportunity to select subpopulation of high quality sperm from damaged spermatozoa and other constituents of the ejaculate. Nevertheless, the application of these techniques for fish sperm is quite challenging and data are very limited. Fish spermatozoa are commonly immotile in seminal fluid and for motility initiation, changes in environment osmolality and ionic composition are required (Cosson, 2004). However, to be able to apply separation technique for fish sperm, spermatozoa should be kept inactivated as the motility period after initiation is very limited. Thus, separation of fish spermatozoa after cryopreservation has potential to be applied in improvement of assisted reproduction in aquaculture, however, it will require more in-depth elaboration than is the case for mammals, and specifically depending on fish species.

Although fish sperm cryopreservation can provoke a broad range of species-specific damage, it can also serve as a useful tool in fields of research and aquaculture production. Meanwhile, cryopreservation is a technique of definite interest with increasing potential for wider application in aquaculture industry. Therefore, the existent cryopreservation protocols must be adapted to find species-specific compromises, where the protocols are better developed to guarantee the benefits of this technique.

AIMS AND OBJECTIVES OF THE STUDY

The present study was aimed to explore the possibilities to increase the fish sperm cryopreservation outcomes via elucidation of reasons for the heterogeneity of fish sperm cryoresistance, standardization of freezing protocols and elaboration of methods allowing dipper understanding of cryodamage.

The specific objectives of the study were:

1. To explore and compare the lipid composition of the plasma membrane in common carp (*Cyprinus carpio*) sperm exhibiting different cryoresistance.
2. To standardize the cryoprotocols needed for fish sperm cryobanking, by means of development of new techniques and tools for cryopreservation of sterlet (*Acipenser ruthenus*) sperm in uncontrolled cooling devices.
3. To examine the cryopreservation effects on motility, viability, and proteome of survived this process spermatozoa subpopulation selected by Percoll density gradient centrifugation in sterlet (*Acipenser ruthenus*) sperm.

REFERENCES

Ahmadi, A, Soon-Chye, N., 1999. Developmental capacity of damaged spermatozoa. Human Reproduction 14(9): 2279-2285.

Aitken, R. J., Baker, M. A. 2006. Oxidative stress, sperm survival and fertility control." Molecular and Cellular Endocrinology 250(1-2): 66-69.

Aramli, M. S., Kalbassi, M. R., Nazari, R. M., Aramli, S. 2013. Effects of short-term storage on the motility, oxidative stress, and ATP content of Persian sturgeon (*Acipenser persicus*) sperm. Animal Reproduction Science 143(1): 112-117.

Ardon, F., Suarez S. S. 2013. Cryopreservation increases coating of bull sperm by seminal plasma binder of sperm proteins BSP1, BSP3, and BSP5. Reproduction 146: 111-117.

Asturiano, J. F., Cabrita E., Horvath, A. 2016. Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. General and Comparative Endocrinology 245: 69-76

Best, B. P. 2015. Cryoprotectant Toxicity: Facts, Issues, and Questions. Rejuvenation Research 18(5): 422-436.

Billard, R., Cosson, J., Noveiri, S. B., Pourkazemi, M. 2004. Cryopreservation and short-term storage of sturgeon sperm, a review. Aquaculture 236(1–4): 1-9.

Bongers, A. B. J., in't Veld E. P. C., Abo-Hashema, K., Bremmer, I. M., Eding, E. H., Komen, J., Richter, C. J. J. 1994. Androgenesis in common carp (*Cyprinus carpio L.*) using UV irradiation in a synthetic ovarian fluid and heat shocks. Aquaculture 122(2): 119-132.

Boryshpolets, S., Dzyuba, B., Rodina, M., Alavi, S. M. H., Gela, D., Linhart, O. 2011. Cryopreservation of sterlet (*Acipenser ruthenus*) spermatozoa using different cryoprotectants. Applied Ichthyology 27(5): 1147-1149.

Bronzi, P., Rosenthal, H., Gessner, J. 2011. Global sturgeon aquaculture production: an overview. Journal of Applied Ichthyology 27(2): 169-175.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V. 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432: 389-401.

Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., Herráez, M.P. 2005. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology 50(2): 144-153.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M. P. 2010. Cryopreservation of fish sperm: applications and perspectives. Journal of Applied Ichthyology 26(5): 623-635.

Chakrabarty, J., Banerjee, D., Pal, D., De, J., Ghosh, A., Majumder, G. C. 2007. Shedding off specific lipid constituents from sperm cell membrane during cryopreservation. Cryobiology 54(1): 27-35.

Chao, N. H., Liao, I. C. 2001. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture 197(1): 161-189.

Cosson, J. 2004. The Ionic and Osmotic Factors Controlling Motility of Fish Spermatozoa. Aquaculture International 12(1): 69-85.

Denniston, R. S., Michelet S., Bondioli, K. R., Godke, R. A. 2011. Principles of embryo cryopreservation. In: Tiersch T. R. and Green C. C. (Eds.), Cryopreservation in Aquatic Species, 2nd Edition. Baton Rouge, Louisiana, World Aquaculture Society, pp. 274-290.

Duarte, C. M., Marbá, N., Holmer, M. 2007. Rapid Domestication of Marine Species. Science 316(5823): 382.

Kopeika, E. F., Williot, P., Goncharov, B. 2000. Cryopreservation of Atlantic sturgeon Acipenser sturio L., 1758 sperm: First results and associated problems. Boletin Instituto Espanol de Oceanografia 16: 167-173.

European Commission. 2016. The Price structure in the supply chain for fresh carp in Central Europe. European Union, Directorate General for Maritime Affairs and Fisheries of the European Commission

Fahning, M. L., Garcia M. A. 1992. Status of cryopreservation of embryos from domestic animals. Cryobiology 29(1): 1-18.

Fahy, G. M., Wowk, B. 2015. Principles of Cryopreservation by Vitrification. In: Wolkers, W., Oldenhof, H. (Eds.), Cryopreservation and Freeze-Drying Protocols. Methods in Molecular Biology, pp. 21-82.

FAO, 2006. The state of world fisheries and aquaculture 2006 , 2007. Food and Agriculture Organization of the United Nations, Rome, p. 162.

FAO, 2014. The state of world fisheries and aquaculture 2014. Food and Agriculture Organization of the United Nations Rome, p. 223.

Freyhof, J., Brooks, E. 2011. European red list of freshwater fishes. Luxembourg, Publications Office of the European Union, p. 61.

Glogowski, J., Kolman, R., Szczepkowskib, M., Horváth, A., Urbányi, B., Sieczyńskia, P., Rzemieniecki, A., Domagaład, J., Demianowicz, W., Kowalski, R., Ciereszko, A. 2002. Fertilization rate of Siberian sturgeon (Acipenser baeri, Brandt) milt cryopreserved with methanol. Aquaculture 211(1–4): 367-373.

Grunina, A. S., Recoubratsky, A. V., Tsvetkova, L. I., Barmintseva, V. A. 2006. Investigation on dispermic androgenesis in sturgeon fish. The first successful production of androgenetic sturgeons with cryopreserved sperm. International Journal of Refrigeration 29(3): 379-386.

Holt, W. V. 2000. Basic aspects of frozen storage of semen. Animal Reproduction Science 62(1): 3-22.

Horokhovatskyi, Y., Rodina, M., Asyabar, H. D., Boryshpolets, S., Dzyuba, B. 2017. Consequences of uncontrolled cooling during sterlet (*Acipenser ruthenus*) sperm cryopreservation on post-thaw motility and fertilizing ability. Theriogenology 95: 89-95.

Horokhovatskyi, Y., Sampels, S., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M., Dzyuba, B. 2016. Lipid composition in common carp (*Cyprinus carpio*) sperm possessing different cryoresistance. Cryobiology 73(2): 282-285.

Judycka, S., Szczepkowski, M., Ciereszko, A., Dietrich, G. J. 2015. New extender for cryopreservation of Siberian sturgeon (*Acipenser baerii*) semen. Cryobiology 70(2): 184-189.

Katkov, I. I., Bolyukh, V. F., Chernetsov, O. A., Dudin, P. I., Grigoriev, A. Y., Isachenko, V., Isachenko, E., Lulat, A. G. M., Moskovtsev, S. I., Petrushko, M. P., Pinyaev, V. I., Sokol, K. M., Sokol, Y. I., Sushko, A. B., Yakhnenko, I. 2012. Kinetic Vitrification of Spermatozoa of Vertebrates: What Can We Learn from Nature? Current frontiers in cryobiology. In: Katkov, I. I., Current Frontiers in Cryobiology, InTech Open Access Publisher, pp. 1-40

Komen, H., Thorgaard, G. H. 2007. Androgenesis, gynogenesis and the production of clones in fishes: A review. Aquaculture 269(1): 150-173.

Kopeika, E., Kopeika, J., Zhang, T. 2007. Cryopreservation of fish sperm. In: Day, J. G., Stacey, G. N. (Eds.), Cryopreservation and freeze-drying protocols, second edition. Totowa, New Jersey, Humana Press. 368, pp. 203-217.

Labbe, C., Crowe, L. M., Crowe, J. H. 1997. Stability of the lipid component of trout sperm plasma membrane during freeze-thawing. Cryobiology 34(2): 176-182.

Xu, L., Rk, L., Chen, L., Zheng, Y. L. 2000. Comparative study on efficacy of three sperm-separation techniques. Asian Journal of Andrology 2: 131-134.

Lessard C, Parent, S, Leclerc, P, Bailey, J. L., Sullivan, R. 2000. Cryopreservation alters the levels of the bull sperm surface protein P25b. Journal of Andrology 21: 700-707.

Li, P., Hulak, M., Koubek, P., Sulc, M., Dzyuba, B., Boryshpolets, S., Rodina, M., Gela, D., Manaskova-Postlerova, P., Peknicova, J., Linhart, O. 2010a. Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, *Cyprinus carpio*. Theriogenology 74: 413-423.

Li, P., Li, Z.H., Dzyuba, B., Hulak, M., Rodina, M., Linhart, O. 2010b. Evaluating the impacts of osmotic and oxidative stress on common carp (*Cyprinus carpio,* L.) sperm caused by cryopreservation techniques. Biology of reproduction 83(5): 852-858.

Zilli, L., Schiavone, R., Zonno, V., Rossano, R., Storelli, C., Vilella, S. 2005. Effect of Cryopreservation on Sea Bass Sperm Proteins. Biology of reproduction 72: 1262-1267.

Martínez-Páramo, S., Diogo, P., Dinis, M.T., Herráez, M.P., Sarasquete, C., Cabrita, E. 2012. Sea bass sperm freezability is influenced by motility variables and membrane lipid composition but not by membrane integrity and lipid peroxidation. Animal Reproduction Science 131(3): 211-218.

Martínez-Páramo, S., Horváth, A., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T. R., Cabrita, E. 2017. Cryobanking of aquatic species. Aquaculture (Amsterdam, Netherlands) 472: 156-177.

Mazur, P. 1996. Chapter 10 Principles of medical cryobiology: The freezing of living cells, tissues, and organs. In: Bittar, E. E., Bittar, N.(Eds.), Principles of Medical Biology., Elsevier. 4, pp. 355-384.

Mazur, P. 2004. Principles of Cryobiology. In: Fuller, B. J., Lane, N., Benson, E. E. (Eds.), Life in the frozen state, Press LLC, pp. 3-67.

Mazur, P., Leibo, S. P., Chu, E. H. Y. 1972. A two-factor hypothesis of freezing injury: Evidence from Chinese hamster tissue-culture cells. Experimental Cell Research 71(2): 345-355.

Nynca, J., Dietrich, G. J., Dobosz, S., Grudniewska, J., Ciereszko, A. 2014. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. Aquaculture 433: 62-65.

Perchec, G., C. Jeulin, Cosson, J., André, F., Billard, R. 1995. Relationship between sperm ATP content and motility of carp spermatozoa. Journal of Cell Science 108(2): 747-753.

Rice, J. C., Garcia, S. M. 2011. Fisheries, food security, climate change, and biodiversity: characteristics of the sector and perspectives on emerging issues. ICES Journal of Marine Science 68(6): 1343-1353.

Richard, S. D., Sherri, M., Bondioli, K. R., Godke, R. A. 2011. Principles of Embryo Cryopreservation. In: Tiersch T. R. and Green C. C. (Eds.), Cryopreservation in Aquatic Species, 2nd Edition. Baton Rouge, Louisiana, World Aquaculture Society, pp. 274-290.

Seki, S., Mazur, P. 2008. Kinetics and activation energy of recrystallization of intracellular ice in mouse oocytes subjected to interrupted rapid cooling. Cryobiology 56(3): 171-180.

Mims, S. D., Tsvetkova, L. I., Wayman, W. R., Horvath, A., Urbanyi, B., Gomelskyi, B. 2011. Cryopreservation of Sturgeon and Paddlefish Sperm. In: Tiersch T. R. and Green C. C. (Eds.), Cryopreservation in Aquatic Species, 2nd Edition. Baton Rouge, Louisiana, World Aquaculture Society, pp. 366-380.

Beydola, T., Agarwal, A. 2014. Sperm Preparation and Selection Techniques. Medical & Surgical Management of Male Infertility 1: 244-251.

Watson, P. F., Holt, W. V.,(Eds.). 2001. Cryobanking the genetic resource: wildlife conservation for the future? London, Taylor & Francis, pp. 1-443

Zilli, L., Schiavone, R., Storelli, C., Vilella, S. 2008. Molecular mechanisms determining sperm motility initiation in two sparids (*Sparus aurata and Lithognathus mormyrus*). Biology of reproduction 79(2): 356-366.

Zilli, L., Schiavone, R., Zonno, V., Storellia, C., Vilella, S. 2003. Evaluation of DNA damage in Dicentrarchus labrax sperm following cryopreservation. Cryobiology 47(3): 227-235.

CHAPTER 2

LIPID COMPOSITION IN COMMON CARP (*CYPRINUS CARPIO*) SPERM POSSESSING DIFFERENT CRYORESISTANCE

Y. Horokhovatskyi, S. Sampels, J. Cosson, O. Linhart, M. Rodina, P. Fedorov, M. Blecha, B. Dzyuba, 2016. Lipid composition in common carp (*Cyprinus carpio*) sperm possessing different cryoresistance. Cryobiology 73, 282–285.

My contribution to this work was 70%.

Cryobiology 73 (2016) 282–285

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Lipid composition in common carp (*Cyprinus carpio*) sperm possessing different cryoresistance

Y. Horokhovatskyi [a, *], S. Sampels [b], J. Cosson [a], O. Linhart [a], M. Rodina [a], P. Fedorov [a], M. Blecha [a], B. Dzyuba [a]

[a] University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic
[b] University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Institute of Aquaculture and Protection of Waters, Na sádkách 1780, 370 05 České Budějovice, Czech Republic

ARTICLE INFO

Article history:
Received 29 April 2016
Accepted 9 August 2016
Available online 27 August 2016

Keywords:
Carp sperm
Cryopreservation
Lipid content
Sperm motility
Cryoresistance

ABSTRACT

The present study examined the lipid composition of plasma membranes in carp sperm with different post-thaw motility. The approach adapted for carp sperm cryopreservation, which involves the selection of the most effective protocol for individual males by comparing two cryoprotective media, was applied to the cryopreservation procedure. Sperm motility prior to freezing was greater than 80% but decreased to 40% in one group and to 10% in another group following cryopreservation. Lipid content of fresh sperm in all groups was analysed by thin layer chromatography and gas chromatography, with significant differences in phospholipid content, cholesterol and free fatty acids detected between groups, whereas the cholesterol/phospholipid ratio was extremely similar between groups (0.52 ± 0.038 and 0.52 ± 0.022). Increasing concentrations of saturated fatty acids, monounsaturated acids and decreasing concentrations of polyunsaturated n-6 fatty acids were negatively correlated ($P < 0.05$) with post-thaw motility of the carp sperm.

Cryopreservation at liquid nitrogen temperatures, which has been used for more than 60 years as a management tool for fish hybridisation and selective breeding, as well as for conservation of biodiversity and endangered species, is considered both suitable and effective for the prolonged storage of fish sperm [6]. However, fish sperm cryopreservation presents some challenges that currently inhibit the applicability of standardised approaches, such as the high degree of variability in cryoresistance between fish species (and even among individual males of the same species) and the absence of effective sperm cryopreservation techniques for some fish species [1].

As an example, the Czech Republic is the largest producer and exporter of carp in Europe, constituting nearly 88% of total aquaculture production on the continent [2]. Carp breeding programs involve many different strains, with the most desirable strains typically used in aquaculture production. To prevent genetic losses of less-desirable carp strains, the National Program on Conservation of Farm Animal Genetic Resources was established, with the fish sperm cryobank located at the Faculty of Fisheries and Protection of Waters of the University of South Bohemia in Ceske Budejovice part of this program. Over the nearly 20 years of its existence, the fish sperm cryobank has developed many effective tools for carp sperm cryopreservation, an approach that includes the selection of the most effective protocol for individual males [3] via two cryoprotective media consisting of either 10% Me2SO or ethylene glycol. Yet this approach cannot produce satisfactory results for the cryopreservation of some individual sperm samples, and the reasons for differences in the cryoresistance of carp sperm remain unclear.

It is well known that spermatozoa are exposed to numerous damaging factors during the freeze-thaw phases of the cryopreservation process, and success depends largely on membrane resistance to these factors [4]. Membrane lipid composition, especially in regard to the cholesterol/phospholipid ratio and free fatty acid composition, influences permeability to water and other molecules, membrane fluidity and lipid phase transitions in the

* Corresponding author.
E-mail addresses: horokhovatskyi@frov.jcu.cz (Y. Horokhovatskyi), sampels@frov.jcu.cz (S. Sampels), cosson@frov.jcu.cz (J. Cosson), linhart@frov.jcu.cz (O. Linhart), rodina@frov.jcu.cz (M. Rodina), fedorp00@frov.jcu.cz (P. Fedorov), blechm00@frov.jcu.cz (M. Blecha), bdzyuba@frov.jcu.cz (B. Dzyuba).

http://dx.doi.org/10.1016/j.cryobiol.2016.08.005
0011-2240/

membrane bilayer [5]. For this reason, lipid composition is believed to be one of the primary determinants of fish sperm cryoresistance. The goal of this study therefore was to explore the lipid composition of the plasma membrane in carp sperm exhibiting different post-thaw motility.

Male carp were collected from aquaculture ponds during the natural spawning period (May–June) and transported to 4-m3 hatchery tanks with a water flow rate of 0.2 L/s, temperature of 18 °C–22 °C and O2 content of 6–7 mg/L. Spermiation was induced using injection of carp pituitary extract (1 mg kg-1). Twenty-four hours after injection, sperm was collected from 25 males and kept in individual plastic syringes stored on ice (2–4 °C, 1–2 h) before freezing. Sperm motility was subjectively estimated using an optical phase contrast microscope (× 200) before and after freezing by mixing 1 μl sperm with 100 μl of activation solution (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.2). Only sperm samples that displayed high motility (>80%) were used for cryopreservation.

The freezing protocols for carp sperm cryopreservation described by Rodina (2010) were followed here. Sperm samples were diluted in two different extenders composed of either 180 mM NaCl, 2.68 mM KCl, 1.36 mM CaCl2·2H2O and 2.38 mM NaHCO3 with 10% of Me2SO; or 59 mM NaCl, 6.3 mM KCl, 0.68 mM CaCl2, 2.1 mM Mg2SO4, 27 mM NaHCO3, 3.4 mM sucrose, 69 mM D-mannitol, 118 mM Tris–HCl, pH 8.1 with 16% ethylene glycol. After dilution with the cryoprotective media, samples were drawn into 0.5-ml straws and frozen in a Styrofoam box filled with liquid nitrogen, with 30 straws placed on a Styrofoam raft (3-cm thick) situated above the surface of the liquid nitrogen. After 10 min, the straws were plunged directly into liquid nitrogen. The thawing procedure was performed in a 40 °C water bath for 6 s, and thawed sperm samples were used immediately for the sperm motility analysis. Samples were separated into those that exhibited high post-thaw sperm motility (40%, PTM-High) and those displaying low post-thaw sperm motility (<10%, PTM-Low).

All samples were prepared for lipid extraction and subsequent analysis of total lipids, phospholipids and free fatty acids. The native sperm samples were concentrated by centrifugation (ThermoFisher Scientific, Heraeus Fresco 17, Germany) at 5000 × g for 15 min at 4 °C, and then frozen at −80 °C until the time of lipid extraction.

Lipid extraction, methylation and analyses by GC were performed following the procedures described by Sampels (2011) [7]. Sperm samples were allowed to thaw at ambient room temperature (about 20 °C), with lipids then extracted using hexane:isopropanol (3:2) and an Ultra Turrax (T25, Janke & Kunkel, IKA Werke, Germany) for homogenisation. For phase separation, an aqueous solution of sodium sulphate (Na2SO4 at 6.67%) was added, and the suspension was then centrifuged for 5 min at 4000 rpm (2103 rcf) (Sorvall Super T21, Sorvall Products L.P., Newton, Connecticut, USA) and the upper hexane-rich layer removed and transferred to new tubes and evaporated under N2. The samples were dissolved in 2 ml of hexane and stored in sealed vials at −80 °C until further analyses.

An automated TLC Sampler (CAMAG, Switzerland) was used to apply the lipids to precoated silica gel plates (20 cm × 10 cm; Silicagel 60; 0.2 mm layer; Merck, Darmstadt, Germany). For total lipids, the plates with lipid fractions were predeveloped to full length in hexane:diethyl ether, whereas methyl-acetate:isopropanol:chloroform:methanol:KCl was used for phospholipids [8]. Plates were subsequently dried by a warm airstream in an automated developing chamber (CAMAG, Switzerland) and then sprayed with a cupric-phosphoric acid charring spray and activated at 110 °C for 20 min. The TLC chromatograms were evaluated using a TLC Scanner, and the scans were processed with winCATS software (CAMAG Scientific, Wilmington, US).

For fatty acid (FA) analyses with gas chromatography, all samples were methylated according to [9]. Two millilitres of 0.01 M NaOH in dry methanol was added to each sample, following which the samples were heated at 60 °C for 10 min. Three millilitres of boron trifluoride (BF3) was then added to the samples, and samples were again heated at 60 °C for 10 min. After cooling the samples under cold running water, 2 ml of a 3.42 M NaCl solution in water and 2 ml of hexane were added, and after 30 min, the upper layer was transferred to a new tube and evaporated under N2. The lipids were dissolved in 0.5 ml hexane and stored at −20 °C for a maximum of 1 week prior to analysis by gas chromatography.

Fatty acid composition was analysed with a gas chromatograph (CP9001, Chrompack, Middelburg, the Netherlands) equipped with a flame ionisation detector and a split injector, as described by Pickova et al. (1997) using a BPX 70 column (SGE, Austin, Texas, USA) with a length of 50 cm, an id of 0.22 mm, and a film thickness of 0.25 μm. Peaks were identified by comparing their retention times with those of a standard mixture (GLC-68A; Nu-Chek Prep, Elysian, USA) and other accepted standards; the response factors were also evaluated via comparisons with the GLC-68A standard [7]. Statistical analyses were performed using Statistica V 12.0 software (Statsoft Inc., Tulsa, OK, USA). As the number of males was small (n = 25), nonparametric statistics were applied, and the Mann–Whitney *U* test was used to compare the two independent groups (PTM-High and PTM-Low). Differences were considered statistically significant at $P < 0.05$.

There were significant differences in phospholipid, cholesterol and free fatty acid proportions between the PTM-High and PTM-Low groups of carp sperm (Fig. 1). The percentage of phospholipids and cholesterol increased in the PTM-Low group in comparison with the PTM-High group; however, the cholesterol/phospholipid ratio was similar for both groups (PTM-High: 0.520 ± 0.038; PTM-Low: 0.520 ± 0.022). Diacylglycerols (DAGs) were found to be present in the PTM-Low group but not in the PTM-High group.

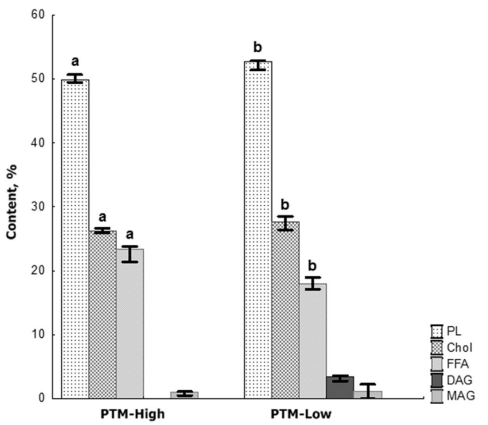


Fig. 1. Content of major lipid classes in carp sperm in the PTM-High and PTM-Low groups. PL: phospholipids; Chol: total cholesterol; FFA: free fatty acids; DAG: diacylglycerols; MAG: monoacylglycerols. Data are presented as median values with 25% and 75% percentiles. Different superscripts indicate statistical differences ($P < 0.05$) between the PTM-High and PTM-Low groups.

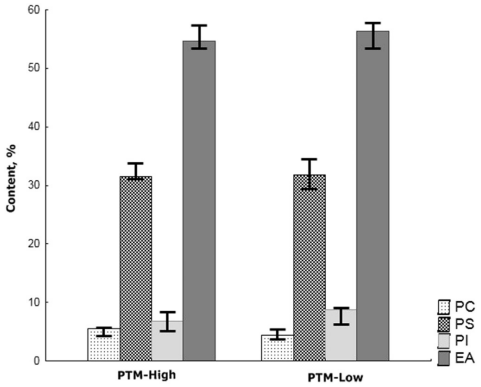


Fig. 2. Phospholipid content of carp sperm in the PTM-High and PTM-Low groups. PC: α-phosphatidylcholine; PS: α-phosphatidylserine; PI: phosphatidylinositol; EA –phosphotidylethanolamine. Data are presented as median values with 25% and 75% percentiles. No significant differences between the PTM-High and PTM-Low groups were detected ($P > 0.05$).

However, no statistically significant differences were found within the phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (EA) phospholipid groups (Fig. 2).

The fatty acid composition of the groups is shown in Table 1. The PTM-High group had significantly lower saturated FA and 18:1 n-9 but significantly higher levels of n-6 FA.

The results of this study demonstrate that there is a negative correlation between changes in the contents of major lipid classes and sperm motility after cryopreservation. The observed changes in major lipid classes are indicative of the increase in phospholipid, cholesterol and free fatty acid levels in the PTM-Low group. The cholesterol/phospholipid ratio is known to be negatively correlated with sperm motility, as at high levels it can influence membrane fluidity and thereby compromise the osmotic regulation and ionic exchange processes involved in motility activation [10], but a negative correlation between cholesterol/phospholipid ratio and post-thaw sperm motility was not observed in the present study for either the PTM-High or PTM-Low group. Thus, it would appear that the cholesterol/phospholipid ratio does not affect the freezability of carp sperm.

Table 1
Fatty acid content (percentage) in carp sperm. Data are presented as mean values ± one standard deviation; bolded values with different superscripted letters in the same row are significantly different ($P < 0.05$).

Fatty acid group	Notation	PTM-low (%)	PTM-high (%)
Saturated FA		**20.47 ± 1.12^a**	**19.29 ± 1.02^b**
	C 14:0	0.21 ± 0.11	0.24 ± 0.09
	C 16:0	1.23 ± 0.21	1.14 ± 0.17
	C 18:0	15.3 ± 0.92	13.5 ± 0.74
	C 21:0	0.35 ± 0.16	0.31 ± 0.07
	C 24:0	3.5 ± 1.28	3.98 ± 0.99
Monounsaturated FA		16.79 ± 3.43	15.46 ± 3.17
	C16:1	1.8 ± 0.76	1.92 ± 0.39
	C16:1 trans	1.23 ± 0.23	1.14 ± 0.17
	C18:1 n-9	**10.68 ± 2.55^a**	**9.09 ± 2.62^b**
	C18: 1 n-7	3.09 ± 0.29	3.32 ± 0.38
Polyunsaturated n-3 FA		24.69 ± 2.46	23.43 ± 3.06
	C18:3 n-3	0.47 ± 0.22	0.36 ± 0.09
	C20:4 n-3	0.25 ± 0.22	0.20 ± 0.05
	C20:5 n-3	9.48 ± 5.06	6.75 ± 2.01
	C22:5 n-3	2.89 ± 1.31	1.17 ± 1.08
	C22:6 n-3	20.8 ± 2.83	21.07 ± 2.81
Polyunsaturated n-6 FA		**28.72 ± 5.30^b**	**34.76 ± 2.16^b**
	C18:2 n-6	**3.7 ± 0.76^a**	**4.27 ± 0.73^b**
	C20:4 n-6	**19.53 ± 3.29^a**	**23.17 ± 1.36^b**
	C22:4 n-6	**2 ± 0.37^a**	**2.75 ± 0.27^b**
	C20:2 n-6	3.49 ± 1.51	4.72 ± 1.21
PUFA		**62.52 ± 3.19^a**	**65.10 ± 3.72^b**

Phospholipid components of sperm plasma membranes could be modified by cryopreservation, leading to changes in cellular homeostasis and to irregularities in sperm function [4], but no differences were observed between the two groups in regard to phospholipid composition. We found that both saturated FA and monounsaturated FA were higher in the PTM-Low group, whereas higher levels of polyunsaturated FA were present in the PTM-High group, suggesting that higher levels of unsaturated FA are associated with higher levels of cryoresistance. In our study, sperm with higher proportions of total n-6 had better cryoresistance, indicating that in carp sperm these FA—especially 20:4 n-6 but also 18:2 n-6 and 22:4 n-6—might play an important role in determining the degree of sperm cryoresistance. These differences could have an influence on plasma membrane fluidity, lipid phase transition, and permeability to water and other molecules [5].

Finally, higher post-thaw motility was also linked to lower lipid content in sperm. In light of the fact that differences in the content of some lipid classes—as for example free fatty acids, which were also statistically significant—do not fully explain the observed variability of post-thaw sperm motility, future studies are required to elucidate the role of each lipid class (and their ratios) in carp sperm freezability.

Conflict of interest

We have no conflict of interest to declare.

Acknowledgements

The authors thank Viktoriya Dzyuba for her valuable comments and discussions. The study was financially supported by the Grant Agency of the University of South Bohemia in České Budějovice (No. GAJU 059/2016/Z), and by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program).

References

[1] L.-Å. Appelqvist, Rapid methods of lipid extraction and fatty acid methyl ester preparation for seed and leaf tissue with special remarks on preventing the accumulation of lipid contaminants, Royal Swedish Academy of Science (Kungliga Svenska Vetenskapsakademien), Stockholm 28, 1968, pp. 551–570.
[2] B. Dzyuba, M. Rodina, S. Boryshpolets, O. Linhart, Freezing sperm of common carp (Cyprinus carpio L.) for the need for preservation of germplasm in practical conditions (Zmrazovani spermatu kapra obecneho (Cyprinus carpio L.) pro potreby uchovani genofondu v praktickych podminkach), Edice Metod. (2010) 6–24.
[3] FAO, National Aquaculture Sector Overview Czech Republic, Fisheries and Aquaculture Department, 2010.
[4] A. Hara, N.S. Radin, Lipid extraction of tissues with low toxicity solvent, Anal. Biochem. 90 (1978) 420–426.
[5] W.V. Holt, Basic aspects of frozen storage of semen, Animal Reproduction Sci. 62 (2000) 3–22.
[6] E. Kopeika, J. Kopeika, T. Zhang, Cryopreservation of Fish Sperm, Cryopreservation and Freeze-drying Protocols Second Edition, 2007, pp. 203–219.
[7] C. Labbe, L.M. Crowe, J.H. Crowe, Stability of the lipid component of trout sperm plasma membrane during freeze–thawing, Cryobiology 34 (1997) 176–182.
[8] S. Martínez-Páramo, P. Diogo, M.T. Dinis, M.P. Herráez, C. Sarasquete, E. Cabrita, Sea bass sperm freezability is influenced by motility variables and

membrane lipid composition but not by membrane integrity and lipid peroxidation, Animal Reproduction Sci. 131 (2012) 211–218.
[9] R.E. Olsen, R.J. Henderson, The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry, J. Exp. Mar. Biol. Ecol. 129 (1989) 189–197.
[10] S. Sampels, J. Pickova, Comparison of two different methods for the separation of lipid classes and fatty acid methylation in reindeer and fish muscle, Food Chem. 128 (2011) 811–819.

CHAPTER 3

CONSEQUENCES OF UNCONTROLLED COOLING DURING STERLET (*ACIPENSER RUTHENUS*) SPERM CRYOPRESERVATION ON POST-THAW MOTILITY AND FERTILIZING ABILITY

Yevhen Horokhovatskyi, Marek Rodina, Hadiseh Dadras Asyabar, Sergii Boryshpolets, Borys Dzyuba, 2017. Consequences of uncontrolled cooling during sterlet (*Acipenser ruthenus*) sperm cryopreservation on post-thaw motility and fertilizing ability. Theriogenology 95, 89–95.

My contribution to this work was 80%.

Theriogenology 95 (2017) 89–95

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Consequences of uncontrolled cooling during sterlet (*Acipenser ruthenus*) sperm cryopreservation on post-thaw motility and fertilizing ability

Yevhen Horokhovatskyi*, Marek Rodina, Hadiseh Dadras Asyabar, Sergii Boryshpolets, Borys Dzyuba

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25, Vodňany, Czech Republic

ARTICLE INFO

Article history:
Received 10 October 2016
Received in revised form
3 February 2017
Accepted 11 March 2017
Available online 15 March 2017

Keywords:
Sturgeon sperm
Cooling device
Freezing rate
Cryopreservation
Sperm motility
Fertilization

ABSTRACT

The significant influence of the number and position of fish sperm sample straws in uncontrolled cooling devices on post-thaw spermatozoa parameters, such as motility and fertilizing ability, is presented in this study. The two most popular uncontrolled cooling devices were used in this study: a Styrofoam box setup with a polystyrene floating raft on liquid nitrogen and the dry shipper setup with a straw holder. We tested the effect of different quantities of straws (6 or 60) placed on the polystyrene floating raft and the position of the straws in the holder (on the periphery or in the centre). Using these cooling methods, sperm of 10 male sterlets diluted with methanol containing cryoprotective medium was frozen. All temperature changes were recorded by a thermocouple inside the straw, and the thermogram intervals were analysed. Spermatozoa motility was evaluated by video microscopy with integrated computer-assisted sperm analysis software. Fertilization trials were conducted at a 10^5 spermatozoa/egg ratio. Post-thaw spermatozoa parameters, including the percent of motile spermatozoa, curvilinear velocity, velocity according to the smoothed path, linearity of track, beat-cross frequency and fertilization rate, were significantly decreased in the 60-straw floating raft setup in comparison to all of the other cooling methods. The freezing rate between −10 °C and −30 °C was significantly decreased by up to 18.6 ± 0.61 °C/min for the 60-straw floating raft setup in comparison to the other freezing conditions. Considering the above results, efforts to standardize cryopreservation protocols using uncontrolled cooling devices should take into account the amount of straws subjected to freezing.

1. Introduction

Sturgeons occupy a specific niche in the modern fish market worldwide. The demand for sturgeon aquaculture is increasing, especially for their caviar and boneless meat, which are valuable worldwide [1]. Current caviar production is estimated at 260 tons per year and could increase to 750 tons in the next 10 years [2]. However, due to the continuous decline of wild sturgeon populations and growing market demand for caviar, they have been included in biodiversity conservation programs for population restoration [3]. Currently, fish sperm cryobanking is considered to be a potentially powerful tool in aquaculture for fish hybridization, selective breeding programs for biodiversity, and also for the restoration of endangered species [4,5].

Many successful protocols for sturgeon sperm cryopreservation have been developed over the past decade [6–9]. Nevertheless, the success of fish sperm cryopreservation still depends on a broad range of factors, including initial sperm quality, extender and cryoprotectant type, dilution ratio, ability to be activated by the freezing/thawing process itself, and appropriate freezing and thawing rates [5,10,11]. Moreover, during freezing, spermatozoa can be injured by physical factors, such as aggregative transition in membranes, formation of ice crystals inside and outside the cell, and osmotic or oxidative stress [12]. It is currently accepted that many damaging effects of cryopreservation depend on the freezing rate, which in turn provokes osmotic stress during slow freezing or

* Corresponding author.
E-mail addresses: horokhovatskyi@frov.jcu.cz (Y. Horokhovatskyi), rodina@frov.jcu.cz (M. Rodina), dadras@frov.jcu.cz (H.D. Asyabar), sboryshpolets@frov.jcu.cz (S. Boryshpolets), bdzyuba@frov.jcu.cz (B. Dzyuba).

http://dx.doi.org/10.1016/j.theriogenology.2017.03.007
0093-691X/

intracellular ice formation during rapid freezing [13]. However, optimal freezing rates can minimize these damaging factors. Achieving optimal freezing rates is possible thorough commercial programmable freezers (Asymptote EF600, Asymptote Ltd.; Cryomed™, Thermo Scientific; PLANER Kryo 550-16, Planer PLC, and others) that deliver stable, controlled, and repeatable freezing rates [14]. However, despite the advantages of programmable cooling devices, many researchers studying fish sperm cryopreservation prefer devices with uncontrolled freezing rates, which are much simpler to use, less expensive, and easily transportable. The most popular uncontrolled freezer devices are Styrofoam boxes containing liquid nitrogen and equipped with a floating raft (which is designed to keep the set of freezing straws above the liquid nitrogen surface) or dry shippers into which straws are loaded directly in a liquid nitrogen-free cold interior space [6,15–18].

Regarding these devices, an exploration of publications in the "ScienceDirect" database (www.sciencedirect.com) using "fish sperm cryopreservation" as key word over the last 10 years shows that 62.5% of such studies used uncontrolled cooling devices and that the remaining 37.5% used programmable cooling devices.

Nevertheless, despite the higher usage rate of uncontrolled cooling devices, standardized protocols for freezing in these devices have not been published. Therefore, there is a risk of negatively modifying cryopreservation procedure by many factors, including the size and geometry of the device as well as different volumes and quantities of straws. All of these factors could influence the convective stream of nitrogen vapours and consequently change the freezing rate.

In the present study, we explore whether the number or position of straws frozen by application of uncontrolled cooling devices in fish sperm cryobanking influence the post-thaw sperm parameters, such as motility and fertilizing ability.

2. Materials and methods

2.1. Sperm collection

During the natural for sturgeon spawning season (April–May), ten sterlet males (3–4 years old, 0.6–2.0 kg body weight, BW) were transferred from fish farming ponds to 4-m^3 plastic tanks with a closed water recirculation system, and the temperature of the water was increased to 15 °C over the subsequent 24 h. Males were injected once with 4 mg/kg BW carp pituitary powder dissolved in a 0.9% (w/v) NaCl solution. Sperm was collected after 24 h using a syringe with an attached 4-mm plastic catheter inserted into the urogenital ducts. Prior to experimentation, sperm was stored at 4 °C on ice [15].

2.2. Sperm motility parameters assessment

Immediately after sperm collection, the motility parameters were evaluated. Motility of sperm samples was initiated (in a 1:100 dilution) in activation medium consisting of 10 mM Tris–HCl buffer and 0.25% Pluronic F-127 (catalogue number P2443, Sigma-Aldrich) at pH 8.0 [19]. Motility was observed using a negative phase-contrast microscope (UB 200i, PROISER, Spain) with an attached ISAS 782M digital camera (PROISER, Germany). As fish sperm tend to swim near the surface [20], motility was recorded on the bottom portion of the activation medium droplet. Video records were obtained at 10-s intervals from 10 to 90 s post-activation and were analysed using the Integrated System for Semen Analysis (ISAS) software (Proiser, Valencia, Spain). CASA analysis included the following parameters: percent of motile cells, VCL (curvilinear velocity over the actual path, in μm/s), VAP (velocity over the smoothed path, in μm/s), LIN (linearity of track, VSL/VCL × 100%) and BCF (beat-cross frequency, in Hz). The motility duration was measured as the time elapsed from activation to end of motility in approximately 95% of spermatozoa. The spermatozoa concentration was evaluated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on Olympus BX 50 phase-contrast microscope (Olympus, Japan) [19].

2.3. Cryopreservation: protocols, techniques and devices

Sperm samples were diluted to a 1:1 ratio (v:v) with a solution of the following composition: 30 mM Tris, 23.4 mM sucrose and 0.25 mM KCl supplemented with 10% methanol [6]. Following dilution, sperm samples were cryopreserved using uncontrolled cooling in either a Styrofoam box or dry shipper. The Styrofoam box (Fig. 1A) (dimensions: 52 × 33 × 30 cm) was filled to a depth of 10 cm with liquid nitrogen, and 6 or 60 straws (volume 0.5 mL) on a polystyrene raft (dimensions: 40 × 20 × 3 cm) were placed on the liquid nitrogen surface. After a 10-min exposure to liquid nitrogen vapour, straws were plunged directly into liquid nitrogen [19].

Before initiating experiments with usage of dry shipper, the device (Model CX 100, Taylor, Wharton, Alabama, USA) was cooled to −196 °C using liquid nitrogen one day before the experiments, and then, a straw holder containing 60 straws was placed inside the device (Fig. 1B). The cylindrical straw holder, which had 120-mm height and 65-mm radius, was purpose-made in our laboratory for freezing samples in a dry shipper. This design prevents the straws from contacting the wall of dry shipper or other straws and also maintains constant conditions for all experiments. After a 10-min cooling inside the dry shipper, straws were removed and

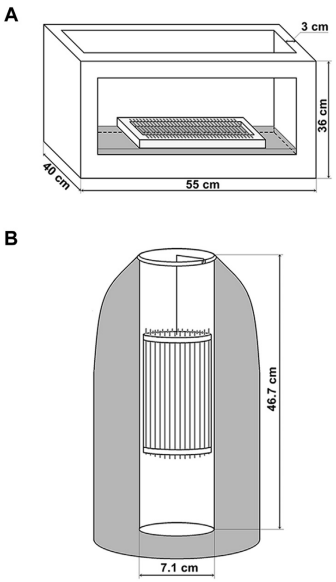


Fig. 1. Schematic illustration of uncontrolled cooling devices. (A) The Styrofoam box method consists in exposure of a 3-cm thick raft with straws to the liquid nitrogen surface (dark grey) inside a Styrofoam box. (B) The dry shipper method consists in insertion of a straw holder with straws in the middle of the interior space of a dry shipper.

immediately plunged into liquid nitrogen.

The thawing procedure was performed by transferring sperm samples to a 40 °C water bath for 6 s, followed by immediate use in motility and fertilization assays.

2.4. Freezing rate measurement

The cooling rates were measured for all of the cooling profiles using a copper-constantan type T thermocouple with a diameter 0.1 mm, (Model L-0044T, Omega, Stamford, USA), which was inserted inside four straws. Temperature values were recorded three times for each method by a 4 Channel Thermocouple Data Logger (OM-CP-QuadTemp2000, OMEGA) in conjunction with Omega_v4.2.7.0 software (OMEGA). The freezing parameters were collected over the following three temperature intervals: 1) freezing rate before initial water crystallization from 10 °C to 0 °C; 2) time of subsequent phase change from melting point to −10 °C; and 3) freezing rate from −10 °C to −30 °C.

2.5. Fertilization

To assess the fertilizing ability of spermatozoa, eggs from three females were pooled in equal parts and used for experiments. Based on the sperm concentration, a specific number of sperm (10^5 spermatozoa/egg ratio) and 8 mL of tank water were added to 2 g of eggs (approximately 180 eggs). After mixing for 2 min, dishes with fertilized eggs were transferred to an incubator with aerated, dechlorinated and UV-sterilized tank water at 16 °C. The development rate was evaluated after four days of incubation, and dead eggs were separated from live embryos. The hatching rate was evaluated at day 8 of incubation. Data are expressed as the percent of live embryos and hatched larvae at days 4 and 8 of incubation [15].

2.6. Statistical analysis

All experiments were conducted with sperm from 10 sterlet males to measure the motility parameters. The values of the percent of motile cells, motility duration, development and hatching rates are expressed as the mean ± standard error of the mean (SEM). Due to the low number of samples (n = 10), a nonparametric Kruskal–Wallis test followed by the Mann–Whitney U-test with Bonferroni correction was applied to identify differences between groups. The criterion for significance was $P < 0.05$.

The VCL, VAP, BCF and LIN parameters obtained from the measurement of the motility of 17 101 spermatozoa were subjected to linear regression analysis to evaluate the relationship between the cooling methods and post-thaw sperm parameters. Lines from linear regression plots were used to measure the slope (a), intercept (b), coefficient of determination (R2) and P-value for R2 using GraphPad Prism version 6 for Windows software (La Jolla, CA, USA). Values obtained after cryopreservation with the raft holding 60 straws (raft_60) were compared with values obtained with use of all other freezing methods. The t-test was calculated by a "difference test calculator" with $P < 0.05$.

Thermograms were recorded three times by four thermocouples for each freezing method. The parameters obtained for the freezing rates were expressed as the mean ± standard deviation (SD) and were compared by the nonparametric Kruskal–Wallis test followed by the Mann–Whitney U-test with Bonferroni correction at $P < 0.05$.

All statistical significance analyses were carried out using Statistica V 12.0 computer program (Statsoft, Inc., Tulsa, OK, USA) [21].

3. Results

3.1. Temperature characteristics of cooling regimes

The cooling techniques and accompanying freezing parameters over different intervals of thermograms are shown in Fig. 2B. The freezing rate from 10 °C to 0 °C was significantly lower in raft_60 compared to the raft holding 6 straws (raft_6), while the freezing rates for straws frozen in the centre (shipper_centre) and on the periphery (shipper_periphery) of the dry shipper were not significantly different from each other. The elapsed time from the melting point to −10 °C was significantly longer in raft_60 compared to raft_6 and shipper_periphery, but not compared to shipper_centre. Furthermore, the lowest freezing rate from −10 °C to −30 °C was observed in raft_60, while the freezing rates for

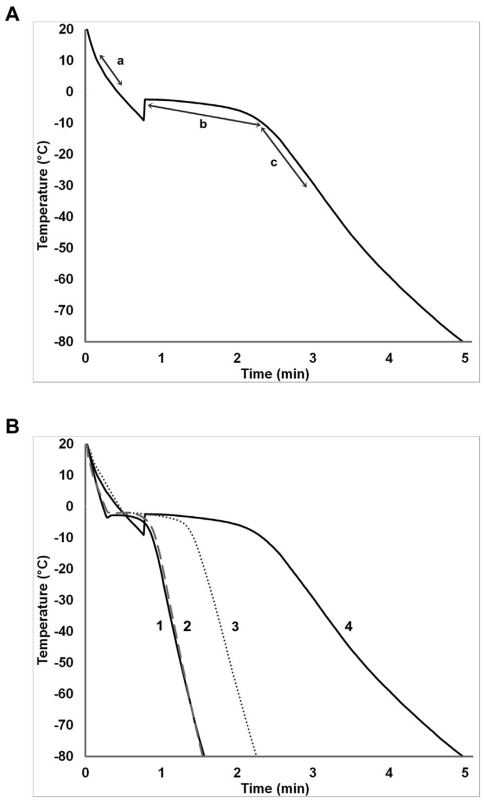


Fig. 2. Cooling curves of 0.5-mL straws obtained in different uncontrolled cooling devices. (A) Schematic representation of the following intervals of interest: freezing rate from 10 °C to 0 °C (region a), duration of relatively constant temperature (release of latent heat) during the subsequent phase change from melting point to −10 °C (region b), and freezing rate from −10 °C to −30 °C (region c). (B) Representative thermograms: 3-cm polystyrene raft floating on the surface of liquid nitrogen in a Styrofoam box with 6 straws (curve 1), the same cooling regime but with 60 straws (curve 4), cooling in the dry shipper with straws in the periphery of the holder (curve 2) and in the centre of the holder (curve 3).

raft_6, shipper_periphery and shipper_centre were significantly higher (Table 1).

3.2. Sperm motility parameters

The cryopreservation procedure significantly decreased the motility percentage of spermatozoa (Fig. 3A) and motility duration (Fig. 3B) in all groups compared to the control group. The highest motility percentage among frozen groups was observed in the Styrofoam box method with 6 straws on the raft and in the dry shipper method with straws in the periphery of the holder. However, both increasing the number of straws on the raft to 60 and placement of straws in the centre of the holder led to a lower motility. By contrast, the motility duration of spermatozoa after freezing was similar for all cooling profiles.

The post-activation time dependencies of VCL, VAP, LIN and BCF are presented as linear regression lines (Fig. 4). Based on CASA post-thaw analysis, the motility parameters significantly decreased for raft_60 compared to all other cooling methods: raft_6, shipper_-periphery and shipper_centre ($P < 0.05$). The parameters of the lines of the best fit are presented in Table 2.

3.3. Development and hatching rates

The development rates (Fig. 5A) and corresponding hatching rates (Fig. 5B) demonstrated the high quality of sperm and eggs used in the experiment. After fertilization, the development rate (9.65 ± 2.71) as well as the hatching rate (11.71 ± 4.22) for raft_60 were significantly lower compared to those of raft_6, shipper_-periphery, shipper_centre and the control group ($P < 0.05$), with the development rate in the range (48.78–59.39) and the hatching rate in the range (46.01–59.12).

4. Discussion

The sperm motility parameters and fertilization ability are among the most widely used and recognized parameters for assessing sperm quality. In our study, these parameters correlated with the applied cooling method for sperm cryopreservation. Each cooling method was characterized by different freezing rates, and the optimal freezing rate was determined based on the highest values of the post-thaw sperm motility parameters and fertilization ability.

Principal cryoinjuries during freezing are known to occur in the range between 0 °C and −40 °C [22]. As the freezing rate in our study varied during the cooling process, three representative intervals of the thermograms were selected for further analysis for the following reasons. The first interval, "a", on the thermograms shows the initial freezing rate at which cooling was started. The time between the melting point and −10 °C on the second interval, "b", was selected because the majority of ice formation occurs during this interval [23]. During this time, the release of latent heat of fusion slows the cooling to the exact point at which intracellular water requires extra time for outward diffusion from the cell [24].

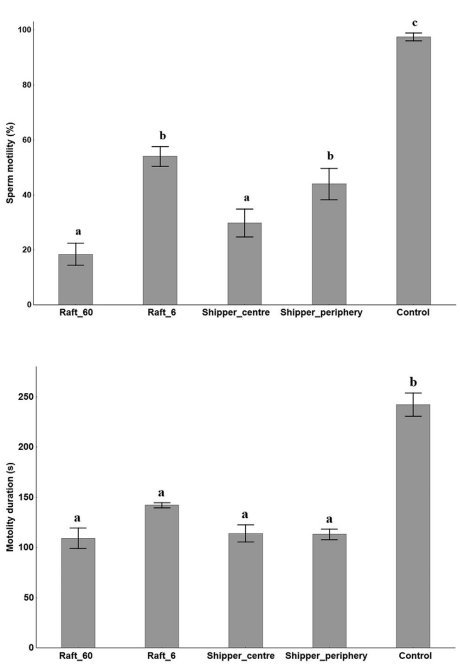


Fig. 3. Sperm motility percentage (A) and motility duration (B) after application of different cooling techniques. The data are presented as the mean ± SEM. Columns with different letters (a, b, c) are significantly different ($P < 0.05$). Multiple comparisons for all groups were done by nonparametric Kruskal–Wallis test followed by the Mann–Whitney U-test with Bonferroni correction; $n = 10$.

The third interval, "c", was selected as the maximal freezing rate during the interval of −10 °C to −30 °C.

Based on the two hypothetical factors responsible for freezing-induced injury [13], the long duration of cell dehydration during slow cooling leads to increased cellular dehydration, which in turn leads to high intracellular salt concentrations and results in shrinkage of the cell membrane. On the other hand, when dehydration occurs for a shorter period of time during fast cooling, the dehydration may not be sufficient to prevent intracellular ice formation. Thus, the optimal freezing rate must be in a range that is neither too fast nor too slow [25].

Currently, a widely used cooling device is the dry shipper, the cooling properties of which were observed in our study. Dry shippers unusually consist of a tank with liquid nitrogen vapour and are

Table 1
Thermogram data obtained during the cryopreservation of sturgeon sperm in 0.5-mL straws under different cooling conditions.

Cryopreservation method	Freezing rate from 10 °C to 0 °C (°C/min)	Time between melting point and −10 °C (S)	Freezing rate from −10 °C to −30 °C (°C/min)
Raft_60	35 ± 1^{a}	98 ± 10^{a}	19 ± 1^{a}
Raft_6	86 ± 3^{b}	34 ± 8^{b}	105 ± 1^{b}
Shipper_centre	37 ± 1^{a}	54 ± 6^{ab}	72 ± 8^{b}
Shipper_periphery	53 ± 3^{b}	33 ± 6^{b}	97 ± 4^{b}

Data are presented as the mean ± SD. Means with different letters (a, b) are significantly different ($P < 0.05$). Multiple comparisons in all groups were done by nonparametric Kruskal–Wallis test followed by the Mann–Whitney U-test with Bonferroni correction.

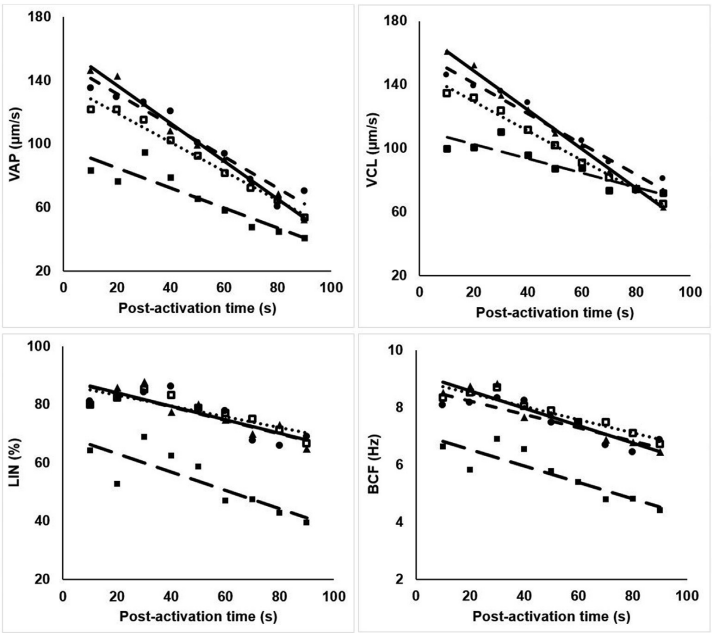


Fig. 4. Lines of linear regression which describe changes of the motility parameters during post-activation time after sperm cryopreservation by different cooling methods. The lines are based on the values of the mean ± SD (■ – – raft with 60 straws; □ ••••• raft with 6 straws; ▲ -- straws frozen in the periphery of straw holder inside the dry shipper; ● - - - straws frozen in the centre of straw holder inside the dry shipper). For the construction of the lines, the motility parameters of 17 101 spermatozoa were used.

used for the safe transportation of a variety of materials at low temperatures (−196 °C) (www.mitegen.com). As a cooling device, dry-shippers were used for the conservation of endangered fish species in Brazil [26]. At the present time, it is regarded as a simple and convenient device for fish sperm cryopreservation and sample shipping in field trials [27]. To our knowledge, our study is the first to apply the dry shipper method for the cryopreservation of sterlet (*Acipenser ruthenus*) sperm. Use of the straw holder was necessary in our experiments to avoid induction of variability arising from the straw positions inside the dry shipper. Indeed, as shown in this study, the position of straws in the holder has an effect on the freezing rate. The effect of the straw position in a holding cane on the temperature has been also observed in other studies [28]. Despite the engineered spacing between the straws, the convective stream of nitrogen vapour was not as intense in the centre of straw holder as in the periphery. The straws located closer to the wall of

Table 2
The parameters of the lines of the best fit from linear regression for the post-thaw motility parameters of 17 101 spermatozoa obtained by the CASA system.

	Cooling method	R^2	P for R^2	a ± SD	b ± SD
VCL	Raft_60	0.8175	0.0008	−0.451 ± 0.081[a]	111 ± 4.5 [a]
	Raft_6	0.9925	<0.0001	−0.926 ± 0.031[b]	147.9 ± 1.711 [b]
	Shipper_periphery	0.9958	<0.0001	−1.230 ± 0.030 [b]	173.3 ± 1.699 [b]
	Shipper_centre	0.9552	<0.0001	−0.954 ± 0.078 [b]	159.9 ± 4.393 [b]
VAP	Raft_60	0.8374	0.0005	−0.631 ± 0.105 [a]	97.4 ± 5.91 [a]
	Raft_6	0.9828	<0.0001	−0.916 ± 0.046 [b]	137.7 ± 2.57 [b]
	Shipper_periphery	0.9904	<0.0001	−1.191 ± 0.044 [b]	160.7 ± 2.49 [b]
	Shipper_centre	0.9408	<0.0001	−0.989 ± 0.094 [b]	151.1 ± 5.27 [b]
LIN	Raft_60	0.6879	0.0057	−0.003 ± 0.001 [a]	0.7 ± 0.05 [a]
	Raft_6	0.7409	0.0029	−0.002 ± 0.001 [b]	0.9 ± 0.02 [b]
	Shipper_periphery	0.7344	0.0032	−0.003 ± 0.001 [b]	0.9 ± 0.03 [b]
	Shipper_centre	0.707	0.0045	−0.002 ± 0.001 [b]	0.9 ± 0.03 [b]
BCF	Raft_60	0.7805	0.0016	−0.029 ± 0.006 [a]	7.1 ± 0.33 [a]
	Raft_6	0.8859	0.0002	−0.023 ± 0.003 [b]	8.9 ± 0.18 [b]
	Shipper_periphery	0.9009	<0.0001	−0.025 ± 0.004 [b]	9.2 ± 0.22 [b]
	Shipper_centre	0.7859	0.0014	−0.023 ± 0.005 [b]	8.7 ± 0.26 [b]

The data are presented as the mean ± SD. Means with different letters (a, b) are significantly different, as calculated using t-test "difference test calculator," with $P < 0.05$.

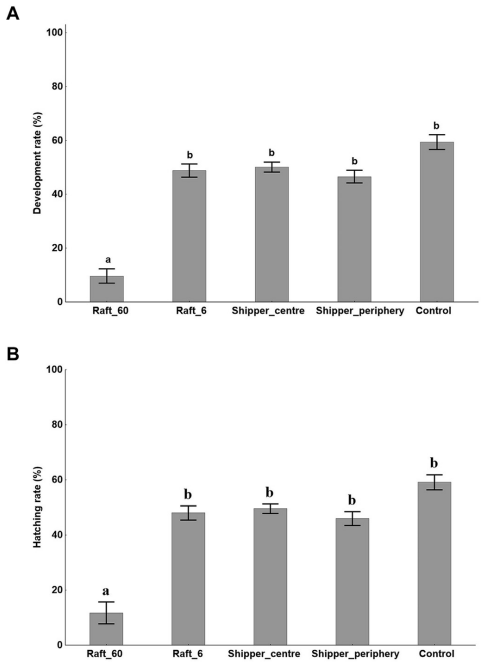


Fig. 5. Percentages of the development (A) and hatching (B) rates obtained after application of the tested cooling methods. The data are expressed as the mean ± SEM; columns with different letters (a, b) are significantly different ($P < 0.05$; multiple comparisons for all groups by the nonparametric Kruskal–Wallis test followed by the Mann–Whitney U-test with Bonferroni correction).

the dry shipper showed faster freezing rates than the straws placed in the centre of the holder. Because the best sperm post-thaw quality parameters were shown after freezing in the periphery of the dry shipper, the initial freezing rate of 53 °C/min on interval "a" can be considered as optimal freezing rate for sterlet sperm cryopreservation in a dry shipper. In contrast, in previous study by another group, an average cooling rate of −30 °C/min in a dry shipper was also adequate to freeze fish spermatozoa [26]. However, average freezing rates presented by Carolsfeld et al. (2003) can vary widely between 0 °C and −60 °C and do not fully describe the physical changes and freezing rate variation during the freezing process.

Despite the different geometry and directionality of the nitrogen vapour convective stream, the thermograms for a raft with 6 straws and peripheral placement of straws in a dry shipper were similar, as well as the post-thaw sperm motility parameters and fertilization ability. Nevertheless, slower freezing rates in the centre of the dry shipper led to a minor decrease of the percentage of motile spermatozoa, while the motility duration was similar for all of the freezing methods.

Using CASA system, multiple indicators of the post-thaw sperm motility parameters (VCL, VAP, LIN and BCF) were collected. As these data consist of measurements from more than 17,000 spermatozoa, regression analysis was considered to be the appropriate tool for statistical analysis. High coefficient of determination, R2, and low P-values for R2 indicate that the obtained data are well-fitted for linear regression analysis. In this model, the intercept can be considered as a criterion of initial value of the motility parameters and the slope corresponds to the decrease of these parameters during post-activation time. However, the dependence of the motility parameters on the post-activation time can be more complicated than a simple linear relationship [29]. As applied in our study, these analyses allowed us to determine the general features of post-thaw spermatozoa. The results of this study confirm the previously found negative effects of cryopreservation on fish sperm [4,12,30] and clearly indicate a relationship between the applied freezing rates and sperm quality parameters after cryopreservation.

In fertilization trials, use of sperm cryopreserved by different cooling methods resulted in rates of development and hatching that approached those observed with fresh sperm. Only sperm frozen with 60-straw raft showed significantly decreased rates of both development and hatching.

5. Conclusions

The results of this study show that the optimal freezing rate can be achieved by each investigated uncontrolled cooling device. However, due to inability to precisely control the cooling process, the freezing parameters can be changed by varying straw number (in the case of freezing in the Styrofoam box) or straw position (in the case of freezing inside the dry shipper). Nevertheless, the freezing in a dry shipper with 60 straws inside is the most convenient as the straws are in more restricted space, that provides more stable cooling conditions and, therefore, less variability in the post-thaw sperm parameters. The freezing rate in the dry shipper, presumably, is in the optimal range for sturgeon spermatozoa. Meanwhile, using the same number of straws on a raft leads to a significantly lower freezing rate. As a consequence, these changes lead to less-than-optimal freezing rates and as a result to the decrease in post-thaw sperm motility and fertilization ability. Therefore, future studies on the standardization of protocols for fish sperm cryopreservation in Styrofoam box (device with uncontrolled freezing rate) should take into consideration the effect of the straw number on the raft during the freezing process.

Acknowledgments

This work was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program), Czech Science Foundation (GACR 16-02407Y), COST (No. LD14119) and by Grant Agency of the University of South Bohemia in Ceske Budejovice (No. GAJU 059/2016/Z).

References

[1] Bronzi P, Rosenthal H, Gessner J. Global sturgeon aquaculture production: an overview. Appl Ichthyol 2011;27(2):69–175.
[2] Bronzi P, Rosenthal H. Present and future sturgeon and caviar production and marketing: a global market overview. Appl Ichthyol 2014;30:1536–46.
[3] Mims SD, Tsvetkova LI, Wayman RW, Horvath A, Urbanyi B, Gomelskyi B. Cryopreservation of sturgeon and paddlefish sperm. In: Tiersch TR, Green CC, editors. Cryopreservation in aquatic species. second ed. Baton Rouge: World Aquaculture Society; 2011. p. 366–80.
[4] Billard R, Cosson J, Noveiri SB, Pourkazemi M. Cryopreservation and short-term storage of sturgeon sperm, a review. Aquaculture 2004;236(1–4):1–9.
[5] Kopeika E, Kopeika J, Zhang T. Cryopreservation of fish sperm. In: Day JG, Stacey GN, editors. Cryopreservation and freeze-drying protocols second edition. New Jersey: Humana Press Totowa; 2007. p. 203–19.
[6] Glogowski J, Kolman R, Szczepkowski M, Horvath A, Urbanyi B, Sieczynski P, et al. Fertilization rate of Siberian sturgeon (Acipenser baeri, Brandt) milt cryopreserved with methanol. Aquaculture 2002;211(1–4):367–73.
[7] Boryshpolets S, Dzyuba B, Rodina M, Alavi SMH, Gela D, Linhart O.

Cryopreservation of sterlet (Acipenser ruthenus) spermatozoa using different cryoprotectants. Appl Ichthyol 2011;27(5):1147–9.

[8] Judycka S, Szczepkowski M, Ciereszko A, Dietrich GJ. New extender for cryopreservation of Siberian sturgeon (Acipenser baerii) semen. Cryobiology 2015;70(2):184–9.

[9] Kopeika EF, Williot P, Goncharov BF. Cryopreservation of Atlantic sturgeon Acipenser sturio L., 1758 sperm: first results and associated problems. Bol Inst Espanol Oceanogr 2000;16:167–73.

[10] Asturiano JF, Marco-Jimenez F, Penaranda DS, Garzon DL, Perez L, Vicente JS, et al. Effect of sperm cryopreservation on the European eel sperm viability and spermatozoa morphology. Reproduct Domest Animals 2006;42(2):162–6.

[11] Dzyuba B, Boryshpolets S, Rodina M, Gela D, Linhart O. Spontaneous activation of spermatozoa motility by routine freeze-thawing in different fish species. Appl Ichthyol 2010;26:720–5.

[12] Cabrita E, Sarasquete C, Martinez-Paramo S, Robles V, Beirao J, Perez-Cerezales S, et al. Cryopreservation of fish sperm: applications and perspectives. Appl Ichthyol 2010;26(5):623–35.

[13] Mazur P, Leibo SP, Chu EHY. A two-factor hypothesis of freezing injury: evidence from Chinese hamster tissue-culture cells. Exp Cell Res 1972;71(2):345–55.

[14] Bernath G, Bokor Z, Kasa E, Varkonyi L, Hegyi A, Kollar T, et al. Comparison of two different methods in the cryopreservation of Eurasian perch (Perca fluviatilis) sperm. Cryobiology 2015;70(1):76–8.

[15] Dzyuba B, Boryshpolets S, Shaliutina A, Rodina M, Yamaner G, Gela D, et al. Spermatozoa motility, cryoresistance, and fertilizing ability in sterlet Acipenser ruthenus during sequential stripping. Aquaculture 2012;356–357:272–8.

[16] Judycka S, Szczepkowski M, Ciereszko A, Dietrich GJ. New extender for cryopreservation of Siberian sturgeon (Acipenser baerii) semen. Cryobiology 2015;70(1):184–9.

[17] Melo FCSA, Godinho HP. A protocol for cryopreservation of spermatozoa of the fish Brycon orthotaenia. Animal Reproduct Sci 2006;3:380–5.

[18] Koh IC, Tanaka D, Itagane T, Tsuji M, Tsuchihashi Y, Ohta H. Dry shipper cryopreservation of seven-band grouper (Epinephelus septemfasciatus Thunberg) spermatozoa. Aquac Res 2013;44:59–66.

[19] Dzyuba B, Boryshpolets S, Cosson J, Dzyuba V, Fedorov P, Saito T, et al. Motility and fertilization ability of sterlet Acipenser ruthenus testicular sperm after cryopreservation. Cryobiology 2014;69(2):339–41.

[20] Boryshpolets S, Cosson J, Bondarenko V, Gillies E, Rodina M, Dzyuba B, et al. Different swimming behaviors of sterlet (Acipenser ruthenus) spermatozoa close to solid and free surfaces. Theriogenology 2013;79:81–6.

[21] Siegel S, Castellan NJ. Nonparametric statistics for the behavioral sciences. second ed. New York: McGraw-Hill; 1988.

[22] Muchlisin ZA. Current status of extenders and cryoprotectants on fish spermatozoa cryopreservation. Biodiversitas 2005;6(1):12–5.

[23] FAO animal production and health guidelines. Basic principles of cryopreservation. Cryoconserv Animal Genet Resour 2012;12:83–93.

[24] Mazur P. Freezing of living cells: mechanisms and implications. Cell Physiol 1984;247(16):125–42.

[25] Zhao G, Luo D, Gao D. Universal model for intracellular ice formation and its growth. AIChE J 2006;52(7):2596–606.

[26] Carolsfeld J, Godinho HP, Zaniboni Filho E, Harvey BJ. Cryopreservation of sperm in Brazilian migratory fish conservation. J Fish Biol 2003;63(2):472–89.

[27] Chew PC, Abd-Rashid Z, Hassan R, Asmuni M, Chuah HP. Semen cryo-bank of the malaysian mahseer (tor tambroides and T. douronensis). Appl Ichthyol 2010;26(5):726–31.

[28] Taitson PF, Chami E, Godinho HP. Gene banking of the neotropical fish Leporinus obtusidens (Valenciennes, 1836): a protocol to freeze its sperm in the field. Animal Reproduct Sci 2008;105(3–4):283–91.

[29] Purchase CF, Moreau DTR. Stressful environments induce novel phenotypic variation: hierarchical reaction norms for sperm performance of a pervasive invader. Ecol Evol 2012;2(10):2567–76.

[30] Nynca J, Dietrich GJ, Dobosz S, Grudniewska J, Ciereszko A. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. Aquaculture 2014;433:62–5.

CHAPTER 4

CRYOPRESERVATION EFFECT ON STERLET (*ACIPENSER RUTHENUS*) SPERM SUB-POPULATIONS OBTAINED BY PERCOLL DENSITY GRADIENT

Horokhovatskyi, Y., Dietrich, M.A., Lebeda, I., Fedorov, P., Rodina, M., Dzyuba, D., Cryopreservation effects on a viable sperm sterlet (*Acipenser ruthenus*) subpopulation obtained by a Percoll density gradient method. PLOS ONE. 10.1371/journal.pone.0202514. (accepted)

My contribution to this work was 70%.

CRYOPRESERVATION EFFECTS ON A VIABLE SPERM STERLET (*ACIPENSER RUTHENUS*) SUBPOPULATION OBTAINED BY A PERCOLL DENSITY GRADIENT METHOD

Yevhen Horokhovatskyi[1]*, Mariola A. Dietrich[2], Ievgen Lebeda[1], Pavlo Fedorov[1], Marek Rodina[1], Borys Dzyuba[1]

[1]Research Institute of fish Culture and Hydrobiology, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Vodnany, Czech Republic

[2]Department of Gametes and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland

**Corresponding author*

E-mail: horokhovatskyi@gmail.com

Abstract

In many fish species, sperm cryopreservation has deleterious effects and leads to a significant decrease in spermatozoa viability. However, the effect of cryopreservation on sperm cells that survive this process and are still viable is not fully understood. The objective of this study was to compare the viability and proteomes of fresh and cryopreserved sterlet (*Acipenser ruthenus*) sperm samples before and after live-dead cell separation using Percoll density gradient centrifugation. Both fresh and cryopreserved sperm samples were divided into two groups (with or without application of Percoll separation). At each step of the experiment, sperm quality was evaluated by video microscopy combined with integrated computer-assisted sperm analysis software and flow cytometry for live-dead sperm viability analysis. Sperm motility and the percentage of live cells were reduced in the cryopreserved group compared to the fresh group from 89% to 33% for percentage of motility and from 96% to 70% for live cells. Straight line velocity and linearity of track were significantly lower in cryopreserved samples than in those separated by Percoll before and after cryopreservation. However, the percentages of motile and live spermatozoa were higher than 90% in samples subjected to Percoll separation. Proteomic analysis of spermatozoa by two-dimensional differences in-gel electrophoresis coupled with matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight mass spectrometry revealed that 20 protein spot abundances underwent significant changes in cryopreserved samples compared to fresh ones. However, only one protein spot was significantly altered when samples before and after cryopreservation followed by Percoll separation were compared. Thus, the results of this study show that cryopreservation leads to minimal proteomic changes in the spermatozoa population, retaining high motility and viability parameters. The results also suggest that global differences in protein profiles between unselected fresh and cryopreserved samples are mainly due to protein loss or changes in the lethal and sublethal damaged cell subpopulations.

Key words: *Cryopreservation effect, fish sperm, live/dead cell viability, Percoll separation, protein, sturgeon*

INTRODUCTION

Worldwide demand for sturgeon boneless meat and caviar has resulted in an increased requirement for sturgeon aquaculture (Bronzi et al., 2011). At the same time, fish sperm cryopreservation is considered to be a powerful tool in aquaculture as it affords an opportunity for gamete availability synchronization of sperm and eggs, the rational use of sperm, a decrease in the number of males needed in a hatchery, simplification of broodstock maintenance and gamete transport between fish farms (Cabrita et al., 2010). However, despite all of these advantages, cryopreservation presents challenges, as it may strongly impair sperm function and survival and thus decrease reproductive capacity. Moreover, the mechanisms of spermatozoa cryo-injuries are still under intensive study and are not fully understood (Medeiros et al., 2002; Cabrita et al., 2010).

To be successfully cryopreserved and used for artificial reproduction in aquaculture, fish sperm must be of the highest quality before freezing as well as after thawing. However, in many fish species, the cryopreservation procedure has deleterious effects and is associated with a significant decrease in sperm viability (Glogowski et al., 2002; Asturiano et al., 2006; Kopeika et al., 2007). During freezing and thawing, spermatozoa are exposed to the influence of numerous physical and chemical factors caused by a high solute concentration during slow freezing or intracellular ice formation during rapid freezing (Mazur et al., 1972). Moreover, water can recrystallize into larger lethal-sized ice crystals during warming as a result of differences in surface free energy and damage the cell (Seki and Mazur, 2008).

Cryo-injuries caused by crystallization of internal and external water have deleterious effects on the spermatozoa plasma membrane due to changes in lipid membrane composition, organization and its properties. Moreover, the freeze-thaw process also leads to alterations in DNA, protein integrity and protein-protein interactions (Zilli et al., 2003; Cabrita et al., 2005; Li et al., 2010b). The defects in sperm protein, in turn, may have a pernicious effect on sperm motility, fertilization ability, and the early stage after fertilization (Lessard et al., 2000; Li et al., 2010b). As a result of all of these deleterious effects, the membrane-bound proteins and intracellular enzymes, as well as other components of the cell, co-elute from spermatozoa when it is damaged (Li et al., 2010b).

As a consequence, all damage to which the spermatozoa are subjected during the freezing-thawing process results in the appearance of viable, lethal and sublethal damaged cell subpopulations in a thawed sperm mixture. Usually, such a frozen-thawed sperm mixture is an object of routine cryo-biological studies aimed at evaluating the cryopreservation-generated damage to fish sperm proteins and lipids (Holt, 2000; Horokhovatskyi et al., 2016). However, according to our hypothesis, the presence of lethal and sublethal damaged cell subpopulations in the sperm mixture can obscure the real cryopreservation effects on the subpopulation of spermatozoa that survive the cryopreservation process and retain suitable motility parameters. Furthermore, this subpopulation is the only one involved in fertilization, and the presence of lethal and sublethal damaged spermatozoa can negatively influence this process if a post-thaw mixture is used (Mortimer and Templeton, 1982; Henkel and Schill, 2003).

In mammals, to obtain a higher number of high-quality spermatozoa even from a poor-quality semen, a variety of different sperm separation techniques are widely used. Foremost among these are swim-up, swim-down, fluorescence cell sorting, electrophoresis, migration-sedimentation, glass wool filtration, magnetic activated cell sorting, and density gradient centrifugation (Henkel and Schill, 2003; Ilaria, 2011; Fleming and Aitken, 2012; Beydola et al., 2014). In principle, all of these sperm separation methods imitate the natural selection process *in vitro* in which higher-quality viable sperm are separated from other constituents of the

ejaculate by actively moving through the cervical mucus. In the case of artificial insemination, fresh mammalian sperm, as well as cryopreserved sperm, needs to be separated because the presence of low-quality, lethal and sublethal damaged spermatozoa subpopulations may become an obstacle during in vitro fertilization (Mortimer and Templeton, 1982).

The application of these separation techniques to fish sperm is a completely new approach that presents some challenges. It is known that fish spermatozoa are commonly immotile in seminal fluid and for motility initiation, changes in environment osmolality and ionic composition are required (Cosson, 2004). Thus, to apply separation techniques to fish sperm, the spermatozoa should be kept immotile because the motility period after initiation is not as long as in mammalian species. Additionally, the data from fish sperm separation techniques are very limited. To our knowledge, several attempts have been made using separation techniques for fish sperm. Magnetic-activated cell sorting was first applied to Senegalese sole (*Solea senegalensis*) sperm to eliminate the apoptotic spermatozoa subpopulation before cryopreservation (Valcarce et al., 2016). This technique obtained a non-apoptotic sperm subpopulation from low-quality samples and improved artificial fertilization and cryopreservation outcomes. Another separation technique was used for common carp (*Cyprinus carpio*) sperm after a conventional freeze-thaw procedure (Li et al., 2010a). The Percoll density gradient centrifugation technique was effective for the removal of most dead spermatozoa and spermatozoa with damaged membranes exposed to the effects of cryopreservation. Therefore, separation of fish spermatozoa has great potential to be applied for the improvement of assisted reproduction in aquaculture; however, the technique requires refinement, specifically depending on the fish species.

In our study, the Percoll density gradient separation technique was used for the first time in sterlet (*Acipenser Ruthenus*) sperm for the selection of a viable spermatozoa subpopulation from a frozen-thawed sperm suspension. The objective of our study was to compare the sperm motility, viability and proteomes of fresh and cryopreserved sterlet sperm before and after cell subpopulation selection using Percoll density gradient centrifugation.

MATERIALS AND METHODS

The Percoll density gradient centrifugation technique has never been used on sterlet (*Acipenser ruthenus*) sperm. Therefore, this procedure was elaborated for the first time through experimental trial and error. To use the Percoll density gradient centrifugation technique on sterlet sperm, the concentration of the immobilizing saline solution, which keeps the spermatozoa immotile during the entire process of separation, the Percoll concentration in column layers, and centrifugation force and time were determined. The successful application of this technique showing constant and repeated results was obtained on samples from more than 30 individual sterlet males.

Ethics Statement

The methodological protocol of the current study was approved by the expert committee of the Institutional Animal Care and Use Committee (IACUC) of the University of South Bohemia (USB) in Ceske Budejovice, Faculty of Fisheries and Protection of Waters (FFPW) in Vodnany according to the law for the protection of animals against cruelty (Act no. 246/1992 Coll., ref. number 16OZ22302/201417214).

Sample collection

Sterlet (*Acipenser ruthenus*) was selected as the model sturgeon species in this study. All manipulations with sterlet individuals were described in a protocol approved by the committee mentioned above. According to this protocol, sturgeon individuals weighing less than 40 kg are not anaesthetized (Chebanov and Galich, 2013), as the anesthesia leads to a fish recovery time and can provoke sperm loss together with fish suffering from the anesthesia itself. Sample collections were performed in March at the genetic fisheries center of the Faculty of Fisheries and Protection of Waters, Vodnany. Thirty sterlet males (3–4 years old, 0.8–1.6 kg body weight) were transferred from fish farming ponds with a water temperature of 8–10 °C to 4 m3 plastic tanks with a closed water recirculating system. The water temperature in the plastic tanks was increased to 15 °C by a 1 °C increment per day. Spermiation was induced by a single intramuscular injection of carp pituitary powder dissolved in a 0.9% (w/v) NaCl solution at a dose of 4 mg/kg body weight. Twenty-four hours post-stimulation, sperm was collected from each of thirty males using a syringe with an attached 4 mm plastic catheter inserted into the urogenital ducts. Before the start of the experiment, the collected sperm was stored in a flat-bottom container on ice at 4 °C (Dzyuba et al., 2012).

Cryopreservation procedure

Freshly collected sperm samples from thirty fish males were diluted at a 1:1 ratio (v: v) using an extender with a buffer consisting of 30 mM Tris, 23.4 mM sucrose, and 0.25 mM KCl supplemented with 10% methanol (Glogowski et al., 2002). During a 10 min equilibration period, 0.5 mL plastic straws were filled with a sperm-extender suspension and placed on a 3 cm thick polystyrene raft (dimensions: 40×20×3 cm). Each set of 20 straws on the raft was then placed in a Styrofoam box (dimensions: 52x33x30 cm) that was filled to a depth of 10 cm with liquid nitrogen. Ten min after exposure to liquid nitrogen vapor (-160 °C), the straws were plunged directly into liquid nitrogen (-196 °C) and stored in plastic goblets prior to thawing. During thawing, samples were exposed to a 40 °C water bath for 6 s (Dzyuba et al., 2012) followed by immediate use for further estimations and sample processing.

Sperm separation by Percoll density gradient centrifugation

To obtain the 90% and 40% Percoll solutions, stock Percoll (catalog number P1644, Sigma-Aldrich, USA) was diluted with a 100 × concentrated artificial seminal fluid to yield a final concentration of 16 mM NaCl, 3 mM KCl, 0.19 mM CaCl2, 10 mM Tris-HCl buffer at pH 8.0 that is similar to native sterlet seminal fluid (Dzyuba et al., 2014). The Percoll density column was set up in a 15 mL plastic tube by smoothly layering 2 mL of 40% Percoll solution on top of 2 mL of 90% Percoll solution. On the top of the column, 1 mL of either fresh or frozen-thawed sperm mixture was gently layered, and then the tubes were centrifuged for 20 min at 2000 × g, 4 °C. A visual presentation of the results of Percoll gradient centrifugation applied to sterlet sperm is shown in S1 Figure. The resulting pellets were washed at a 1:10 ratio with artificial seminal fluid and centrifuged at 3000 × g for 10 min at 4 °C. After resuspension, one part of each pellet underwent sperm parameter evaluation and a live/dead sperm cells ratio estimation, while the other part was frozen at -80 °C prior to proteome analysis. The information regarding sperm concentration of fresh and cryopreserved samples before and after Percoll separation is presented in S2 Table.

Sperm motility parameters

For sperm motility activation, fresh, fresh-separated, cryopreserved and cryopreserved-separated sperm samples from thirty fish males were diluted at 1:50 in a medium containing 1 mM CaCl2, 10 mM Tris-HCl (pH 8.0), and 0.25% Pluronic F-127 (catalog number P2443, Sigma-Aldrich, USA) under a phase contrast microscope (UB 200i, PROISER, Spain). Immediately after activation, the motility was recorded in duplicate with an ISAS 782M digital camera (PROISER, Spain) in AVI format. The resulting video records were analyzed using an Integrated System for Semen Analysis (ISAS) software (PROISER, Spain). Sperm motility evaluations included several parameters: 1.) percentage of motile sperm cells (%MOT); 2.) curvilinear velocity over the actual path in μm/s (VCL); 3.) straight line velocity in μm/s (VSL); and 4.) linearity of track, VSL/VCL * 100% (LIN). All computer-assisted sperm analysis (CASA) parameters were obtained at 10 s intervals from 10 to 90 s post-activation. To describe the changes in VCL, VSL, and LIN over the post-activation time, these parameters were analyzed using linear regression. In this approach, the intercept with the Y-axis can be viewed as a criterion describing the initial value of the sperm motility parameters while the slope can be considered as a value describing the decrease in these parameters over the post-activation time.

Sperm viability analysis

The live/dead sperm cell ratio was determined for all sperm samples (n=30) by flow-cytometric analysis using the membrane-permeant SYBR-14 for live cell nucleic acid staining in combination with propidium iodide (PI) for dead cell nucleic acid staining that penetrates through damaged plasma membrane. Prior to measurements, live untreated sperm (negative control) and sperm subjected to repeated freezing-thawing (positive control) were used to calibrate the sensitivity of each fluorescent channel, thresholds, and set-up regions of interest. Artificial seminal fluid samples diluted to a concentration of 10,000 spermatozoa/mL were at first incubated with 5 μL of 20 × diluted from initial concentration SYBR-14 dye (initial concentration: 10,000 × in DMSO, catalog number S9430, Sigma-Aldrich, USA) at 4 °C for 5 min. Thereafter, 5 μL of 4.8 mM propidium iodide was added to the same suspension and incubated for an additional 30 minutes at 4 °C (Probes, 2001). A minimum of 3,000 sperm cells was analyzed using a CUBE 8 (Partec, Germany) flow cytometer at flow speed 0.2 μL/s. The data were processed using CyView 1.3 (Partec, Germany) software. For each sample, the populations with different intensities in the PI channel were compared and correlated with membrane damage; thus, populations with high PI fluorescent signals were considered dead cells. Based on the ratio between populations with low and high PI fluorescence intensity, the percentage of live and dead sperm cells was calculated.

Sperm samples preparation for proteomic analysis

Fresh and cryopreserved sperm from sterlet males, either with or without application of the Percoll separation, were used to extract proteins. The resulting sperm pellets were thawed at room temperature (23 °C) and resuspended in a protein extraction buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 40 mM DTT, 0,1% Triton, 2.5% (v/v) protease inhibitor cocktail, and 30 mM Tris at pH 8.0) (Sigma-Aldrich, USA). The diluted suspension was sonicated on ice six times for 5 s at 30% amplitude using a VCX-130 Ultrasonic Processor (Sonics & Materials, Inc., Newtown, CT, USA). After sonication, the suspension was kept on ice for 1 h and then centrifuged in a 4 °C cooled centrifuge at 14,000 × g for 10 min. Thereafter, protein lysates containing approximately 500 μg of sperm proteins were processed with the use of a Clean-

Up Kit (GE Healthcare, Sweden) according to the manufacturer's protocol. All samples were resuspended in 40 µL of lysis buffer 7 M Urea, 2 M Thiourea, 4% w/v CHAPS, and 30 mM Tris, pH 8.0) to a protein concentration of 5 mg/mL. The protein concentration before and after the cleaning procedure was measured by a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, Waltham, USA) (Dietrich et al., 2014).

Fluorescent labelling of sample groups with CyDyes and 2D-DIGE

Protein labelling with CyDye DIGE fluors (Cy2, Cy3, Cy5) and two-dimensional (2D) electrophoresis were performed using the same parameters as described by Dietrich et al. (Dietrich et al., 2016). Aliquots of 50 µl of protein extracted from each sample group (fresh, fresh-separated, cryopreserved, and cryopreserved-separated) were dissolved in a labelling buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 30 mM Tris, pH 8.0) and labelled at a concentration of 400 pmol dye/50 µg of protein using CyDye DIGE Fluor minimal dyes (GE Healthcare, Uppsala, Sweden) which were reconstituted in fresh 99.8% anhydrous dimethylformamide (Table 1).

Table 1. *Mixing and Cy-dying scheme of fresh and cryopreserved samples with or without application of Percoll separation; n = 3 for each group.*

Gel no.	Cy2 (50 µg)	Cy3 (50 µg)	Cy5 (50 µg)
1	Pooled standard	Fresh 1	Fresh-separated 3
2	Pooled standard	Fresh-separated 1	Cryopreserved-separated 2
3	Pooled standard	Cryopreserved 1	Fresh 3
4	Pooled standard	Cryopreserved -separated 1	Cryopreserved 3
5	Pooled standard	Fresh 2	Cryopreserved -separated 3
6	Pooled standard	Fresh-separated 2	Cryopreserved 2

The Cy2 dye labelled the internal standard while Cy3 and Cy5 dyes were used to label the experimental sample groups in which each group has three replicates. An internal standard was obtained by mixing equal parts of protein from each group. On each electrophoresis gel, extracts from two of the various groups after different treatments (Cy3 and Cy5) and internal standard (Cy2) were separated. The labelling reaction was performed in the dark on ice for 30 min.

Differently labelled samples (50 µg of each labelled samples with Cy2, Cy3, and Cy5) were mixed together according to the scheme presented in Table 1. Passive rehydration was performed using a rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 130 mM DTT, 2% Pharmalyte, pH 3–10 NL, and 0.002% bromophenol blue), which was added to each sample mixture to reach a final volume of 350 µL and loaded onto immobilized pH gradients strips (IPG, 24 cm, pH 3–10 NL). The strips were rehydrated for 18 h. Isoelectric focusing (IEF) was performed with an Ettan IPGphor device (GE Healthcare) as described by Dietrich et al. (Dietrich et al., 2016). The device was operating at 20 °C with the limited current to 50 A per strip using the following voltage program: 1.) 100 V/1 h; 2.) 500 V/2 h; 3.) 500 V/4 h; 5.) a linear gradient to 1,000 V over 1 h; 6.) a linear gradient to 8,000 V over 3 h; and 7.) an 8,000 V constant for 2.5 h. After IEF, the strips were equilibrated with an SDS equilibration buffer (6 M urea, 75 mM Tris–HCl, pH 8.8, 29.3% glycerol, 2% SDS and a trace of bromophenol blue) containing 10 mg/mL DTT for 15 min in the first step and in the second step with SDS equilibration buffer containing 25 mg/mL iodoacetamide for another 15 min.

In the process of a 2D gel electrophoresis, the equilibrated strips were then transferred to a 12.5% SDS-PAGE (gel size 25.5 × 19.6 cm, 1 mm thickness; GE Healthcare) and sealed with 0.5% agarose. The gel was run at 1.5 W/gel in an Ettan Dalt-Six device (GE Healthcare) for 16 h (Dietrich et al., 2016). Once the bromophenol blue had reached the anode, electrophoresis was finished.

Image acquisition and analysis of two-dimensional gels

After electrophoresis, the stained 2D gels were scanned on a Typhoon 9500 FLA scanner (GE Healthcare) using the parameters recommended by the manufacturer for 2D-DIGE experiments. Image analysis was performed using the DeCyder Differential In Gel Analysis version 5.02 software (GE Healthcare) in order to identify fluorescent areas. The DeCyder biological variation analysis module was applied to detect protein spots and concurrently match all twelve protein spot maps from six gels using several parameters: 1.) estimated number of spots at 10,000 and 2.) minimum spot size at 3,000. Only protein spots with a $P<0.05$ by t-test analysis that showed at least a 1.2-fold increase or decrease in their relative intensities in any comparison between all groups were significantly different. To properly pick and identify the selected spots, DIGE gels were stained using Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA).

Protein identification using MALDI-TOF/TOF

All spot preparations and identification were performed according to Dietrich et al. (Dietrich et al., 2016). Spots of interest were excised from Coomassie stained two-dimensional gels, placed in Eppendorf tubes and washed with 50 μL of 50 mM ammonium bicarbonate. The washing solution was discarded, and gel particles were destained again with 50 μL of 50 mM ammonium bicarbonate in 50% acetone solution. After washing, the solution was discarded while spots were dried. Thereafter, 2 μL of 0.2 μg/μL modified sequencing grade trypsin (Promega, Madison, WI, USA) solution and 2 μL of 50 mM ammonium bicarbonate were added to Eppendorf tube and incubated overnight at 37 °C. After digestion, the spots were placed in 100 μL of 0.1% trifluoroacetic acid (TFA) and desalted with Zip-Tip C-18 pipette tips (Millipore, Billerica, MA, USA). Each Zip-Tip was first washed with 100% acetonitrile (ACN) and equilibrated with 50% ACN in 0.1% TFA and 0.1% TFA in water. When Zip-Tip washing and equilibration were finished, the peptides were loaded onto the Zip-tip and then eluted with 2 μL of 50% ACN in 0.1% TFA.

After elution, sample was mixed with 2 μL of the matrix solution (5 mg α-cyano-4-hydroxycinnamic acid [Bruker Daltonics, Bremen, Germany] in 1 mL of 50% acetonitrile in 0.1% TFA), and half of this mixture was spotted onto the matrix-assisted laser desorption/ionization target plate (MT 34 Target Plate Ground Steel; Bruker Daltonics) and left to dry.

Matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) analysis was performed using a matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometer (Autoflex Speed TM, Bruker Daltonics). The MS and MS/MS LIFT spectra of selected ions were collected and calibrated externally using monoisotpoic protonated ion peptide calibration standards (Bruker Daltonics) and imported to BioTools (Bruker Daltonics). The data were searched using MascotServer (Matrix Sciences) in the INCIr database (2016.04.26) with the specific MASCOT settings: 1.) cleavage enzyme: trypsin, max missed cleavages: 2 and mass tolerance mono: 50 ppm, fragment ion mass tolerance of 0.5 Da; 2.) parent ion mass tolerance of 200 ppm; 3.) alkylation of cysteine by carbamidomethylation as a fixed modification; and 4.) oxidation of methionine as a variable

modification. For the PMF and MS/MS ion search, statistically significant (P ≤0.05) matches by MASCOT were regarded as correct hits. Hypothetically identified proteins were searched in terms of protein sequence similarity using the Basic Local Alignment Search Tool (BLAST) (Dietrich et al., 2017).

Ingenuity pathway analysis of differentially abundant proteins

An Ingenuity Pathway Analysis (IPA, Ingenuity, Mountain View, CA) was performed to uncover the significance of potential biological pathways. As IPA only accepts gene or protein accession numbers representing human, mouse, and rat genes or proteins; orthologs of the identified sturgeon proteins belonging to those three species were first identified and the accession numbers of the top blast hits were uploaded to IPA. Each identifier was associated with the IPA knowledge base and used to perform functional and canonical pathway analysis. Fisher's exact test and Benjamini–Hochberg multiple testing corrections were used to calculate the significance ($P < 0.05$) of functional and canonical pathways of proteins changed upon cryopreservation.

Protein-protein interaction analysis

A protein-protein interaction analysis was performed using a web-based bioinformatic Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) software (https://www.string-db.org/), version 10.5. The STRING is a database of known and predicted protein-protein interactions with physical and functional associations which are based on computational prediction, from knowledge transfer between organisms, and from interactions aggregated from other databases. The identified sperm proteins that underwent significant changes in their abundance after cryopreservation process were entered into STRING for analysis of potential protein-protein interactions. The search of interactions was restricted to Homo sapiens (Human, 9606) protein pairs. The reliability of the interactions between proteins was assessed by a combined score (edge score).

Statistical analysis

The experiments evaluating sperm motility, live/dead sperm cells ratio and the Percoll separation success were conducted with sperm from thirty sterlet males. The values of the percent of motile spermatozoa and live/dead cells were expressed as the mean ± standard deviation (±SD). Due to the low number of samples in experimental group, a nonparametric Kruskal-Wallis test followed by the Mann-Whitney U-test with Bonferroni correction was applied to determine differences between groups. The criterion for significance was $P < 0.05$ (Siegel and Castellan, 1988). VCL, VSL, and LIN parameters obtained from the motility measurement from more than 10,000 spermatozoa were subjected to an estimation of mean and standard deviation at 10–90 s (in 10 sec increments) post-activation times. The resulting mean and standard deviations values were used to plot linear regression lines and determination of the slope (a), intercept (b), coefficient of determination (R2), and P-value for R2, whereas testing the hypothesis for the equality of regression slopes by ANCOVA were performed using GraphPad Prism version 6 for Windows software (La Jolla, CA, USA). When ANCOVA concluded that the slopes were not all equal, the t-test with Bonferroni correction was applied to detect the significance of difference between coefficients of individual lines (the criterion for significance was $P < 0.05$) (Jerrold, 2014). All statistical significance analyses for sperm motility parameters and live/dead cell percentages were carried out using the Statistica V 12.0 computer program (Statsoft, Inc., Tulsa, OK, USA).

Statistical analysis of changes in protein abundance was performed using the Biological Variance Module of DeCyder Differential In-Gel Analysis version 5.02 software (GE Healthcare) on three biological replicates (individual fishes). The data are expressed as log standardized abundance to ensure a normal distribution of the data. A t-test and average ratio test were performed. Changes in protein spot abundance were considered statistically significant at $P < 0.05$ with a fold change of 1.2.

Principal component analysis (PCA) was performed in the Extended Data Analysis module of the DeCyder software to visualize the differences between fresh, fresh-separated, cryopreserved and cryopreserved-separated sperm groups. PCA creates and coordinates the points representing samples by using the total log standardized abundance of spots on a specific gel. This analysis emphasizes the variation and brings out strong data patterns obtained from protein spots in the form of principal components in which the first component explains the largest proportion of the variance.

RESULTS

Sperm motility parameters and viability before and after cryopreservation

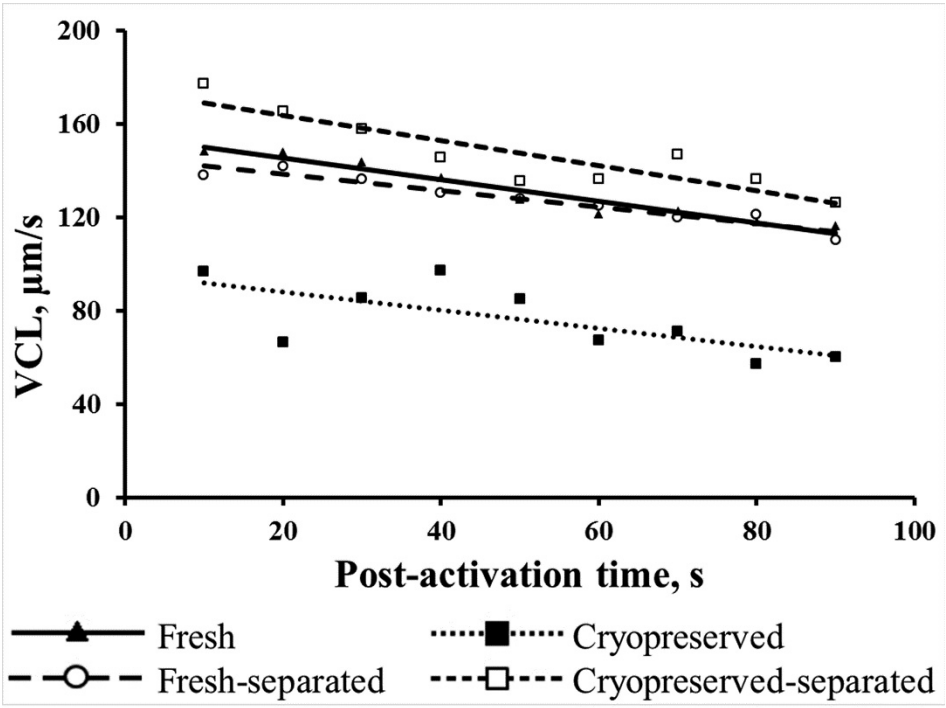
The cryopreservation procedure led to a significant decrease in sperm motility percentage in frozen-thawed samples compared to fresh ones (Table 2). The highest percentage of motile sperm cells was observed in samples that underwent the Percoll separation procedure. The value increased from 88% to 98% for separated fresh sperm, while the value of separated samples after cryopreservation samples significantly increased from 24% to 95%. The Percoll separation caused a significant increase in the sperm motility percentage (Table 2) and motility parameters (Fig 1). The significantly lowest value of live sperm cells was observed in cryopreserved samples compared to the fresh, fresh-separated and cryopreserved-separated groups. No significant differences were observed between the fresh, fresh-separated and cryopreserved-separated sample groups.

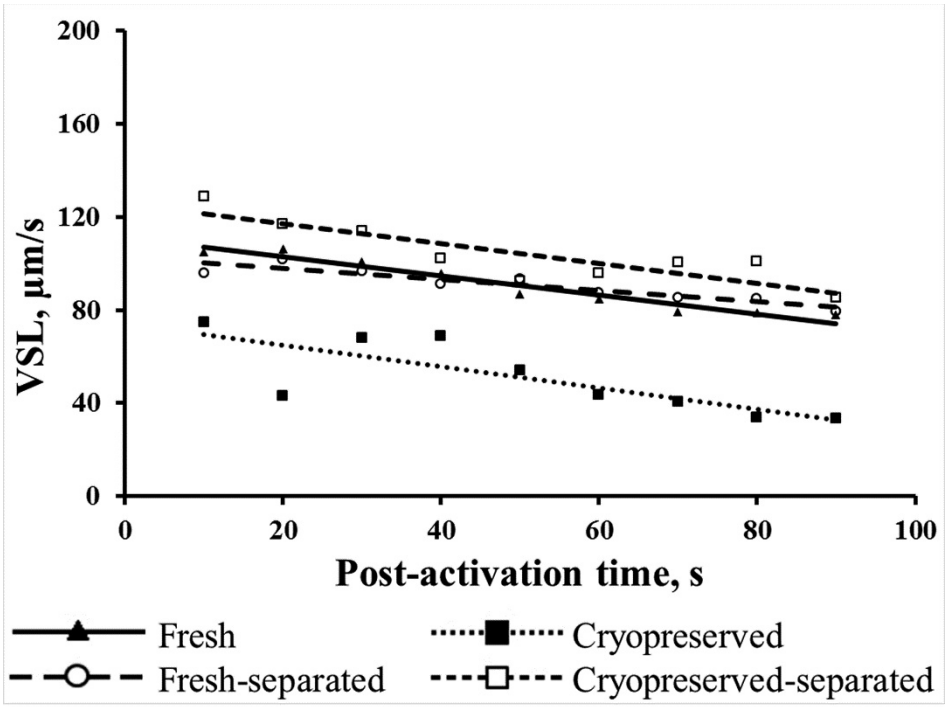
Table 2. *Sperm motility and viability percentages of samples before and after cryopreservation and Percoll separation.*

[a-b]Means within a row with different superscripts differ ($P < 0.05$).

Parameter	Treatment			
	Fresh	Fresh-separated	Cryopreserved	Cryopreserved-separated
Motility, %	88 ± 3[a]	98 ± 2[a]	24 ± 9[b]	95 ± 5[a]
Live cell, %	97 ± 1[a]	99 ± 1[a]	74 ± 2[b]	95 ± 3[a]

The dependencies of VCL, VSL, and LIN over the post-activation time are presented as linear regression lines (Fig 1). The curvilinear velocity (VCL) showed a similar downward slope in all groups ($P > 0.05$) over the post-activation time, while a sharper decrease ($P < 0.05$) in track linearity (LIN) was observed in samples after cryopreservation (Table 3). Moreover, the b values showed that the VCL and VSL parameters were initially lower in the cryopreserved samples than in the fresh samples and samples after Percoll separation. As a result of the application of Percoll separation, the highest VCL and VSL parameters were achieved in cryopreserved samples, while the LIN parameter recovered to similar levels in the fresh and Percoll-separated groups.


VCL, µm/s
200
160
120
80
40
0
0
20
40
60
80
100
Post-activation time, s
Fresh
Cryopreserved
Fresh-separated
Cryopreserved-separated


VSL, µm/s
200
160
120
80
40
0
0
20
40
60
80
100
Post-activation time, s
Fresh
Cryopreserved
Fresh-separated
Cryopreserved-separated

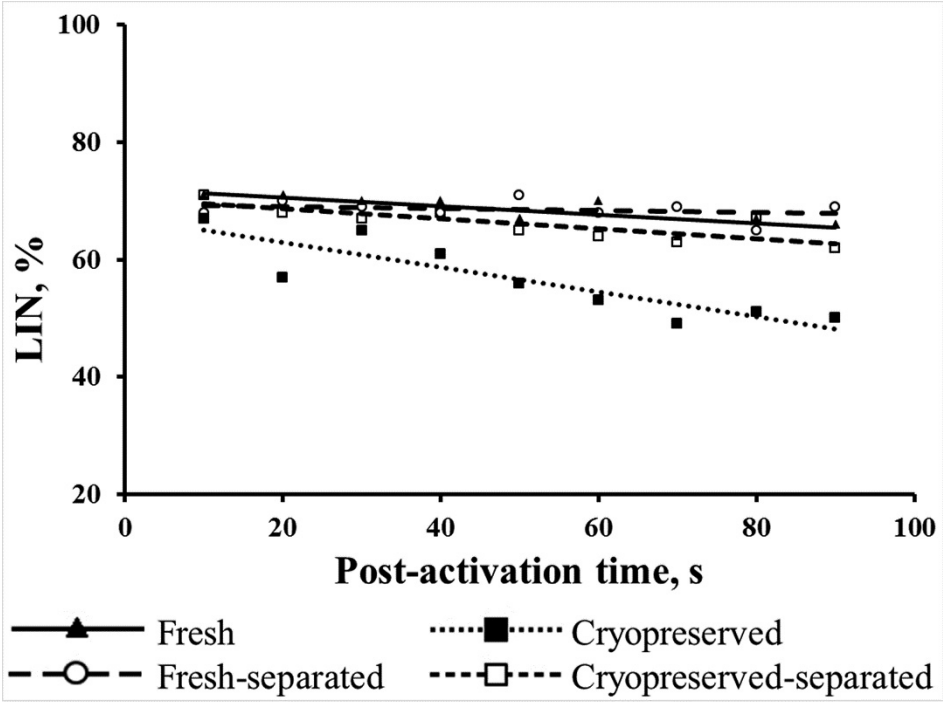


Figure 1. *Linear regression lines describing changes in sperm motility parameters over the post-activation time.*

VCL = curvilinear velocity over the actual path, in μm/s; VSL = straight line velocity in μm/s; LIN = linearity of track, VSL/VCL * 100%. The lines are plotted on the values of the mean ± SD.

Table 3. *The parameters of the linear regression lines describing the sperm motility characteristics obtained with CASA system.*

[a-c]Means within a column with different superscripts are differ (P < 0.05).

[1]CASA: VCL = curvilinear velocity over the actual path, in μm/s; VSL = straight line velocity in μm/s; LIN = linearity of track, VSL/VCL * 100%.

[2]Parameters: R^2 = coefficient of determination; a = slope, which can be considered as a value describing the decrease of sperm motility parameters over the post-activation time; b = intercept, initial value of the sperm motility parameters.

CASA[1]	Treatment	Parameter[2]			
		R^2	P	a ± SD	b ± SD
VCL	Fresh	0.9450	0.0001	-0.461 ± 0.042[a]	154.6 ± 2.4[a]
	Fresh-separated	0.9265	0.0001	-0.353 ± 0.038[a]	145.6 ± 2.1[b]
	Cryopreserved	0.8557	0.0041	-0.538 ± 0.125[a]	95.7 ± 9.1[c]
	Cryopreserved-separated	0.7749	0.0017	-0.536 ± 0.109[a]	174.0 ± 6.1[d]
VSL	Fresh	0.9399	0.0001	-0.407 ± 0.039[a]	111.0 ± 2.2[a]
	Fresh-separated	0.8705	0.0002	-0.238 ± 0.035[b]	102.7 ± 1.9[b]
	Cryopreserved	0.8764	0.0006	-0.611 ± 0.094[c]	84.2 ± 5.5[c]
	Cryopreserved-separated	0.7475	0.0026	-0.426 ± 0.094[a]	125.5 ± 5.3[d]
LIN	Fresh	0.7439	0.0044	-0.073 ± 0.021[a]	72.1 ± 1.2[a]
	Fresh-separated	0.6980	0.0051	-0.052 ± 0.017[a]	71.1 ± 1.1[a]
	Cryopreserved	0.9431	0.0001	-0.317 ± 0.029[b]	70.2 ± 1.7[a]
	Cryopreserved-separated	0.6681	0.0071	-0.085 ± 0.023[a]	70.4 ± 1.3[a]

Proteins expression in 2D-DIGE and identification of different protein spots by MALDI-TOF/TOF

Analysis of the DIGE gels from fresh, fresh-separated cryopreserved and cryopreserved-separated sperm sample groups revealed 1566 matched spots; among these, 60 spots had significant differences (P < 0.05). A quantitative comparison of the proteome profiles of fresh and cryopreserved sperm samples revealed that 20 protein spots underwent significant changes (P < 0.05) in abundance (Fig 2A, C) while differences for only one protein spot were significant when comparing fresh and cryopreserved sperm after Percoll separation (Fig 2B, D). The comparison of fresh samples before and after Percoll separation showed significant changes in 11 proteins (Fig 2A, B), while 28 proteins underwent significant changes in their contents due to Percoll separation of cryopreserved samples (Fig 2C, D). A representative 2D-DIGE image of an overlay of fresh compared to cryopreserved spermatozoa, fresh compared to cryopreserved sperm after Percoll separation, fresh compared to fresh-separated spermatozoa, and cryopreserved compared to cryopreserved-separated spermatozoa is presented in Fig 3.

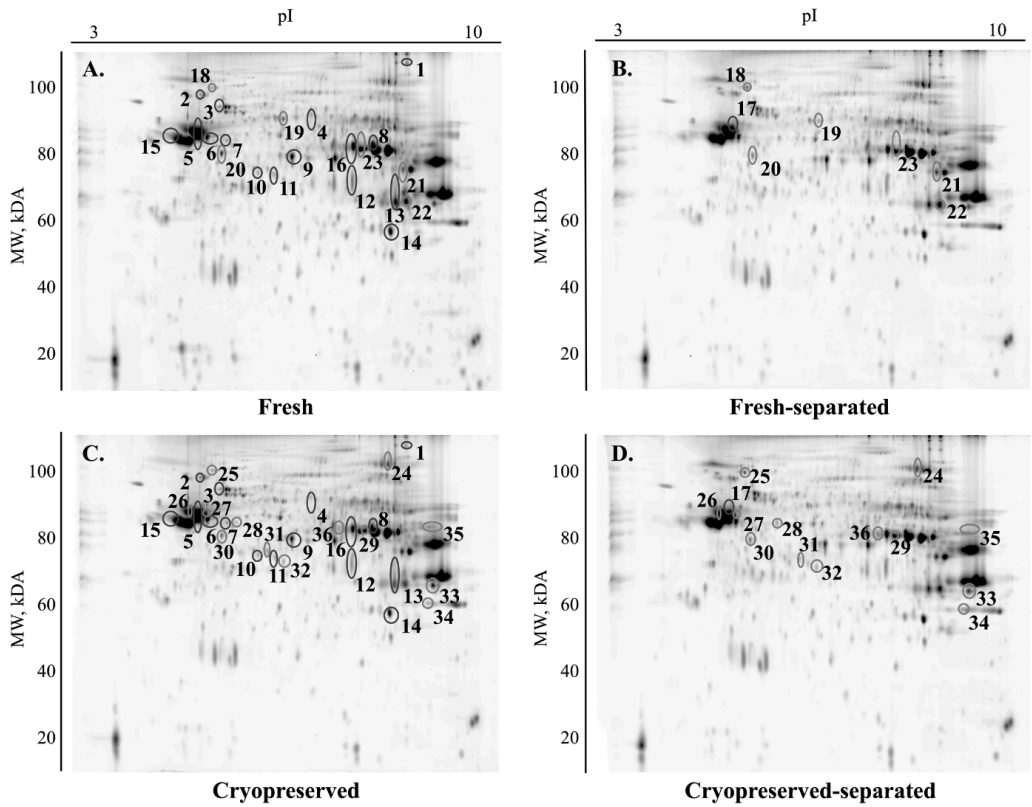


Figure 2. *Representative 2D differences in-gel electrophoresis gels showing comparison of proteomic profiles of the investigated groups.*

The protein spots were identified because they underwent significant changes in their abundance are marked with different colors: 1.) fresh compared to cryopreserved sperm groups (blue color A, C); 2.) fresh-separated compared to cryopreserved-separated sperm groups (blue color B, D); and 3.) fresh compared to fresh separated sperm groups (red color A, B); 4.) cryopreserved compared to cryopreserved-separated sperm groups (red color C, D). pI = isoelectric point; MW = molecular weight.

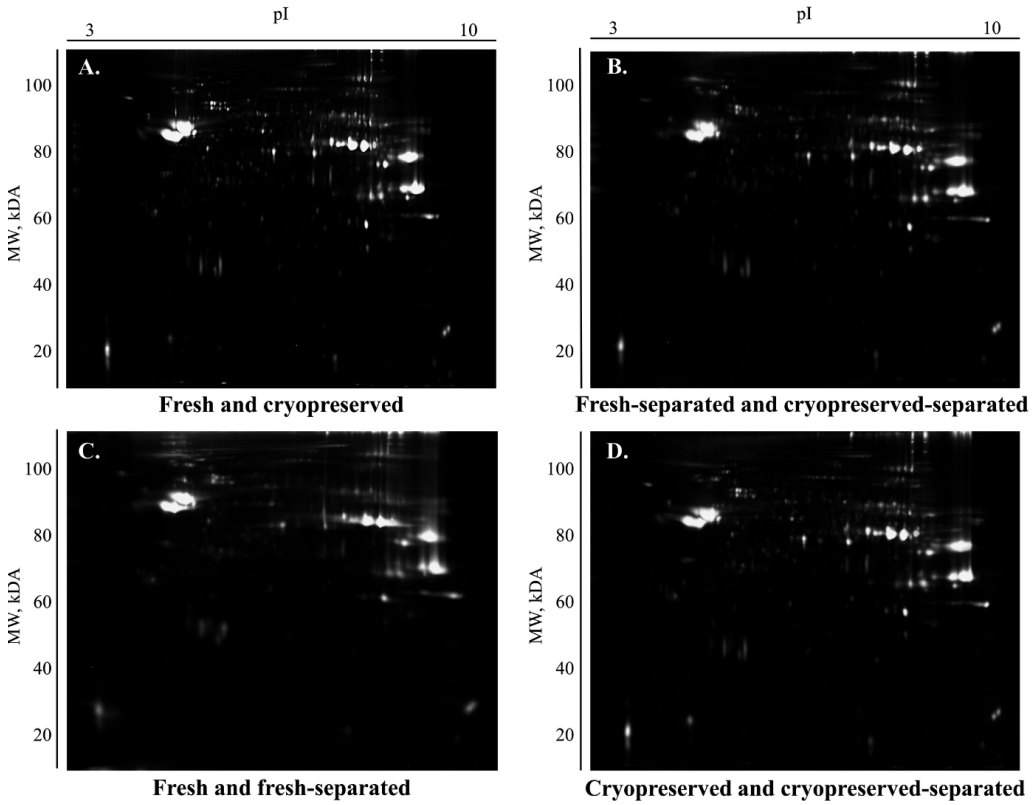


Figure 3. *Representative 2D differences in-gel electrophoresis gels.*
Showing the overlay of fresh (Cy5 dye, red) and cryopreserved (Cy3 dye, green) sperm groups (A), fresh-separated (Cy3 dye, green), and cryopreserved-separated (Cy5 dye, red) sperm groups (B), fresh (Cy3 dye, green) and fresh separated (Cy5 dye, red) sperm groups (C), cryopreserved (Cy5 dye, red), and cryopreserved-separated (Cy3 dye, green) sperm groups (D). Protein spots appearing with yellow color have similar intensities. pI = isoelectric point; MW = molecular weight.

MALDI-TOF/TOF analysis allowed the identification of 16 out of 20 selected protein spots (Table 4) when comparing fresh and cryopreserved sperm, while one protein spot was identified after comparison of fresh and cryopreserved sperm after Percoll separation. Out of 11 different protein spots, six were identified when comparing fresh samples before and after separation, and 13 of 28 selected protein spots were identified by comparison of cryopreserved samples before and after separation.

Table 4. *List of identified sterlet sperm proteins whose abundance changes significantly (P < 0.05; fold change ± 1.2) during cryopreservation or Percoll separation and identified by matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight mass spectrometry and fold change of protein abundance between fresh and cryopreserved, fresh-separated and cryopreserved separated, cryopreserved and cryopreserved-separated, fresh and fresh-separated sperm groups.*

[a]GI = GenInfo Identifier;

[b]MW = molecular weight;

[c]pI = isoelectric point.

Spot no.	Protein name	Gene name	GI[a] number	Organism	Protein score	MW[b],Da/pI[c]	Number of peptides (ion score ≥ 30)	Sequence coverage, %	Fold change	P-value	Protein function
Fresh compared to cryopreserved											
1	L-lactate dehydrogenase A chain	LDHA	gi|975115072	*Gekko japonicus*	103	36866/7.71	1	17	-1.25	0.005	Carbohydrate metabolic process
2	Histone H3-like	H3F3A	gi|688563016	*Branchiostoma belcheri*	218	59409/10.9	4	16	1.34	0.008	Transcription regulation
3	heat shock protein 70	HSPA8	gi|302566321	*Acipenser baerii*	355	71158/5.28	3	25	1.25	0.024	Regulatory role in autophagy
4	Serine/threonine-protein kinase SIK2, partial	SIK2	gi|565316864	*Ophiophagus hannah*	80	8509/6.25	1	61	-1.64	0.006	Cell viability processes
5	tubulin alpha chain, testis-specific	TUBA4A	gi|831271688	*Clupea harengus*	1010	46473/5.01	8	61	1.28	0.027	Microtubule cytoskeleton organization
6	mitochondrial H+-transporting ATP synthase F1 complex beta polypeptide	ATP5A1	gi|296802112	*Rousettus leschenaultii*	750	51384/5.11	7	50	1.39	0.002	ATP metabolic process
7	cytochrome b-c1 complex subunit 1, mitochondrial-like	UQCRC1	gi|591370457	*Chelonia mydas*	210	50681/5.7	2	14	1.38	0.001	Respiratory chain protein
8	phosphoglycerate kinase	PGK1	gi|46849425	*Acipenser baerii*	616	42021/5.76	7	44	-1.4	0.004	Glycolytic process

Spot no.	Protein name	Gene name	GI[a] number	Organism	Protein score	MW[b],Da/pI[c]	Number of peptides (ion score ≥ 30)	Sequence coverage, %	Fold change	P-value	Protein function
Fresh compared to cryopreserved											
9	creatine kinase B-type-like isoform X1	CKB	gi|1020521224	*Sinocyclocheilus grahami*	498	43152/5.42	5	27	-1.2	0.044	Phosphocreatine metabolic process, ATP binding
10	isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	IDHA1	gi|45361551	*Xenopus tropicalis*	220	40297/6.33	2	22	1.21	0.001	Tricarboxylic acid cycle, NAD binding
11	isocitrate dehydrogenase 3 (NAD+) alpha	IDH3A	gi|148227952	*Xenopus laevis*	240	40595/6.76	2	14	1.16	0.011	Tricarboxylic acid cycle, NAD binding
12	glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	GPD1	gi|663288276	*Calypte anna*	155	34729/6.66	3	16	-1.28	0.020	Weight-loss-responsive gene
13	L-lactate dehydrogenase A chain	LDHA	gi|725549051	*Saimiri boliviensis boliviensis*	185	36796/7.08	3	29	-1.23	0.037	Carbohydrate metabolic process
14	triose phosphate isomerase	TPI1	gi|46849427	*Acipenser baerii*	437	22890/6.1	5	51	-1.24	0.011	Glycolytic process
15	tubulin beta-4B chain	TUBB4B	gi|831324610	*Clupea harengus*	1110	50253/4.79	9	56	1.32	0.031	The major constituent of microtubules
16	phosphoglycerate kinase	PGK1	gi|46849425	*Acipenser baerii*	520	42021/5.76	6	56	-1.26	0.011	Glycolytic process
Fresh-separated compared to cryopreserved-separated											
17	tubulin alpha-8 chain-like	TUBA8	gi|927153352	*Thamnophis sirtalis*	92	32528/4.48	1	13	1.15	0.042	The major constituent of microtubules
Fresh compared to fresh-separated											

Spot no.	Protein name	Gene name	GI[a] number	Organism	Protein score	MW[b],Da/pI[c]	Number of peptides (ion score ≥ 30)	Sequence coverage, %	Fold change	P-value	Protein function
Fresh compared to cryopreserved											
18	transitional endoplasmic reticulum ATPase	SEC16B	gi|1025248139	*Sinocyclocheilus rhinocerous*	225	89982/5.14	3	15	1.21	0.034	Endoplasmic reticulum export factor to deliver to newly synthesized proteins and lipids
19	keratin type IIE	KRT3	gi|32452105	*Acipenser baerii*	171	51448/5.06	1	28	-2.1	0.005	Structural framework
20	beta-actin	ACTB	gi|119943232	*Misgurnus anguillicaudatus*	730	42038/5.29	7	41	-1.96	0.020	Cell mtility
21	creatine kinase U-type, mitochondrial	CKMT1A	gi|38488694	*Danio rerio*	236	47133/8.05	2	15	1.31	0.041	Energy transduction
22	L-lactate dehydrogenase A chain	LDHA	gi|573909562	*Lepisosteus oculatus*	216	36934/7.12	2	19	-1.1	0.025	Carbohydrate metabolic process
23	phosphoglycerate kinase	PGK	gi|46849425	*Acipenser baerii*	520	42021/5.76	6	56	1.06	0.045	Glycolytic process
Cryopreserved compared to cryopreserved-separated											
24	glycogen phosphorylase, muscle form	GPH1	gi|946678688	*Pelodiscus sinensis*	408	97539/6.77	6	28	1.4	0.048	Catalyzes the hydrolysis of glycogen
25	transitional endoplasmic reticulum ATPase	tER	gi|1025248139	*Sinocyclocheilus rhinocerous*	225	89982/5.14	3	15	1.23	0.003	Termination of DNA replication and function by binding to DNA replication terminator sequences
26	tubulin alpha chain, testis-specific	TUBA	gi|831271688	*Clupea harengus*	1010	46473/5.01	8	61	-1.3	0.040	Microtubule cytoskeleton organization

Spot no.	Protein name	Gene name	GI[a] number	Organism	Protein score	MW[b],Da/pI[c]	Number of peptides (ion score ≥ 30)	Sequence coverage, %	Fold change	P-value	Protein function
				Fresh compared to cryopreserved							
27	ATP synthase subunit beta, mitochondrial-like	ATP2	gi|432849647		714	55238/5.1	7	47	-1.26	0.002	ATP metabolic process
28	cytochrome b-c1 complex subunit 1, mitochondrial-like	MZM1	gi|591370457	*Chelonia mydas*	210	50681/5.7	2	14	-1.36	0.006	
29	phosphoglycerate kinase	PGK1	gi|46849425	*Acipenser baerii*	616	42021/5.76	7	44	1.34	0.019	Glycolytic process
30	beta-actin	ACTB	gi|119943232	*Misgurnus anguillicaudatus*	730	42038/5.29	7	41	-1.22	0.015	Cell mtility
31	isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	IDP2	gi|45361551	*Xenopus tropicalis*	220	40297/6.33	2	22	-1.3	0.001	Tricarboxylic acid cycle, NAD binding
32	isocitrate dehydrogenase 3 (NAD+) alpha	IDH3A	gi|148227952	*Xenopus laevis*	240	40595/6.76	2	14	-1.38	0.012	Tricarboxylic acid cycle, NAD binding
33	voltage-dependent anion-selective channel protein 2 isoform X1	VDAC	gi|1020410464	*Sinocyclocheilus grahami*	672	30351/8.83	5	38	-1.21	0.004	Cell signaling events
34	phosphoglycerate mutase 2	PGAM2	gi|471409135	*Trichechus manatus latirostris*	304	29037/8.84	3	30	1.68	0.015	Glycolytic process
35	fructose-bisphosphate aldolase A-2	IOIJ	gi|46849415	*Acipenser baerii*	118	36299/8.1	1	24	1.34	0.029	
36	phosphoglycerate kinase	PGK1	gi|46849425	*Acipenser baerii*	393	42021/5.76	5	30	1.22	0.042	Glycolytic process

Principal component analysis (PCA) distinguished differences in the abundance of 43 protein spots in fresh, fresh-separated, cryopreserved, and cryopreserved-separated sperm protein groups (Fig 4). The first principal component accounted for 47.9% of the total variance, suggesting that the greatest differences in protein abundance occurred between cryopreserved semen and the three remaining groups. The highest changes in protein abundance within the groups were between cryopreserved and fresh sperm, then between cryopreserved and fresh-separated sperm, and finally between the cryopreserved and cryopreserved-separated groups. The second principal component accounted for 24.4% of the total variance and showed minimal differences in protein abundance between the fresh and fresh-separated groups and between the fresh and cryopreserved-separated groups. Differences between the fresh-separated and cryopreserved-separated groups were not observed.

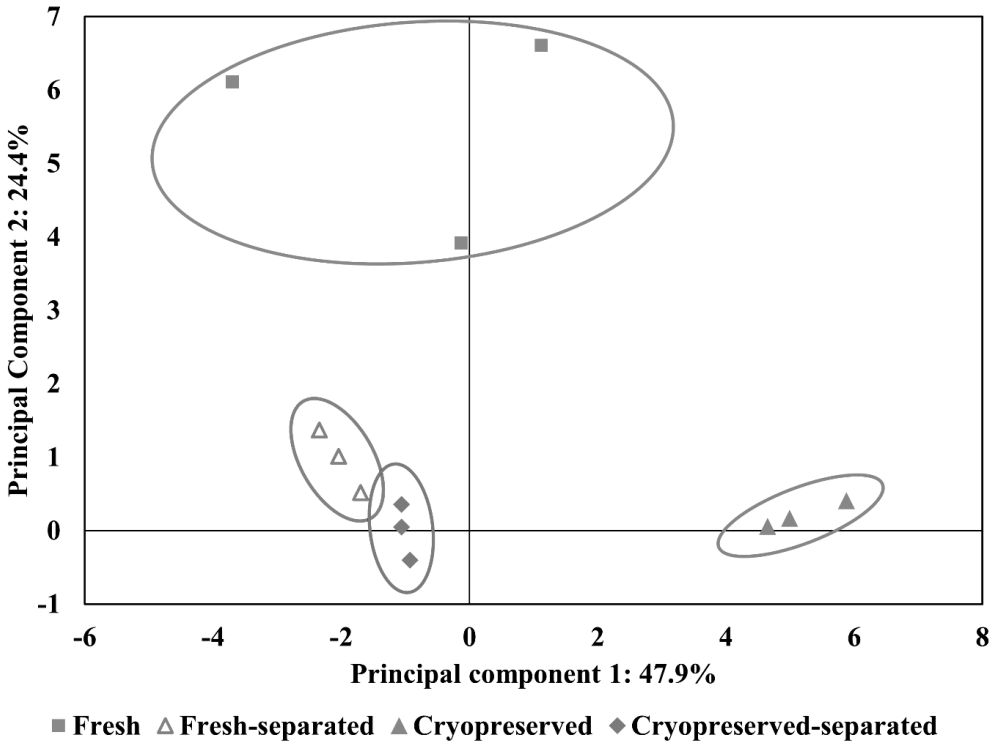


Figure 4. *Principal component analysis of abundance data from sturgeon sperm proteins significantly changed during the cryopreservation. Each symbol represents a gel image of one sample and groups of samples yielded by principal component analysis are enclosed in circles.*

Ingenuity pathway analysis of differentially abundant proteins

The gene name for each identified protein spot that underwent a significant change in abundance due to the cryopreservation process was subjected to IPA analysis to identify its signaling pathway and function (Table 5). These changed sperm proteins were associated with the generation of precursor metabolites and energy, nucleic acid metabolism, and cellular assembly and organization in the top molecular and cellular function category.

Furthermore, IPA demonstrated that the sirtuin signaling pathway, glycolysis and oxidative phosphorylation were the canonical pathways that were most affected by differentially abundant proteins.

Table 5. *Ingenuity pathway analysis of differentially abundant proteins between fresh and cryopreserved sperm groups.*

[a]Proteins: LDHA – l-lactate dehydrogenase A chain; H3F3A – histone H3-like; HSPA8 – heat shock protein 70; TUBA4A – tubulin alpha chain, testis-specific; ATP5A1 – mitochondrial H+-transporting ATP synthase F1 complex beta polypeptide; UQCRC1 – cytochrome b-c1 complex subunit 1, mitochondrial; PGK1 – phosphoglycerate kinase; IDH3A – isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial; GPD1 – glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic; TPI1 – triose phosphate isomerase.

Top molecular and cellular functions	**P-value range**	**No. of molecule**	**Protein[a]**
Generation of precursor metabolites and energy	2.01e-05 – 2.75e-07	6	GPD1, TPI1, PGK1, ATP5A1, IDH3A, LDHA
Nucleic acid metabolism	2.21e-05 – 2.65e-07	5	ATP5A1, GPD1, HSPA8, IDH3A, PGK1
Cellular assembly and organization	2.01e-05 – 2.75e-07	5	GPD1, TPI1, PGK1, ATP5A1, IDH3A, LDHA
Top canonical pathways	p-Value	No. of molecule	Protein[a]
Sirtuin Signaling Pathway	1.53e-05	4	ATP5A1, H3F3A, PGK1, TUBA4A
Glycolysis	9.34e-05	4	PGK1, TPI1, LDHA, GPD1
Oxidative phosphorylation	1.65e-03	2	ATP5A1, UQCRC1

Protein-protein interaction analysis

The protein-protein interactions network showed medium edge (score 0.4–0.6) confidence for 43 pairs of interactions and high interactions (score 0.7–0.9) for 10 protein pairs when comparing fresh and cryopreserved sperm groups (Fig 5). The highest interaction (score 0.9) was observed between l-lactate dehydrogenase A and phosphoglycerate kinase 1, mitochondrial H+-transporting ATP synthase F1 complex beta polypeptide and cytochrome b-c1 complex subunit 1, tubulin alpha chain, testis-specific and tubulin beta-4B chain, and phosphoglycerate kinase and triose phosphate isomerase. Such high interactions with high scores indicate functional protein linkages.

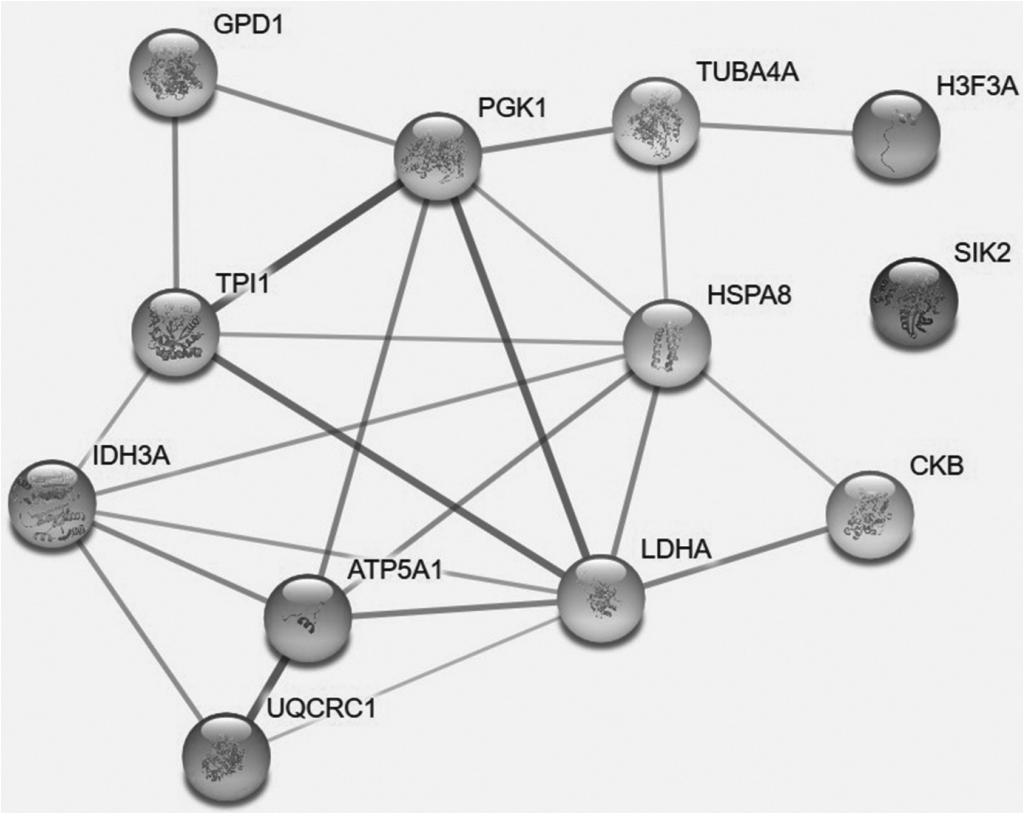


Figure 5. *STRING analysis of proteins-protein interaction of differentially abundant proteins.*

The interaction between fresh and cryopreserved sperm samples is presented. The line thickness indicates the strength of data support. LDHA – l-lactate dehydrogenase A chain; H3F3A – histone H3-like; HSPA8 – heat shock protein 70; SIK2 – serine/threonine-protein kinase SIK2; TUBA4A – tubulin alpha chain, testis-specific; ATP5A1 – mitochondrial H+-transporting ATP synthase F1 complex beta polypeptide; UQCRC1 – cytochrome b-c1 complex subunit 1, mitochondrial; PGK1 – phosphoglycerate kinase; CKB – creatine kinase B-type; IDH3A – isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial; GPD1 – glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic; TPI1 – triose phosphate isomerase; TUBB4B – tubulin beta-4B chain.

DISCUSSION

In the present study, for the first time, the Percoll density gradient centrifugation method was used for the selection and analyses of spermatozoa possessing high viability and motility in sterlet (*Acipenser ruthenus*) sperm. Moreover, it is the first study that focused on the investigation of changes in protein profiles of the spermatozoa population containing more than 95% of viable cells after the cryopreservation process.

Selection of high-quality, high-density spermatozoa that maintain their fertilization capacity is a crucial factor for the success of assisted reproduction techniques, since dead and abnormal spermatozoa as well as sperm debris may be an obstacle to successful fertilization in *in vitro* conditions (Poe-Zeigler et al., 1997). In mammals, a broad range of techniques for sperm

separation (sperm selection) is widely used for the improvement of assisted reproduction technologies. Sperm selection techniques mainly separate good-quality spermatozoa, which are usually high density, from the immotile sperm and other constituents of the seminal fluid (Arias et al., 2017). This approach significantly improves sperm quality, thereby increasing the amount of progressively motile spermatozoa. For in vitro insemination application, both fresh and cryopreserved mammalian sperm should be separated or sorted (Drobnis et al., 1991; Henkel and Schill, 2003; Ozkavukcu et al., 2008). The main rationale for such sorting takes into account the fact that the active separation of the most actively swimming spermatozoa that occurs during migration through the cervical mucus is the normal process under in vivo conditions (Mortimer and Templeton, 1982) but that this process does not occur in vitro.

In contrast to these technologies applied to mammalian sperm, the separation of fish sperm is a totally new approach that has never been developed to this extent and presents some challenges. Due to characteristics specific to fish spermatozoa, such as motility activation dependency on environmental osmolality and ionic composition, and the necessity to keep spermatozoa immotile before fertilization (Gallis et al., 1991; Cosson et al., 2000), the application of these separation techniques can be quite challenging. However, despite such challenges, several attempts have been made in this direction. Previously, Valcarce et al. (Valcarce et al., 2016) implemented a sperm selection method for non-apoptotic sperm subpopulation selection in Senegalese sole (Solea senegalensis) sperm before cryopreservation. In particular, magnetic-activated cell sorting was used to eliminate the apoptotic spermatozoa subpopulation before cryopreservation. Another example of the application of sperm separation techniques is in common carp (*Cyprinus carpio*) sperm. Li et al. (Li et al., 2010a), working with cryopreservation of carp spermatozoa, obtained a fraction of higher-quality spermatozoa using Percoll density gradient centrifugation after cryopreservation. They observed a significantly greater proportion of motile spermatozoa with higher velocity and intact membranes in cryopreserved carp sperm after Percoll separation. Similarly, an increased percentage of motile sperm cells with higher motility parameters after Percoll selection were observed in our study. Moreover, the motility percentage of cryopreserved and fresh sperm following the separation by Percoll was higher than the percentage recorded for fresh sperm motility. This indicates that Percoll separation results in the selection of the spermatozoa population presenting the best motility parameters, even in the case of fresh sperm.

A decrease in sperm motility parameters due to the impact of cryopreservation is a well-known phenomenon, and our findings are consistent with those described previously in several fish species (Billard et al., 2004; Asturiano et al., 2006; Nynca et al., 2014; Judycka et al., 2015; Horokhovatskyi et al., 2017). Our results show a downward slope in the parameters VCL, VSL and LIN over the post-activation time. Nevertheless, the most substantial decrease in the initial VCL and VSL parameters according to the intercept value was observed for cryopreserved samples. These changes were associated with the presence of a large number of lethal and sublethal damaged spermatozoa that appeared due to the damage engendered by the cryopreservation process and that had an impact on the mean values of these parameters. However, along with an overall decline in the movement characteristics of cryopreserved sperm, the parameters of cryopreserved and Percoll separated sperm showed the highest values of VCL and VSL. A similar phenomenon was observed by Boryshpolets et al. (Boryshpolets et al., 2011), in which the cryopreserved sperm showed higher average velocity parameters than fresh sperm. We suppose that the sperm population possessing lower motility parameters was presented in samples after cryopreservation and then was removed from the post-thaw sperm mixture by Percoll separation.

The application of SYBR-14 and PI fluorescent dyes was successful to determine the live/

dead cell ratio in sturgeon sperm samples. A low motility percentage and quite high live cell percentage was observed in the cryopreserved group. These can be attributed to the presence of sperm cells that saved an intact permeability barrier (cell membrane) but that have lost the ability for movement activation. A similar phenomenon was reported by Drobnis et al. (Drobnis et al., 1991) in human semen after cryopreservation. Nevertheless, the determination in our study of the percentage of live sperm was one of the parameters showing the efficiency of the Percoll separation technique.

There are many probable causes for sperm cryo-damage. Some authors have reported damage to sperm structure or function during the cryopreservation process due to osmotic stress, oxidative stress or ice crystal formation both within the sperm cells and in the external medium (Mazur, 2004; Cabrita et al., 2014). It was also noted that much damage can occur at the level of the sperm plasma membrane through changes in lipid membrane composition, organization, and properties (Müller et al., 2008; Martínez-Páramo et al., 2012). However, the freeze-thaw process also leads to alterations in DNA integrity and protein profiles (Zilli et al., 2003; Cabrita et al., 2005; Li et al., 2010a). The defects appearing in sperm proteins may in turn have a pernicious effect on sperm motility and fertilization ability and even on the resulting embryo at the early stages of development following fertilization (Lessard et al., 2000; Li et al., 2010b). For example, after cryopreservation of bull semen, 16 proteins underwent significant changes in abundance (Westfalewicz et al., 2015), while in common carp sperm following cryopreservation, 14 proteins were significantly altered (Li et al., 2010b). Similarly, in the present study, using 2D-DIGE coupled with MALDI-TOF/TOF, significant changes in the abundance of 20 proteins were detected in sterlet sperm after cryopreservation. According to the IPA, most of the affected proteins, such as L-lactate dehydrogenase A chain, phosphoglycerate kinase, creatine kinase B-type-like isoform X1, isocitrate dehydrogenase [NAD] subunit alpha, and mitochondrial proteins, are related to various metabolic and energy production processes. Furthermore, some of the proteins, such as the tubulin alpha chain and tubulin beta-4B chain, are part of the cytoskeleton and are involved in spermatozoon motility initiation and maintenance (Dietrich et al., 2016). As a consequence, all of these changes are involved in structural integrity, various biological processes, and the main functions of sperm, ultimately decreasing their fertilization capacity.

The significant decrease in protein abundance or even spot disappearance resulting from the cryopreservation procedure may be due to leakage of proteins from inside the spermatozoa to the extracellular medium (Huang et al., 1998; Li et al., 2010b). Nevertheless, it has been shown that cryopreservation can also increase the contents of some proteins in spermatozoa through their modification (Ollero et al., 1998; Ardon and Suarez, 2013). This suggests that cryopreservation has a complex effect on sperm proteins.

Nevertheless, many previously published results are not as clear, as they are based on analysis of cryopreserved sperm suspensions that contain not only the fraction of interest (viable spermatozoa) but also other constituents generated by the cryopreservation of semen. Usually, under the influence of cryo-damage, to which low-quality spermatozoa could be more susceptible, the membrane-bound proteins and intracellular enzymes, in addition to other components of the spermatozoon, co-elute from spermatozoa into the global sperm suspension. Thus, after analysis of such a suspension, the output results will be quite probably based on the parameters of a mixture of viable, lethal and sublethal damaged spermatozoa subpopulations.

To validate this hypothesis, the analysis of only sperm subpopulations that contain more than 95% of viable spermatozoa selected by Percoll separation was performed in our study. The analysis of this particular sperm fraction showed only one significantly altered protein after the cryopreservation procedure. The identified protein, tubulin alpha-8 chain-like, is

linked to cytoskeletal proteins and is involved in sperm motility initiation and maintenance (Dietrich et al., 2016). This result shows a much lower number of changes in proteins from cryopreserved and Percoll separated spermatozoa. However, it is still remains a question if this sperm subpopulation can be more cryoresistant than one that underwent pernicious changes in the proteins. Additionally, the differences in protein abundance when fresh and fresh-separated and cryopreserved and cryopreserved-separated groups were compared are related to the application of Percoll separation. This could result from the fact that the separation technique also removes the proteins that were initially present in the seminal fluid.

Additionally, principal component analysis showed that the greatest changes in protein abundance occurred in the group of sperm samples after cryopreservation compared to the three other groups. The highest differences were observed between fresh sperm and the cryopreserved group, suggesting to what extent cryopreservation can influence the protein profile of spermatozoa, leading to possible protein modification or their leakage to the external medium. However, the changes in protein abundance in Percoll separated samples are present to a much lower extent. These findings suggest that Percoll separation is relevant for both fresh and cryopreserved sperm, as it can remove the low-density spermatozoa and debris present in fresh samples or induced by cryopreservation. Moreover, both fresh and cryopreserved sperm after Percoll separation showed the lowest possible variance in multidimensional space. This indicates that the protein content of cryopreserved Percoll separated samples may not be changed due to cryopreservation and remains the same as in Percoll separated fresh sperm samples. However, the question remains as to whether the separation technique for only selected spermatozoa could be used for the improvement of the fertilization procedure.

In summary, our study, which was performed with four experimental groups of samples, has confirmed that ryopreservation has a deleterious effect on fish sperm motility, viability and the functional state of many sperm proteins in the cryopreserved group when compared to the fresh group. Furthermore, the differences in many proteins found in these groups are related to the presence of lethal and sublethal damaged sperm. Additionally, the differences in protein abundance when the fresh and fresh-separated groups and cryopreserved and cryopreserved-separated groups were compared are related to the application of Percoll separation. This could result from the fact that the separation technique also removes the proteins that were initially present in the seminal fluid.

However, through the application of Percoll separation method to fresh and cryopreserved groups, it is possible to select and analyze only the sperm population that contains more than 95% of viable cells. This selected sperm subpopulation retained high motility parameters before the cryopreservation procedure (fresh-separated group) and after freeze-thawing (cryopreserved-separated group). No differences in sperm motility, viability, or sperm proteins were found in these groups. All of the detected changes in post-thaw sperm motility parameters, viability, and the protein profiles of viable spermatozoa after the freeze-thaw process provide a background for further directions of investigations aimed at a deeper understanding of the mechanism responsible for the cryodamage of sperm. Further studies should also address whether sperm separation techniques can serve as useful tools for both a better understanding of non-lethal sperm damage and the improvement of in vitro fertilization results in sturgeon species.

REFERENCES

Ardon, F., Suarez, S. S. 2013. Cryopreservation increases coating of bull sperm by seminal plasma binder of sperm proteins BSP1, BSP3, and BSP5. Reproduction 146:111-117.

Arias, M. E., Andara, K., Briones, E., Felmer, R. 2017. Bovine sperm separation by Swim-up and density gradients (Percoll and BoviPure): Effect on sperm quality, function and gene expression. Reproductive Biology 17(2):126-132.

Asturiano, J. F., Marco-Jimenez, F., Penaranda, D. S., Garzon, D. L., Perez, L., Vicente, J. S., Jover, M. 2006. Effect of sperm cryopreservation on the European eel sperm viability and spermatozoa morphology. Reproduction in Domestic Animals 42(2):162-166.

Beydola, T., Sharma, R. K., Agarwal, A. 2014. Sperm preparation and selection techniques. In: R. Botros, editor, Medical and surgical management of male Infertility No. 1. Jaypee Brothers Medical Publishers, Philadelphia. p. 244-251.

Billard, R., Cosson, J., Noveiri, S. B., Pourkazemi, M. 2004. Cryopreservation and short-term storage of sturgeon sperm, a review. Aquaculture 236(1–4):1-9.

Boryshpolets, S., Dzyuba, B., Rodina, M., Alavi, S. M. H., Gela, D., Linhart, O. 2011. Cryopreservation of sterlet (*Acipenser ruthenus*) spermatozoa using different cryoprotectants. Applied Ichthyology 27(5):1147-1149.

Bronzi, P., Rosenthal, H., Gessner, J. 2011. Global sturgeon aquaculture production: an overview. Journal of Applied Ichthyology 27(2):169-175.

Cabrita, E., Martínez-Páramo, S., Gavaia, P. J., Riesco, M. F., Valcarce, D. G., Sarasquete, C., Herráez, M. P., Robles, V. 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432:389-401.

Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., Herráez, M. P. 2005. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology 50(2):144-153.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M. P. 2010. Cryopreservation of fish sperm: applications and perspectives. Journal of Applied Ichthyology 26(5):623-635.

Chebanov, M. S., Galich, E. V. 2013. Sturgeon hatchery manual. Ankara: Food and Agriculture Organization of United Nations

Cosson, J. 2004. The Ionic and Osmotic Factors Controlling Motility of Fish Spermatozoa. Aquaculture International 12(1):69-85.

Cosson, J., Linhart, O., Mims, S. D., Shelton, W. L., Rodina, M. 2000. Analysis of motility parameters from paddlefish and shovelnose sturgeon spermatozoa. Journal of Fish Biology 56(6):1348-1367.

Dietrich, M. A., Arnold, G. J., Fröhlich, T., Ciereszko, A. 2014. In-depth proteomic analysis of carp (*Cyprinus carpio*) spermatozoa. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 12:10-15.

Dietrich, M. A., Dietrich, G. J., Mostek, A., Ciereszko, A. 2016. Motility of carp spermatozoa is associated with profound changes in the sperm proteome. Journal of Proteomics 138:124-135.

Dietrich, M. A., Irnazarow, I., Ciereszko, A. 2017. Proteomic identification of seminal plasma proteins related to the freezability of carp semen. Journal of Proteomics 162:52-61.

Drobnis, E. Z., Zhong, C. Q., Overstreet, J. W. 1991. Separation of Cryopreserved Human Semen Using Sephadex Columns, Washing, or Percoll Gradients. Journal of Andrology 12(3):201-208.

Dzyuba, B., Boryshpolets, S., Shaliutina, A., Rodina, M., Yamaner, G., Gela, D., Dzyuba, V., Linhart, O. 2012. Spermatozoa motility, cryoresistance, and fertilizing ability in sterlet *Acipenser ruthenus* during sequential stripping. Aquaculture 356:272-278.

Dzyuba, B., Cosson, J., Boryshpolets, S., Bondarenko, O., Dzyuba, V., Prokopchuk, G., Gazo, I., Rodina, M., Linhart, O. 2014. In vitro sperm maturation in sterlet, *Acipenser ruthenus*. Reproductive Biology 14(2):160-163.

Fleming, S., Aitken, R. J. 2012. Electrophoretic sperm separation. In: Z. Nagy, A. Varghese and A. Agarwal, editors, Practical manual of in vitro fertilization. Springer, New York. p. 259-263.

Gallis, J. L., Fedrigo, E., Jatteau, P., Bonpunt, E., Billard, R. 1991. Siberian sturgeon spermatozoa: effects of dilution, pH, osmolality, sodium and potassium ions on motility. Acipenser:143-151.

Glogowski, J., Kolman, R., Szczepkowski, M., Horváth, Á., Urbányi, B., Sieczyński, P., Rzemieniecki, A., Domagała, J., Demianowicz, W., Kowalski, R., Ciereszko, A. 2002. Fertilization rate of Siberian sturgeon (Acipenser baeri, Brandt) milt cryopreserved with methanol. Aquaculture 211(1–4):367-373.

Henkel, R., Schill, W. B. 2003. Sperm preparation for ART. Reproductive biology and endocrinology 1:108-108.

Holt, W. V. 2000. Basic aspects of frozen storage of semen. Animal Reproduction Science 62(1):3-22.

Horokhovatskyi, Y., Rodina, M., Asyabar, H. D., Boryshpolets, S., Dzyuba, B. 2017. Consequences of uncontrolled cooling during sterlet (*Acipenser ruthenus*) sperm cryopreservation on post-thaw motility and fertilizing ability. Theriogenology 95:89-95.

Horokhovatskyi, Y., Sampels, S., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M., Dzyuba, B. 2016. Lipid composition in common carp (*Cyprinus carpio*) sperm possessing different cryoresistance. Cryobiology 73(2):282-285.

Huang, S. Y., Kuo, Y. H., Lee, W. C., Tsou, H. L., Lee, Y. P., Chang, H. L., Wu, J. J., Yang, P. C. 1998. Substantial decrease of heat-shock protein 90 precedes the decline of sperm motility during cooling of boar spermatozoa. Theriogenology 51(5):1007-1016.

Ilaria, N. 2011. Sperm Preparation Techniques for Artificial Insemination - Comparison of sperm Washing, Swim Up, and Density Gradient Centrifugation Methods. In: M. Manafi, editor, Artificial insemination in farm animals. InTech, Rijeka, Croatia. p. 115-122.

Jerrold, H. Z. 2014. Biostatistical analysis. Pearson Education Inc., New Jersey.

Judycka, S., Szczepkowski, M., Ciereszko, A., Dietrich, G. J. 2015. New extender for cryopreservation of Siberian sturgeon (*Acipenser baerii*) semen. Cryobiology 70(2):184-189.

Kopeika, E., Kopeika, J., Zhang, T. 2007. Cryopreservation of Fish Sperm. In: J. G. Day and G. N. Stacey, editors, Cryopreservation and Freeze-Drying Protocols No. 368. Humana Press, Totowa, New Jersey. p. 203-217.

Lessard, C., Parent, S., Leclerc, P., Bailey, J., Sullivan, R. 2000. Cryopreservation alters the levels of the bull sperm surface protein P25b. Journal of Andrology 21:700-707.

Li, P., Dzyuba, B., Hulak, M., Rodina, M., Boryshpolets, S., Li, Z. H., Linhart, O. 2010a. Percoll gradient separation of cryopreserved common carp spermatozoa to obtain a fraction with higher motility, velocity and membrane integrity. Theriogenology 74(8):1356-1361.

Li, P., Hulak, M., Koubek, P., Sulc, M., Dzyuba, B., Boryshpolets, S., Rodina, M., Gela, D., Manaskova-Postlerova, P., Peknicova, J., Linhart, O. 2010b. Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, *Cyprinus carpio*. Theriogenology 74:413-423.

Martínez-Páramo, S., Diogo, P., Dinis, M. T., Herráez, M. P., Sarasquete, C., Cabrita, E. 2012. Sea bass sperm freezability is influenced by motility variables and membrane lipid composition but not by membrane integrity and lipid peroxidation. Animal Reproduction Science 131(3):211-218.

Mazur, P. 2004. Principles of Cryobiology Life in the frozen state. p 3-67.

Mazur, P., Leibo, S. P., Chu, E. H. Y. 1972. A two-factor hypothesis of freezing injury: Evidence from Chinese hamster tissue-culture cells. Experimental Cell Research 71(2):345-355.

Medeiros, C. M. O., Forell, F., Oliveira, A. T. D., Rodrigues, J. L. 2002. Current status of sperm cryopreservation: why isn't it better? Theriogenology 57(1):327-344.

Mortimer, D., Templeton, A. A. 1982. Sperm transport in the human female reproductive tract in relation to semen analysis characteristics and time of ovulation. Journal of Reproduction and Fertility 64:401-408.

Müller, K., Müller, P., Pincemy, G., Kurz, A., Labbe, C. 2008. Characterization of sperm plasma membrane properties after cholesterol modification: consequences for cryopreservation of rainbow trout spermatozoa. BIOLOGY OF REPRODUCTION 78(3):390-399.

Nynca, J., Dietrich, G. J., Dobosz, S., Grudniewska, J., Ciereszko, A. 2014. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. Aquaculture 433:62-65.

Ollero, M., Bescós, O., Cebrián-Pérez, J. A., Muiño-Blanco, T. 1998. Loss of plasma membrane proteins of bull spermatozoa through the freezing–thawing process. Theriogenology 49:547-555.

Ozkavukcu, S., Erdemli, E., Isik, A., Oztuna, D., Karahuseyinoglu, S. 2008. Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. Journal of Assisted Reproduction and Genetics 25(8):403-411.

Poe-Zeigler, R., Nehchiri, F., Hamacher, P., Boyd, C., Oehninger, S., Muasher, S., Lanzendorf, S. E. 1997. Effects of sperm viability on fertilization and embryo cleavage following intracytoplasmic sperm injection. Journal of Assisted Reproduction and Genetics 14(5):277-281.

Probes, M. 2001. Live/Dead sperm viability kit (L-7011). Molecular Probes, Inc.

Seki, S., Mazur, P. 2008. Kinetics and activation energy of recrystallization of intracellular ice in mouse oocytes subjected to interrupted rapid cooling. Cryobiology 56(3):171-180.

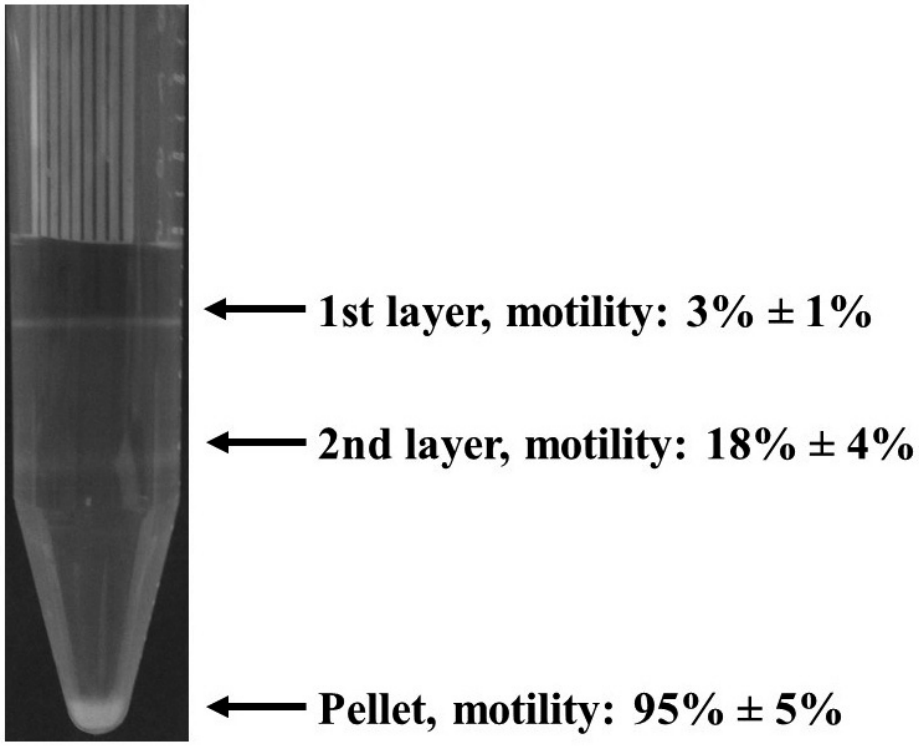
Siegel, S., Castellan, N. J. 1988. Nonparametric statistics for the behavioral sciences. McGraw-Hill Humanities, New York.

Valcarce, D. G., Herráez, M. P., Chereguini, O., Rodríguez, C., Robles, V. 2016. Selection of nonapoptotic sperm by magnetic-activated cell sorting in Senegalese sole (*Solea senegalensis*). Theriogenology 86(5):1195-1202.

Westfalewicz, B., Dietrich, M. A., Ciereszko, A. 2015. Impact of cryopreservation on bull (Bos taurus) semen proteome. Journal of Animal Science 93(11):5240-5253.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., Vilella, S. 2003. Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology 47(3):227-235.

SUPPORTING INFORMATION



S1 Figure. *Visual presentation of results of Percoll gradient centrifugation applied to sterlet sperm.*
Data on sperm motility in each layer are presented as the mean ± SD, (n=7).
1st layer: this layer contained mass of spermatozoa and debris; after transferring into activating medium, an extremely low motility percentage (3 ± 1%) was observed;
2nd layer: this layer contained mainly dead spermatozoa and a small amount of debris; after transferring into activating medium, the motility percentage was (18 ± 4%);
Pellet: the pellet contained spermatozoa, debris was not detected; after transferring into activating medium, the motility percentage was 95-100%.

S2 Table. *Sperm concentration of fresh and cryopreserved samples before and after Percoll separation in billions per milliliter.*
Data are presented as median values with the 25% and 75% percentiles (n=11).

Parameter	Median	Percentile 25%	Percentile 75%
Fresh	0.84	0.49	1.24
Fresh-separated	0.68	0.40	1.05
Fresh-separated/Fresh * 100%	80.95	78.84	84.16
Cryopreserved	0.43	0.33	0.86
Cryopreserved-separated	0.13	0.11	0.24
Cryopreserved-separated/Cryopreserved * 100%	28.00	28.00	31.00

CHAPTER 5

GENERAL DISCUSSION

Fish sperm cryopreservation is becoming more attractive due to the increasing number of potential applications and it has been considerably developed over the past decades. Cryopreservation of fish sperm is expected to improve the broodstock management in hatcheries, simplify hybridization and selective breeding, and serve as a useful tool for biodiversity and conservation of endangered species programs. Nevertheless, despite all these advantages, the application of cryotechniques still presents challenges, as it may strongly impair the quality and survival of sperm and thus decrease the fertilization capacity. Therefore, a better understanding of the mechanisms of spermatozoa cryo-injuries and the development of new and more effective methods for cryopreservation of fish sperm are of primary importance.

INVESTIGATION OF CAUSES OF INDIVIDUAL VARIABILITY IN COMMON CARP SPERM CRYORESISTANCE

In the first part of this dissertation (Chapter 2), we aimed to investigate the individual variability of sperm cryoresistance in common carp (*Cyprinus carpio*) especially regarding the lipid composition of the plasma membrane (Horokhovatskyi et al. 2016). It is well known that lipids are an important constituent of spermatozoa and are involved in different spermatozoan functions. During the cryopreservation process, spermatozoa are exposed to numerous physical and chemical factors and their resistance to any harmful influence depends largely on several membrane properties that are mainly determined by lipid composition. (Hara, 1978; Cabrita, et al., 2010). The lipid composition, especially the cholesterol/phospholipid ratio and free fatty acid composition, can influence several membrane properties such as permeability to water and other molecules, membrane fluidity and lipid phase transitions within membrane bilayer. Therefore, lipid composition is considered as one of the key factor which determines fish sperm cryoresistance.

Many studies have established a clear relationship between membrane lipid composition and survival success after cryopreservation (Cerolini et al., 2001; Martínez-Páramo et al., 2012). In mammals, it has been shown that lipids play a key role in the variety of cryoresistance among spermatozoa, showing the importance of some lipid classes in spermatozoa protection during cryopreservation (Parks and Lynch, 1992; White, 1993). For this reason, we examined the lipid composition of plasma membranes in fresh carp spermatozoa usnder different post-thaw motility levels. We found a negative correlation between changes in the contents of major lipid classes and sperm motility after cryopreservation. In particular, an increased level of phospholipid, cholesterol and free fatty acid in samples with low post-thaw motility percentage was observed. Previously, it was also reported that phospholipid, cholesterol and free fatty acid composition of sperm can vary not only between different animals, but also between different species, and even between individuals of the same species (Mandal et al., 2014).

Phospholipids are one of the most representative lipid fractions of the sperm cell membranes and they could be modified by cryopreservation, leading to changes in cellular homeostasis and to irregularities in sperm function (Hara, 1978). At the same time, many researchers have suggested that especially; ratio between cholesterol and phospholipid is negatively correlated with sperm motility, as at high levels it can influence membrane fluidity and thereby compromise restoration of membrane viability, osmotic regulation and ionic exchange processes involved in motility activation (Blesbois et al., 2005). A greater proportion of cholesterol is positively correlated with spermatozoa surviving in mammals (Moore et al.,

2005; Mocé and Graham 2006), while in rainbow trout spermatozoa, it has been shown that cholesterol stabilized the plasma membrane of fresh sperm, but did not improve spermatozoa cryo-resistance (Müller et al., 2008).

However, inconsistent with the past results, we found no significant differences in phospholipid composition or in cholesterol/phospholipid ratio in relation to post-thaw sperm motility showing that these components do not affect freezing ability of carp sperm. At the same time, we found higher levels in both saturated and mono-unsaturated fatty acids, especially with regard to oleic acid (C18:1n-9), in samples possessing low post-thaw motility. Similarly, higher level of oleic acid showed a negative correlation with cryoresistance in European sea bass sperm (Martínez-Páramo et al., 2012). In our study, a higher amount of polyunsaturated fatty acids was present in samples possessing high post-thaw motility, suggesting that higher levels of unsaturated FA are associated with higher levels of cryoresistance. Sperm possessing higher proportions of total n-6 polyunsaturated fatty acids had better cryoresistance, indicating that, in carp sperm, these fatty acids especially arachidonic acid (20:4 n-6), linoleic acid (18:2 n-6) and adrenic acid (22:4 n-6) might play an important role in determining the degree of sperm cryoresistance.

These differences show that individual variability of spermatozoa cryoresistance in common carp sperm samples can be related to the sperm lipid composition as it may have an influence on plasma membrane fluidity, lipid phase transition, and permeability to water and other molecules.

STANDARDIZATION OF STERLET (*ACIPENSER RUTHENUS*) SPERM CRYOPRESERVATION PROCEDURE IN UNCONTROLLED COOLING DEVICES

In Chapter 3, we explored whether the number or position of straws frozen by application of uncontrolled cooling devices in fish sperm cryobanking influenced the post-thaw sperm parameters, such as motility and fertilizing ability. Styrofoam box containing liquid nitrogen and equipped with a floating raft (which is designed to keep the set of freezing straws above the liquid nitrogen surface) and dry shipper into which straws are loaded directly in a liquid nitrogen-free cold interior space were selected as the most commonly used freezing device in fish cryobanking practice.

It is known that the principal cryoinjuries occur during freezing within a range of 0 °C and – 40 °C (Mazur, 2004). However, spermatozoa can also be damaged depending on freezing rate. According to the "Two factor hypothesis of freezing injury", there are different causes of injury during rapid freezing as well as during slow freezing (Mazur et al., 1972). It has been suggested that slow cooling results in osmotic effects caused by transient enormous concentrations of solutes, while rapid freezing leads to intracellular ice formation. However, the application of optimal freezing rates can minimize the influence of these potentially damaging factors (Zhao, 2006).

In our study the optimal freezing rate for sturgeon cryopreservation was determined according to the highest values of the post-thaw sperm motility parameters and fertilization capacity. It has been shown that the optimal freezing rate can be achieved by each uncontrolled cooling device under investigation. However, due to inability to precisely control the cooling process, the freezing rate can be changed by varying straw number (in the case of freezing in the Styrofoam box) or straw position (in the case of freezing inside the dry shipper). The percentage of motile spermatozoa, duration of sperm movement, VCL, VAP, LIN and BCF have been affected by cryopreservation in both devices. These parameters were highly dependent on applied freezing protocol showing a relationship between the applied freezing rates and post-thaw sperm quality parameters. The decrease in sperm motility parameters is a well-

known phenomenon, and our findings were consistent with those described previously in several fish species (Billard et al., 2004; Asturiano et al., 2006; Nynca et al., 2014). The highest motility parameters and fertilization success were achieved during freezing in a dry shipper with 60 straws inside and at the initial freezing rate of 53 °C /min. Due to more restricted interior space, the dry shipper provides more stable cooling conditions and, therefore, less variability in the post-thaw sperm parameters. Previously, Carolsfeld et al. (Carolsfeld et al., 2003) showed that an average freezing rate of 30 °C /min achieved in a dry shipper was also adequate to freeze fish spermatozoa. Similarly, Viveiros et al. (Viveiros et al., 2012) reported a successful cryopreservation of Brazilian fish at a freezing rate of approximately 35 °C/min between 21 °C and −170 °C. To our knowledge, our study is the first to apply the dry shipper for the cryopreservation of sterlet (Acipenser ruthenus) sperm and the freezing rate achieved in this uncontrolled cooling device, presumably, is in the optimal range for sturgeon spermatozoa.

Despite the different geometry and directionality of the nitrogen vapor convective stream, similar high post-thaw sperm parameters were obtained during freezing on a raft with 6 straws placed in the Styrofoam box containing liquid nitrogen. The initial freezing rate in this case was 86 °C /min, showing that ther is a wide range of optimal freezing rates for sturgeon spermatozoa. Meanwhile, a ten-fold increase of straw numbers on the raft, up to 60, led to a significantly lower initial freezing rate of 35 °C/min. This, probably, shifted the optimal freezing rates needed for successful sturgeon sperm cryopreservation resulting in a decrease in post-thaw sperm motility and fertilization ability. In contrast, a 40 °C/min freezing rate, has been shown suitable for beluga sturgeon (*Huso huso*) sperm cryopreservation (Aramli et al., 2015). Similar observation was reported by Frankel et al. (Frankel et al., 2013), when four different freezing rates were tested (10 °C, 15 °C, 20 °C, 40 °C/min) for striped bass (*Morone saxatilis*) sperm cryopreservation; they reported that the highest motility and fertility were observed at a freezing rate of −40 °C/min. In contrast, a 0% fertilization success at 10 °C/min freezing rate and 22% at 4 °C/min were observed in *Acippenser stellatus* sperm cryopreservation (Yamaner et al., 2015). All these observations suggest that the optimal freezing rate would be species-specific and must be determined for each species separately. However, future studies on the standardization of protocols for fish sperm cryopreservation in Styrofoam box (device with uncontrolled freezing rate) should take into consideration the effect of the straw number on the raft during the freezing process.

CRYOPRESERVATION EFFECT ON VIABLE STERLET (*ACIPENSER RUTHENUS*) SPERM SUBPOPULATION OBTAINED BY PERCOLL DENSITY GRADIENT

The Chapter 4 is relates investigation of cryopreservation effect on a subpopulation of sterlet (*Acipenser ruthenus*) spermatozoa that survived this process and retained suitable motility parameters.

In order to enhance the spermatozoa parameters of ejaculate possessing low sperm quality, a sperm subpopulation selection technique is required. A broad range of sperm selection techniques have been widely used in mamms to improve success in assisted reproductive technologies (Henkel and Schill, 2003; Matás et al., 2011; Gil et al., 2013), but few of these technologies have been uses in fish. We optimized and applied Percoll density gradient centrifugation method for the first time with sterlet (*Acipenser ruthenus*) sperm.

Many studies on sturgeon sperm cryopreservation have reported a significant decrease in parameters of frozen-thawed sperm compared to fresh ones (Ciereszko et al., 1996; Billard et al., 2004); our findings were consistent with the previous results. The most substantial decrease in sperm parameters after cryopreservation was observed in the curvilinear velocity

and straight-line velocity. However, an increased percentage of motile sperm cells with high motility parameters after application of Percoll gradient selection was observed in our study. Moreover, the motility percentage of cryopreserved and fresh sperm following the selection by Percoll was higher than the motility percentage recorded for fresh sperm. This indicates that application of the Percoll density gradient centrifugation leads to a selection of the spermatozoa population which presents the highest motility parameters over fresh sperm. Similarly, Percoll separation produced a fraction of spermatozoa which had significantly higher motility and curvilinear velocity in common carp sperm (Li et al., 2010a), bovine sperm (Parrish et al., 1995) and boar sperm (Matás et al., 2011).

The application of SYBR-14 and PI fluorescent dyes was successful to determine live/dead cell ratio in sturgeon sperm samples. The percentage of live cells was not strongly correlated with percentage of motile cells and always exceeded it. Similar observations were reported by Drobnis et al. (Drobnis et al., 1991) when the percentage of live cells were evaluated in human sperm; they showed that spermatozoa which had lost the ability for activation still had intact permeability barrier (cell membrane). Nevertheless, in our study the percentage of live sperm was one of the parameters showing the efficiency of the Percoll separation technique.

It is known, that the freezing-thawing process has a complex effect on spermatozoa. As a first option, it may alter the DNA integrity and protein profile so as to have a pernicious effect on sperm motility, fertilization ability, and even in embryonic development in their early stages after fertilization (Lessard et al., 2000; Zilli et al., 2003). In our study on sterlet sperm, significant changes in the abundance of 20 proteins were detected after cryopreservation. The proteins were L-lactate dehydrogenase A chain, phosphoglycerate kinase, creatine kinase B-type-like isoform X1, isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial which are related to various metabolic and energy production processes in spermatozoa. Furthermore, some of the proteins such as tubulin alpha chain and tubulin beta-4B chain are part of cytoskeleton and are involved in spermatozoon motility initiation and maintenance (Dietrich et al., 2016).

For example, in data published by Li et al. (Li et al., 2010b) on common carp sperm, cryopreservation significantly altered fourteen proteins, while Nynca et al. (Nynca et al., 2015) showed that 73 proteins were altered in rainbow trout semen after cryopreservation. Most of the proteins were mitochondrial, cytoskeletal, nuclear, and cytosolic which have a relation to sperm structure and function, and metabolism of sperm cells. Such alteration in sperm protein profiles after cryopreservation may be due to either leakage of proteins from the inside of spermatozoa to the extracellular medium or through their modification, leading to a decrease or an increase of their abundance respectively (Zilli et al., 2005; Ardon and Suarez, 2013).

Nevertheless, many previously published results have not been clear, as they are based on analysis of frozen/thawed sperm suspension that contained not only the fraction of interest in viable spermatozoa, but also non-viable and cryodamaged spermatozoa which, actually are not involved into the fertilization process. The analysis of such suspensions could obscure real cryopreservation effects on survived spermatozoa subpopulation showing suitable motility parameters and involved into the fertilization process. In fish, no information is available concerning the protein profile changes in spermatozoa that survive the cryopreservation process. To investigate this, the analysis of only viable sperm population selected by Percoll gradient centrifugation was performed in our study. As a result, we found that only one protein was significantly altered after the cryopreservation procedure. This protein was tubulin alpha-8 chain-like and is linked to cytoskeletal proteins and is involved in sperm motility initiation and maintenance (Dietrich et al., 2016). These results show a much lower number of changes in proteins for viable spermatozoa after the cryopreservation process. Thus, suggesting that

frozen-thawed and viable sperm populations are more cryoresistant than the population that underwent pernicious changes in the proteins.

CONCLUSIONS

This current PhD thesis contributes to further improvements in fish sperm cryopreservation by following conclusions:

1. The variability in sperm cryoresistance of common carp is related to differences in lipid composition of fresh sperm.
2. The optimal freezing rate for sturgeon sperm cryopresrvation can be achieved in either commonly used uncontrolled cooling devices: Styrofoam box containing liquid nitrogen and equipped with a floating raft, and dry shippers. However, due to inability to precisely control cooling process, the freezing parameters can be changed by varying straw number (in the case of freezing in the Styrofoam box) or straw position (in the case of freezing inside the dry shipper). As a consequence, these changes lead to less-than-optimal freezing rates and as a result decrease post-thaw sperm motility and fertilization ability.
3. The alterations of spermatozoa proteome and low motility, and viability parameters in frozen-thawed sperm suspension is related to the presence of non-viable and sperm wreckage spermatozoa subpopulation. Application of Percoll density gradient centrifugation permits the selection of spermatozoa subpopulations which have survived cryopreservation and retained high motility and viability parameters together with minimal changes in proteome.

REFERENCES

Aramli, M. S., Golshahi, K., Nazari, R. M., Aramli, S. 2015. Effect of freezing rate on motility, adenosine triphosphate content and fertilizability in beluga sturgeon (*Huso huso*) spermatozoa. Cryobiology 70(2):170-174.

Ardon, F., Suarez, S. S. 2013. Cryopreservation increases coating of bull sperm by seminal plasma binder of sperm proteins BSP1, BSP3, and BSP5. Reproduction 146:111-117.

Asturiano, J. F., Marco-Jimenez, F., Penaranda, D. S., Garzon, D. L., Perez, L., Vicente, J. S., Jover, M. 2006. Effect of sperm cryopreservation on the European eel sperm viability and spermatozoa morphology. Reproduction in Domestic Animals 42(2):162-166.

Billard, R., Cosson, J., Noveiri, S. B., Pourkazemi, M. 2004. Cryopreservation and short-term storage of sturgeon sperm, a review. Aquaculture 236(1–4):1-9.

Blesbois, E., Grasseau, I., Seigneurin, F. 2005. Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. Reproduction 129(3):371-378.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M. P. 2010. Cryopreservation of fish sperm: applications and perspectives. Journal of Applied Ichthyology 26(5):623-635.

Carolsfeld, J., Godinho, H. P., Filho, E. Z., Harvey, B. J. 2003. Cryopreservation of sperm in Brazilian migratory fish conservation. Journal of Fish Biology 63(2):472-489.

Cerolini, S., Maldjian, A., Pizzi, F., Gliozzi, T. 2001. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction 121(3):395-401.

Ciereszko, A., Toth, G. P., Christ, S. A., Dabrowski, K. 1996. Effect of cryopreservation and theophylline on motility characteristics of lake sturgeon (*Acipenser fulvescens*) spermatozoa. Theriogenology 45(3):665-672.

Dietrich, M. A., Dietrich, G. J., Mostek, A., Ciereszko, A. 2016. Motility of carp spermatozoa is associated with profound changes in the sperm proteome. Journal of Proteomics 138:124-135.

Drobnis, E. Z., Zhong, C. Q., Overstreet, J. W. 1991. Separation of Cryopreserved Human Semen Using Sephadex Columns, Washing, or Percoll Gradients. Journal of Andrology 12(3):201-208.

Frankel, T. E., Theisen, D. D., Guthrie, H. D., Welch, G. R., Woods, L. C. 2013. The effect of freezing rate on the quality of striped bass sperm. Theriogenology 79(6):940-945.

Gang Zhao, D. L., Dayong Gao. 2006. Universal Model for Intracellular Ice Formation and Its Growth. AIChE Journal 52(7):2596-2606

Gil, M., Sar-Shalom, V., Melendez Sivira, Y., Carreras, R., Checa, M. A. 2013. Sperm selection using magnetic activated cell sorting (MACS) in assisted reproduction: a systematic review and meta-analysis. Journal of Assisted Reproduction and Genetics 30(4):479-485.

Hara, A., Radin N. S. 1978. Lipid Extraction of Tissues with a Low-Toxicity Solvent. Analytical Biochemistry 90(1):420-426

Henkel, R., Schill, W. B. 2003. Sperm preparation for ART. Reproductive biology and endocrinology 1:108-108.

Holt, W. V. 2000. Basic aspects of frozen storage of semen. Animal Reproduction Science 62(1):3-22.

Horokhovatskyi, Y., Sampels, S., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M., Dzyuba, B. 2016. Lipid composition in common carp (*Cyprinus carpio*) sperm possessing different cryoresistance. Cryobiology 73(2):282-285.

Lessard, C., Parent, S., Leclerc, P., Bailey, J., Sullivan, R. 2000. Cryopreservation alters the levels of the bull sperm surface protein P25b. Journal of Andrology 21:700-707.

Li, P., Dzyuba, B., Hulak, M., Rodina, M., Boryshpolets, S., Li, Z. H., Linhart, O. 2010a. Percoll gradient separation of cryopreserved common carp spermatozoa to obtain a fraction with higher motility, velocity and membrane integrity. Theriogenology 74(8):1356-1361.

Li, P., Hulak, M., Koubek, P., Sulc, M., Dzyuba, B., Boryshpolets, S., Rodina, M., Gela, D., Manaskova-Postlerova, P., Peknicova, J., Linhart, O. 2010b. Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, *Cyprinus carpio*. Theriogenology 74:413-423.

Mandal, R., Badyakar, D., Chakrabarty, J. 2014. Role of Membrane Lipid Fatty Acids in Sperm Cryopreservation. Advances in Andrology 2014:9.

Martínez-Páramo, S., Diogo, P., Dinis, M. T., Herráez, M. P., Sarasquete, C., Cabrita, E. 2012. Sea bass sperm freezability is influenced by motility variables and membrane lipid composition but not by membrane integrity and lipid peroxidation. Animal Reproduction Science 131(3–4):211-218.

Matás, C., Vieira, L., García-Vázquez, F. A., Avilés-López, K., López-Úbeda, R., Carvajal, J. A., Gadea, J. 2011. Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. Animal Reproduction Science 127(1):62-72.

Mazur, P. 2004. Principles of Cryobiology. In: Fuller, B. J., Lane, N., Benson, E. E. (Eds.), Life in the frozen state, Press LLC, pp. 3-67.

Mazur, P., Leibo, S. P., Chu, E. H. Y. 1972. A two-factor hypothesis of freezing injury: Evidence from Chinese hamster tissue-culture cells. Experimental Cell Research 71(2):345-355.

Mocé, E., Graham, J. K. 2006. Cholesterol-loaded cyclodextrins added to fresh bull ejaculates improve sperm cryosurvival. Journal of Animal Science 84(4):826-833.

Moore, A. I., Squires, E. L., Graham, J. K. 2005. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology 51(3):241-249.

Müller, K., Müller, P., Pincemy, G., Kurz, A., Labbe, C. 2008. Characterization of Sperm Plasma Membrane Properties after Cholesterol Modification: Consequences for Cryopreservation of Rainbow Trout Spermatozoa1. Biology of reproduction 78(3):390-399.

Nynca, J., Arnold Georg, J., Fröhlich, T., Ciereszko, A. 2015. Cryopreservation-induced alterations in protein composition of rainbow trout semen. PROTEOMICS 15(15):2643-2654.

Nynca, J., Dietrich, G. J., Dobosz, S., Grudniewska, J., Ciereszko, A. 2014. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. Aquaculture 433:62-65.

Parks, J. E., Lynch, D. V. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology 29(2):255-266.

Parrish, J. J., Krogenaes, A., Susko-Parrish, J. L. 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology 44(6):859-869.

Viveiros, A. T. M., Isaú, Z. A., Caneppele, D., Leal, M. C. 2012. Sperm cryopreservation affects postthaw motility, but not embryogenesis or larval growth in the Brazilian fish Brycon insignis (Characiformes). Theriogenology 78(4):803-810.

White, I. G. 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reproduction Fertility and Development 5(6):639-658.

Yamaner, G., Ekici, A., Tuncelli, G., Memis, D. 2015. A brief overview on cryopreservation method of sturgeon sperm. Journal of Fisheries & Aquatic Science 30(2):14-20.

Zilli, L., Schiavone, R., Zonno, V., Rossano, R., Storelli, C., Vilella, S. 2005. Effect of Cryopreservation on Sea Bass Sperm Proteins. Biology of reproduction 72:1262-1267.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., Vilella, S. 2003. Evaluation of DNA damage in Dicentrarchus labrax sperm following cryopreservation. Cryobiology 47(3):227-235.

ENGLISH SUMMARY

Applied aspects of fish sperm cryopreservation

Yevhen Horokhovatskyi

Applications of fish sperm cryopreservation has received noticeable attention due to an increasing number of potential applications and it has been considerably developed over the past decades. Nevertheless, its current status is highly contentious due to absence of specific techniques and standardization in the developed protocols. Moreover, the heterogeneity of cryoresistance in sperm of some fish individuals and strong impairment of the quality of sperm and survival after cryopreservation has presented some challenges and has inhibited wide applicability of this approach. In the current work, the causes of individual variability in sperm cryoresistance and influence of cryopreservation process on sperm quality parameters regarding application of uncontrolled cooling devices were investigated.

Chapter 2 is related to an investigation on the individual variability of sperm cryoresistance in common carp (*Cyprinus carpio*) males. The objective was to investigate lipid content of fresh sperm which had different post-thaw motility percentage. As a result, a negative correlation between changes in the contents of major lipid classes and sperm motility after cryopreservation was found. In particular, the increased level of phospholipid, cholesterol and free fatty acid in samples with low post-thaw motility percentage were observed and related to a decrease in sperm cryoresistance in common carp sperm.

Chapter 3 focused on an investigation of the consequence of application of uncontrolled cooling devices on post-thaw motility and fertilizing ability of sterlet sperm. The Styrofoam box and dry shipper were selected as the most commonly used uncontrolled cooling devices in fish sperm cryobanking. The results of our study demonstrated that sterlet sperm could be cryopreserved using both devices studied. However, with these devices no precisel control of cooling is possible, but the cooling conditions can be affected by varying straw number during freezing in Styrofoam box or by straw positioning during freezing in dry shipper. As a consequence, these manipulations can change the freezing rates needed for successful sturgeon sperm cryopreservation resulting in decrease in post-thaw sperm motility and fertilization ability. These results suggest that by selecting appropriate straw numbers or by arranging the position of the straws provides some increased standardization of protocol when uncontrolled cooling devices are used.

To explore the cryopreservation effects on fish spermatozoa, the Percoll density gradient centrifugation technique was applided for the first time to sterlet (*Acipenser ruthenus*) sperm (Chapter 4). This technique allows the selection and evaluation of only the sperm population that survives cryopreservation and thus retained high motility parameters. It was found that this spermatozoa subpopulation possesses minimal changes in protein profile in comparison to native sperm. Moreover, the differences in many proteins which were found in sperm suspension after cryopreservation without separation is related to the presence of non-viable and sperm wreckage subpopulations in the frozen-thawed sperm suspension. Collectively, all of the detected changes in sperm motility parameters, viability, and protein profiles of viable spermatozoa after the freeze-thaw process suggests some explanations for the mechanism responsible for cryodamage of sperm and provides the background for further development of an efficient cryopreservation protocols. However, it should be also further studied if the Percoll separation technique could serve as a useful tool for both better understanding of non-lethal sperm cryodamages and improvement of assisted reproduction practice in sturgeon species.

CZECH SUMMARY

Aplikované aspekty kryokonzervace rybích spermií

Yevhen Horokhovatskyi

Aplikování kryokonzervace spermatu ryb získalo značnou pozornost kvůli rostoucímu počtu potenciálních aplikací a v několika posledních desetiletích se značně rozvinulo. Nicméně status kryokonzervace spermatu je značně sporný kvůli absenci specifických technik a standardizaci rozvinutých protokolů. Kromě toho heterogenita kryorezistence spermií u některých individuálních ryb a silné narušení kvality spermatu a přežití po kryokonzervaci představuje určitou výzvu a brání širší aplikovatelnosti tohoto přístupu. V této práci byly zkoumány příčiny individuální variability kryorezistence spermatu, stejně tak byl zkoumán vliv procesu kryokonzervace na parametry kvality spermatu v souvislosti s využíváním nekontrolovaných chladicích zařízení, tj. chladicích přístrojů, které nejsou schopny kontrolovat rychlost zmrazování.

Kapitola 2 této práce je spojena se zkoumáním individuální variability kryorezistence u mlíčáků kapra obecného (*Cyprinus carpio*). Cílem této studie bylo zjistit obsah lipidů čerstvého spermatu, které má různé procento motility po rozmrazení. Výsledkem toho byla nalezena negativní korelace mezi změnami v obsahu hlavních lipidových skupin a motility spermatu po kryokonzervaci. Zvláště byla pozorována zvýšená hladina fosfolipidů, cholesterolu a volných mastných kyselin u vzorků s nízkým procentem motility po rozmražení spojená se sníženou kryorezistencí u spermatu kapra obecného.

Kapitola 3 je zaměřená na zkoumání vlivu důsledků aplikace nekontrolovaného chladicího zařízení na motilitu a fertilitu po následném rozmražení u jesetera malého (*Acipenser ruthenus*). Jakožto nejběžněji používané nekontrolované chladicí vybavení rybích kryobank byla vybrána polystyrenová krabice a suchý přepravní kontejner. Výsledky naší studie ukazují, že sperma jesetera malého může být zmraženo oběma zkoumanými pomůckami. Nicméně kvůli nemožnosti přesně řídit proces ochlazování v těchto dvou zařízeních mohou být chladicí podmínky změněny rozdílným množstvím pejet v polystyrenové krabici během mrazení nebo pozicí pejety v suchém přepravním kontejneru. Jako důsledek tyto změny posunují optimální rychlost mrazení potřebnou pro úspěšnou kryokonzervaci spermatu jesetera malého, což vede k poklesu motility spermatu a schopnosti oplození po následném rozmražení. Tyto výsledky naznačují způsob standardizace protokolů kryokonzervace rybího spermatu pomocí volby vhodného množství či pozice pejet, pokud jsou využívány nekontrolovaná chladicí zařízení.

Za účelem zjištění účinků kryokonzervace na rybí sperma byla rozpracována technika centrifugace s hustotním gradientem za použití Percollu a poprvé aplikována u spermatu jesetera malého (*Acipenser ruthenus*) (Kapitola 4). Tato technika umožňuje vybírat a používat pouze populaci spermatu, která přežila proceduru kryokonzervace spermatu a zároveň zachovává vysoké parametry motility. Bylo zjištěno, že tato subpopulace spermatu vykazuje minimální změny proteinového profilu ve srovnání s čerstvým spermatem. Navíc, rozdíly v mnoha proteinech, které byly nalezeny v suspenzi spermií po zmražení bez separace, jsou spojené s přítomností subpopulací nežádoucích neživotaschopných spermií ve zmražené-rozmražené suspenzi spermatu. Souhrnně, všechny tyto detekované změny v parametrech motility spermatu, jeho životaschopnosti a proteinového složení životaschopného spermatu po zmražení a rozmražení naznačují některé vysvětlení pro mechanizmy odpovědné za kryo poškození spermatu a poskytují pozadí pro další vývoj účinných protokolů kryokonzervace. Nicméně by mělo být dále zkoumáno, jestli by separační technika pomocí Percollu mohla sloužit jakožto užitečný nástroj pro lepší porozumění neletálnímu kryo poškození a zlepšení praktiky asistované reprodukce u jeseterů.

ACKNOWLEDGEMENTS

I dedicate this thesis to my mother, who has always been understanding and supporting me in all the various life situations. I would like to express my deepest and sincere gratitude for her love, concern, and patience throughout my life.

It is my pleasure to acknowledge all people who have given me help, guidance, and encouragement during my doctoral study.

I would like to express my deep appreciation to my supervisor Borys Dzuyba for his continuous support, patience, insightful comments, constructive criticism, and kindness. His guidance helped me in all the time of research, writing of articles, and other fields of scientific life. Besides my supervisor, I would like to thank all colleagues from the Laboratory of Reproductive Physiology and other laboratories of the Faculty of Fisheries and Protection of Waters, who helped me with my experiments and laboratory work. Especially, I would like to thank Marek Rodina, for his valuable support and providing all the necessary facilities for my experiments.

Special thanks go to Jacky Cosson for his support in all questions, valuable and stimulating discussion, and his great sense of humor.

My sincere thanks also go to Sabine Sampels who helped me to start my progress at the beginning of my doctoral study and I am so grateful for her precious support and kindness which she shared with me working together.

I would like to sincerely acknowledge Andrzej Ciereszko and all his team, especially Mariola Dietrich, for their comprehensive assistance and kindness during foreign internship to Poland. I also would like to express my deep appreciation to Jane M Morrell for her support in all endeavors and comprehensive assistance in the work during my stay in her laboratory in Sweden. My sincere thanks also go to Catherine Labbe and her research team for their precious support and kindness during my foreign internship in France.

I express my thanks to all managers of the Faculty of Fisheries and Protection of Waters for their kindness and help. I am grateful to Markéta Flajšhansová, Václav Klimeš and Willem Westra for their great contribution to my language skills improvement and simplification of communication with other people and scientific world.

The research was supported by the following financial sources:

- Aquaculture infrastructures for excellence in European fish research towards 2020, a Horizon 2020 research infrastructure project - "AQUAEXCEL 2020" (No.652831);
- Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program);
- Reproductive and genetic procedures for preserving fish biodiversity and aquaculture (C Z.02.1.01./0.0/0.0/16_025/0007370);
- Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 059/2016/Z);
- Czech Science Foundation (No.: GACR 16-02407Y);
- COST (No. LD14119);
- COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE).

LIST OF PUBLICATIONS

Peer-reviewed journals with impact factor

Horokhovatskyi, Y., Dietrich, M.A., Lebeda, I., Fedorov, P., Rodina, M., Dzyuba, B., 2018. Cryopreservation effects on a viable sperm sterlet (*Acipenser ruthenus*) subpopulation obtained by a Percoll density gradient method. PLOS ONE. 10.1371/journal.pone.0202514. (accepted) (IF 2017 = 2.766)

Horokhovatskyi, **Y.,** Rodina, M., Dadras Asyabar, H., Boryshpolets, S., Dzyuba, B., 2017. Consequences of uncontrolled cooling during sterlet (*Acipenser ruthenus*) sperm cryopreservation on post-thaw motility and fertilizing ability. Theriogenology 95, 89–95. (IF 2016 = 1.986)

Horokhovatskyi, **Y.**, Sampels, S., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M., Dzyuba, B., 2016. Lipid composition in common carp (*Cyprinus carpio*) sperm possessing different cryoresistance. Cryobiology 73, 282–285. (IF 2015 = 1.920)

Book chapters

Shigaeva, M.I., Talanov, E.Y., Venediktova, N.I., **Horokhovatskyi, Y.A.**, Honcharenko, M.S., Murzaeva, S.V., Mironova, G.D., 2014. The role of the calreticuline in mitochondrial ATP – dependent potassium transport. Biological motility: new facts and hypotheses, Institute of Theoretical and Experimental Biophysics RAS, Pushichino, Russia, pp. 260–264.

Abstracts and conference proceedings

Horokhovatskyi, Y., Dietrich, M.A., Lebeda, I., Fedorov, P., Rodina, M., Dzyuba, B., 2018. Cryopreservation effect on sterlet sperm viability and protein content after Percoll separation. "CRYO2018: Scientific Challenges of Cryobiology", July 10–13, 2018, Madrid, Spain.

Dadras, H., Boryshpolets, S., Cosson, J., **Horokhovatskyi, Y.,** Dzyuba B., 2017. A preliminary study of temperature effect on fertilization success in sterlet, *Acipenser ruthenus.* In Book of abstracts "*Aquaculture Europe 2017*", October 17–20, 2017, Dubrovnik, Croatia, p. 253.

Horokhovatskyi, Y., Dietrich, M. A., Lebeda, I., Boryshpolets, S., Fedorov, P., Rodina, M., Dzyuba, B., 2017. Cryopreservation effect on sterlet sperm viability and protein content after live/dead cells separation by Percoll density gradient. In Book of abstracts "6th International Workshop on Biology of Fish Gametes", September 4–7, 2017, Ceske Budejovice, Czech Republic, p. 84.

Horokhovatskyi, Y., Sampels, S., Dzyuba, B., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M., 2016. Heterogeneity of cryoresistance in common carp sperm. In Book of abstracts "10th Biennial Conference of the Association for Applied Animal Andrology", June 24–26, 2016, Tours, France; Animal Reproduction Science 169, 114–115

Bondarenko, O., Yamaner, G., Repina, S., Cherkashina, Y., Nardid, O., **Horokhovatskyi, Y.,** Dzyuba, B., 2015. Fish sperm storage before freezing: the effect on ability for volume changes and cryoresistance. In Book of abstracts "CRYO 2015", July 26–29, 2015, Ostrava, Czech Republic; Cryobiology 71, 564.

Horokhovatskyi, Y., Dzyuba, B., Rodina, M., Cosson, J., Linhart, O., 2015. Investigation of variability of cooling profiles in two freezing methods applied to Sterlet (*Acipenser ruthenus*) sperm cryopreservation. In Book of abstracts "5th International Workshop on Biology of Fish Gametes", September 7–11, 2015, Ancona, Italy, p. 94–95.

Horokhovatskyi, Y., Samples, S., Dzyuba, B., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M., 2015. Cryoresistance in relation with lipid composition in sperm of common carp *Cyprinus carpio*. In Book of abstracts "CRYO 2015", 26–29 July, 2015, Ostrava, Czech Republic; Cryobiology 71, 547.

TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Yevhen Horokhovatskyi
Research department	2014–2018 – Laboratory of Reproductive Physiology of FFPW
Supervisor	Assoc. Prof. Borys Dzyuba
Period	30th September 2014 until 30th September 2018

Ph.D. courses	**Year**
Pond aquaculture	2015
Applied hydrobiology	2015
Fish reproduction	2016
Basic of scientific communication	2016
Ichthyology and fish taxonomy	2016
English language	2017

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2015 2016 2017 2018

International conferences	**Year**
CRYO2015 – the 52nd Annual Meeting of the Society for Cryobiology, July 26–29, Ostrava, Czech Republic. **Horokhovatskyi, Y.**, Samples, S., Dzyuba, B., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M., 2015. Cryoresistance in relation with lipid composition in sperm of common carp *Cyprinus carpio*. Cryobiology 71, 547.	2015
The 5th International Workshop on Biology of Fish Gametes, September 7–11, Ancona, Italy. **Horokhovatskyi, Y.**, Dzyuba, B., Rodina, M., Cosson, J., Linhart, O. Investigation of variability of cooling profiles in two freezing methods applied to Sterlet (*Acipenser Ruthenus*) sperm cryopreservation, p. 94–95.	2015
The 10th Biennial Conference of the Association for Applied Animal Andrology, June 24–26, Tours, France. **Horokhovatskyi, Y.**, Sampels, S., Dzyuba, B., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M. Heterogeneity of cryoresistance in common carp sperm. Animal Reproduction Science 169, 114–115.	2016
The 6th International Workshop on the Biology of Fish Gametes, Ceske Budejovice, Czech Republic. **Horokhovatskyi, Y.**, Dietrich, M. A., Lebeda, I., Boryshpolets, S., Fedorov, P., Rodina, M., Dzyuba, B. Cryopreservation effect on sterlet sperm viability and protein content after live/dead cells separation by Percoll density gradient, p. 84.	2017
CRYO2018 - the 55th Annual Meeting of the Society for Cryobiology, July 10 - 13, Madrid, Spain **Horokhovatskyi, Y.**, Dietrich, M. A., Lebeda, I., Fedorov, P., Rodina, M., Dzyuba, B. Cryopreservation effect on sterlet sperm viability and protein content after Percoll separation. p.60.	2018

Foreign stays during Ph.D. study at RIFCH and FFPW	Year
[1 month] Prof. Andrzej Ciereszko, Investigation of protein content in sturgeon (*Acipenser ruthenus*) sperm after cryopreservation applying Percoll density gradient separation. Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland,	2016
[1 month] Prof. Jane M Morrell, Elaboration of a single layer colloid centrifugation for the selection of high quality sturgeon (*Acipenser ruthenus*) sperm fraction after cryopreservation. Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden	2016
[2 months] Prof. Catherine Labbe, Restoration of European sea sturgeon (*Acipenser sturio*) population and assessment of gamete quality after cryopreservation. Fish Physiology and Genomics Institute, Rennes, France	2017

CURRICULUM VITAE

PERSONAL INFORMATION

Name: Yevhen
Surname: Horokhovatskyi
Title: M.Sc.
Born: 13th October 1992, Lozova, Ukraine
Nationality: Ukrainian
Languages: English, Ukrainian, Russian, Czech
Contact: horokhovatskyi@gmail.com

EDUCATION

2014–2018	Ph.D. student, Laboratory of Reproductive Physiology, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic
2013–2014	M.Sc., Faculty of Radiophisics, Department of Biological and Medical Physics, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine (Biophysicist, Lecturer)
2010–2013	B.Sc., Faculty of Radiophisics, Department of Biological and Medical Physics, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine (Applied Physics, Medical Biophysics)
1999–2009	Gymnasium №1, Vovchansk, Kharkiv region, Ukraine

PH.D. COURSES

Pond aquaculture, Applied hydrobiology, Fish reproduction, Basic of scientific communication, Ichthyology and fish taxonomy, Czech language, English language

LEADING OF PROJECTS

2016–2017	Grant Agency of the University of South Bohemia GAJU 059/2016/Z. Fish sperm cryoresistance heterogeneity: influence of freezing rates and sperm lipid composition

COMPLETED COURSES AND TRAINING

26/09–30/09 2016	Oral presentation and training - The 7th Training School of the COST Action FA1205: AQUAGAMETE, Fish proteomics, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland
29/02–04/03 2016	Basics of scientific comunicatiion, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Vodnany, Czech Republic
13/01–15/01 2015	Oral presentation and training – The 3rd Training School of the COST Action FA1205: AQUAGAMETE, Techniques in Fish Reproductive Biology and Cryobanking, Centre of Marine Sciences of Algarve (CCMAR), University of Algarve, Faro, Portugal

2014–2018	Research member of the Aquaculture infrastructures for excellence in European fish research towards 2020, a Horizon 2020 research infrastructure project – "AQUAEXCEL 2020" (No. 652831)
06–07/2018	International summer school: Repeated cryopreservation of sturgeon sperm after Percoll density gradient separation

RESEARCH STAY AND COLLABORATIONS

06/03–05/05 2017	Prof. Catherine Labbe, Restoration of European sea sturgeon (Acipenser sturio) population and assessment of gamete quality after cryopreservation. Fish Physiology and Genomics Institute, Rennes, France
31/10–29/11 2016	Prof. Jane M Morrell, Elaboration of a single layer colloid centrifugation for the selection of high quality sturgeon (Acipenser ruthenus) sperm fraction after cryopreservation. Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden
01/04–30/04 2016	Prof. Andrzej Ciereszko, Investigation of protein content in sturgeon (Acipenser ruthenus) sperm after cryopreservation applying Percoll density gradient separation. Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland