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Diploma thesis

INFLUENCE OF SPERM DENSITY ON OUTCOMES OF CRYOPRESERVATION IN CARP

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Zásady pro vypracování

Application of low-temperature sperm storage (cryopreservation) is considered nowadays as a powerful tool in agriculture, medicine and conservation biology. This methodological approach is aimed to preserve gene pool of unique populations protecting them from loss of genetic variability and extinction. However, in aquaculture practice it is not widely used because of lack of cheap and standardized protocols.

Common carp Cyprinus Carpio is a species which is the main object of Czech pisciculture, and at the same time the diversity of common carp breeds is a national heritage. That is why common carp sperm cryobanking is already an actual component of National Programme on Conservation of Farm Animal Genetic Resources and future development of its application can be beneficial for aquaculture.

As during cryopreservation spermatozoa are localized in small volumes of vitrified parts of samples, sperm density (concentration of spermatozoa) is a crucial parameter for success of cryopreservation. For carps, the value of appropriate for cryopreservation sperm concentration was not precisely determined and this parameter should be defined for establishment of fisheries practise oriented standardized protocol. This master study project is aimed to determine the optimal concentration of spermatozoa during cryopreservation of common carp sperm. During the realization of the project the review of current knowledge related to the topic will be done as a theoretical part of the study. The specific objectives for the experimental part are: 1) elaboration of quick method of sperm concentration estimation in common carp; 2) determination of sperm concentration range, which is optimal for sperm cryopreservation in common carp.

Methodologically the study will be realized via application of methods: 1) photometry and microscopy for determination of cell concentration in suspensions, 2) video microscopy for determination of sperm motility parameters, 3) sperm cryopreservation by device without cooling rate control, 4) data analysis.

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

Jamieson B.G.M. (1991) Fish evolution and systematics: evidence from spermatozoa. Cambridge University Press.

Holt W.V., Penfold L.M. Fundamental and practical aspects of semen cryopreservation. In Animal Andrology: theories and applications. Edited by Peter J. Chenoweth, Steven P. Lorton. 2014, pp 76-99.

Tiersch T.R., Green C.C. (Eds), (2011) Cryopreservation in aquatic species. 2nd Edition. World aquaculture society. Baton Rouge, Louisiana, USA.
Watson P., Holt W.V. (Eds), (2001) Cryobanking the genetic resource: wildlife conservation for the future. Taylor&Francis, London, New York, USA.
Morris J., Murray A, Fonseca, B. (2012) Freezing injury: The special case of the sperm cell. Cryobiology 64, 71-80.
Judycka, S., Nynca, J., Liszewska, E., Dobosz, S., Grudniewska, J., & Ciereszko, A. (2018) Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa. Aquaculture, 486, 90-97.

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Declaration

I declare that I am the author of this qualification thesis and that I have prepared it using only the sources and literature listed in the list of references used.

03.05.2024 ……

Taisiya Stechkina

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Content

1. Introduction

The main object of the present study is common carp (*Cyprinus carpio*), which presents the most widely cultured cyprinid fish (Woynarovich et al., 2011) and the primary subject of Czech pisciculture (FAO 2024). Carp has a long-standing history of breeding in the Czech Republic, resulting in many unique local strains (Flajšhans et al., 1999; Kocour et al., 2008). Some old, less productive breeds are conserved in situ as a part of national heritage and a source of genes for future breeding as a part of The Czech National Programme for the conservation of genetic resources for commercially important fish species (Flajšhans et al., 2015). However, in addition to in situ approaches, sperm cryobanking is also included in this Programme, and today cryobank in the Research Institute of Fish Culture and Hydrobiology (Faculty of Fisheries and Protection of Waters in Vodnany) includes sperm samples from 11 common carp breeds (Flajšhans et al., 2015).

Cryopreservation today represents the only possible procedure to conserve cells' functional properties for the long term (Jamieson, 1991) wherein the most commonly used method of cryopreservation in fish is slow freezing (Katkov et al., 2012) and sperm remains the main object of this research (Martínez-Páramo et al., 2017). A lot of studies were focused on cyprinids, particularly on common carp, which led to quite well-developed cryopreservation techniques (Martínez-Páramo et al., 2017). One of the critical parameters for the study is the dilution ratio of sperm prior to freezing, which often differs among methods (according to Bernath et al., 2016 applied values varied from 1:1 to 1:9 sperm per cryoprotective medium respectively). Manipulations with this parameter can lead to high heterogeneity of cryopreservation results among different research and make it impossible to compare different studied approaches (Kime et al., 2001). Dilution with a cryoprotective medium modifies the sperm density (also known as "sperm concentration", and these terms can be used interchangeably) in samples, which represents one of the parameters that directly influences sperm survival due to the packing effect that arises during the freezing process (Morris et al., 2012). Different dilution rates also affect the economic side of the cryopreservation method through the increase or reduction in cryostorage space and required chemicals.

Despite the critical role of sperm density during cryopreservation, in many studies, this parameter is ignored (Tan et al., 2010; Pataki et al., 2022). The sperm concentration

optimum for cryopreservation varies among different species. According to Nascimento et al. (2021), the dependency between sperm concentration in the samples subjected to freeze-thawing and cryopreservation outcomes in fish was studied in several species of Salmonidae, Esocidae, Percidae, and Cyprinidae. The aforementioned publication studied the optimum concentration during cryopreservation for sterlet. For common carp, an optimal value of the sperm concentration during freezing remains undetermined. Sperm concentration is also relevant during artificial fish reproduction and can differ concerning the purpose of the fertilization trial. With the growing justifiable interest in this parameter, an elaboration of a reliable tool for its assessment is needed. Traditionally sperm concentration estimation in fish is performed with a counting chamber or haemocytometer (Rurangwa et al., 2004; Tan et al., 2010; Yang et al., 2015). However, due to the time consuming as a main disadvantage of this method, the elaboration of other methods is of high demand for practice (Tan et al., 2010; Judycka et al., 2019a; Pataki et al., 2022). Measurement of sperm suspension absorbance for determination of sperm concentration is one of the approaches already used for different fish species (Ciereszko and Dabrowski, 1993; Tan et al., 2010; Leclercq et al., 2012).

The current study is aimed to understand the importance of sperm concentration during cryopreservation and to elaborate a quick and reliable method for sperm concentration determination in common carp.

The objectives of this diploma thesis are:

1) establishing a regression between light absorbance at different wavelengths of sperm suspension and sperm concentration counted by a haemocytometer;
2) determination of the optimal concentration range for sperm cryopreservation in common carp in terms of post-thaw sperm motility;
3) specifying sperm per egg ratio during artificial propagation in common carp for improvement of the development and hatching rates of common carp progeny obtained with cryopreserved sperm.

2. Literature review

2.1. Common carp

It is assumed that common carp as a species appeared in the region of the present Black, Caspian, and Aral seas. Under the conditions of the postglacial thermal optimum, the species spread westward to the Danube basin and eastward to continental Asia (Balon, 1995). Nowadays it has been introduced practically everywhere outside its native geographical and climatic range. Carp has a different status in the world. In non-native territories, especially in North America and Australia, carp is considered an undesirable invasive species due to its wide distribution and severe impacts in shallow lakes and wetlands (Fanson et al., 2024).

On the other side common carp is the most widely cultured cyprinid species (Woynarovich et al., 2011). During the thousand-year history of domestication carp farming has evolved from a haphazard polyculture of carp through improved rearing methods to artificial reproduction with deep knowledge of genetics. According to FAO statistical data, carp production increased until the year 2021 and now indicates that common carp global production may have come close to its limit.

In the Czech Republic, carp breeding takes a special role. It is the dominant fish produced (88 percent). The Czech Republic is the largest exporter of carp in Europe although the role of fisheries in the Czech national economy is rather marginal. Annual fish production currently ranges from 19 000 to 20 000 tonnes (FAO 2024). Aquaculture production in the Czech Republic is generally characterized by extensive and semi-intensive fish farming in ponds that plays an extremely important non-production role in terms of water management, landscape formation, culture, and protection, and its importance often goes beyond the production of fish itself (FAO 2024).

As well as a worldwide trend the production of carp in Czech Republic is tending towards stagnation (FAO 2024). Recently, however, ornamental fish (this group, among other fish species, also includes koi carp) have also begun to play a very important and integral role in the total aquaculture production of the Czech Republic, although production figures are not included in the official aquaculture statistics. According to production statistics, the Czech Republic is the world's fourth-largest producer and exporter of ornamental and aquarium freshwater fish (FAO 2024).

Common carp has a long-standing history of culture in the Czech Republic, dating back to the 13th century with a peak in the 16th century (Kocour et al., 2008). As a result, many unique local strains that differ in many ways including body shape, scaling pattern, and productive traits have been created. During artificial selection, attention was brought to the improvement of various productive traits like survival in ponds, the usefulness for artificial propagation, and the issue of disease resistance against a wide range of pathogens (Napora-Rutkowski et al., 2017). Eventually, numerous breeds in different countries reflect the adaptation of fish populations to local environmental conditions and local production systems. A list of pure breeds of commercially important freshwater fish species, considered gene resources in the Czech Republic includes nine carp local breeds (Flajšhans et al., 1999).

The Czech National Programme for the in situ conservation of genetic resources for commercially important fish species was established in 1996. It included nine rare and endangered breeds of common carp (*Cyprinus carpio*) (Flajšhans et al., 1999). The objective was to keep old less productive breeds as a part of national heritage and a source of genes for future breeding. At the same time, cryobanking of farmed fish sperm was launched in the Czech Republic as a part of the National program of conservation and use of farm animal genetic resources. The Cryobank was established in the Research Institute of Fish Culture and Hydrobiology (RIFCH), part of the nowadays Faculty of Fisheries and Protection of Waters in Vodnany. Sperm samples from 11 breeds of carp are stored (Flajšhans et al., 1999).

2.2. Long-term storage of common carp sperm

2.2.1. Basic information about cryopreservation of fish sperm

Cryopreservation is the long-term storage of living biological objects with the possibility of restoring their biological functions after thawing (Fuller, 2004). The typical storage temperature is -196 °C (the temperature of liquid nitrogen, which is convenient for low temperature storage) (Jamieson, 1991). At this temperature, the thermodynamic movement of molecules becomes very low, and all biochemical reactions are inhibited leading to extreme suppression of cell ageing (Mazur, 2017). Below -130 °C water exists only in crystalline or glassy (the vitreous) states, and the latter is achieved mainly due to the enormous increase in viscosity of the surrounding milieu (Katkov et al., 2012; Mazur, 2017). Despite the concerns that some events at the atomic level can still take place in

these low temperatures, or genetic information may be damaged by existing background radiation, it is a recognized fact that ultralow temperature storage is the only possibility for today to conserve cells' functional properties for long term (Garman, 2003; Cugia et al., 2011).

Methods of cryopreservation include slow freezing, equilibrium vitrification, and kinetic vitrification (Katkov et al., 2012). The primary goal in the application of all the above methods is to achieve a glassy state inside the cells and within their close vicinity (or also an achievement of a glassy state of the extracellular milieu). The method of cryopreservation implies the use of extender solutions containing permeable or non-permeable cryoprotective agents, which in turn play different roles during the cryopreservation process. In slow freezing, they serve mainly as osmotic buffers and in vitrification, they play the role of thickeners which increase viscosity and deplete growth of ice crystals (Katkov et al., 2012).

To the year 1995, the method of permeable cryoprotectant-free kinetic vitrification was rarely applied to fish semen, while classical equilibrium vitrification had already been successful in the cryopreservation of many fish species (Rana, 1995). However, the majority of cryoprotocols were performed using slow freezing methods. Today slow freezing remains the most commonly used method of fish sperm cryopreservation. To avoid uncertainties, it is necessary to clarify that in this review the term cryopreservation would refer to the mentioned above so-called slow freezing.

Sperm cryopreservation is the most established and commercialized method (Asturiano et al., 2017). The choice of sperm as a cell for cryopreservation can be explained by its simple cellular structure and size, small enough to ensure cryoresistance, its mobility as an indicator of viability, the relative ease of obtaining samples, as well as its reproductive qualities, which are especially important in connection with the advent of the artificial insemination method (Katkov et al., 2012; Asturiano et al., 2017).

The lack of standardization in methods and reporting represents the most important barrier to the expanded application of cryopreservation to practice, which is basically the main objective of the development of this methodology (Tiersch, 2008). It is well-known that there is no single universal cryopreservation protocol for sperm cells from different species because of a large range of cell cryobiological and physiological parameters, so a particular cell needs its own optimal cryopreservation protocol (Rana, 1995). Gametes within one species show more or less sensitive species-specific fitness to biophysical and

chemical processes that occur during cryopreservation (Cabrita et al., 2014). However also in intraspecific levels, the variations in inorganic and organic seminal plasma composition were observed, which signed up for the different cryoresistance of samples from individual fish.

2.2.2. Cryopreservation of carp sperm as a tool

Many researchers in their works have written about the possible contributions that sperm cryopreservation can bring to aquaculture. These include (modified from Jamieson, 1991; Tiersch, 2008):

1) cost savings for keeping brood fish; 2) improvement of existing hatchery activities by providing sperm on demand, simplifying the time management of artificial spawning; 3) genetic improvement of aquaculture fish breeding lines; 4) making affordable transportation of genetic materials between hatcheries; 5) the restoration of genetic material (valuable genetic lines, e.g., from endangered species, research models or improved farmed strains) in case of broodstock loss; 6) a potential to become an entirely new industry itself.

Artificial breeding of carp in the Czech Republic is a technological procedure put on stream. Typically, hatcheries do not have a shortage of males and females for mating and due to elaborated technology of controlled reproduction and hormonal stimulation both sexes spawn at the same programmed time. Males, as a rule, have no problems with spermiation and the amount of their gametes is sufficient for fertilization (Asturiano et al., 2017). From practice, it is known that for successful fertilization approximately 2 mL of milt is enough for the insemination of 1 kg of eggs in common carp (subject to the recommendations and using quality sex products). However, in case of the excess gametes, the utilized amount can be 10 times higher than the mentioned above minimum. Moreover, individual selection is not as advanced in fish as it is observed in terrestrial livestock (Asturiano et al., 2017). Therefore, cryopreservation can hardly be considered as a method that can significantly improve the existing routine process. On the other hand, long-time storage of sperm can open the doors to a wide range of experimental works, international scientific cooperation, commercial purposes and primarily conservation programs (Flajšhans et al., 2015).

Cryopreservation represents an excellent way to preserve the centuries-old work of fish farmers by freezing the sperm of local breeds. There is always the possibility of losing valuable strains under unforeseen circumstances or through irresponsible crossing. For

example, Pokorny in 1995 wrote about 12 original local breeds that were considered to be lost or crossbred. Crossbreds of carp strains are widely used for commercial stock production due to the heterosis effect which can be observed in the first generation of hybrids. It used to be difficult to keep records of fish broodstock before the possibility of using PIT tags and most breeds currently maintained have been characterized using only morphometrical measures (Pokorný et al., 1995). To the year 1998 genetic characterization studies of Czech common carp breeds based on the protein (allozymes) variability studies were started under the gene resources conservation programme but were incomplete at the time (Flajšhans et al., 1998; Hulak et al., 2010). Later it was found that allozyme markers had limited variability, and attention shifted to microsatellite markers (Kohlmann et al., 2003).

2.2.3. Cryopreservation of carp sperm: development and obstacles

According to Magyary et al. (2011), the beginning of carp sperm cryopreservation was laid in the second half of the 20th century when several studies were carried out on freezing sperm (Sneed and Clemens, 1956; Kossman, 1973; Moczarski, 1977; Stein and Bayrle, 1978), but the most known among the first attempts is the report of Kurokura et al. (1984). The use in this work original Extender-2, which consists of 128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, and 2.4 mM $NaHCO_3$, provided promising results, and since then, cryopreservation of common carp sperm has been extensively studied and much research has been conducted to improve freezing methods (Magyary et al., 2011).

Many factors influence the result of cryopreservation, and over time, new ones are discovered, the effect of which was not taken into account in previous studies. For example, Cognie et al. (1989) reported high post-thaw motility of sperm 70 – 80 %, but the fertilization rate was relatively low (30 – 40 % of the control). Later, Magyary et al. (1996) assumed that the low fertilization rate reported by these researchers possibly occurred due to the application of a fertilization solution instead of water for fertilization. They found that both Woynarovich-solution, which is normally used at a high rate in fish farming for fertilization and the avoidance of stickiness in fertilized carp eggs, and its components reduce fertilization rate drastically using frozen-thawed carp sperm.

This circumstance leads to the impossibility of comparing methods and their evaluation. Although in some experiments, technical improvements in sperm freezing methods resulted in improved fertilization and hatching rates in common carp, not

different from those of control treatment, however, this result could not consistently be reproduced (Magyary et al., 1996; Horváth et al., 2003; Magyary et al., 2011).

Warnecke and Pluta (2003) in their work cited uncertainties related to the use of dimethyl sulfoxide (DMSO), methanol, glycerol, dimethyl-acetamide (DMA) and other cryoprotective agents. The results of these medium applications are contradictory to each other in some research.

Highly heterogeneous cryopreservation outcomes and the lack of standardization represent a significant and known problem recognized by many authors who have devoted their work to this area (Rana, 1995; Flajšhans et al., 2015; Asturiano et al., 2017).

2.2.4. Factors affecting the cryopreservation outcomes

Many publications have examined the reasons for the heterogeneity in fish sperm cryopreservation results. Successful cryopreservation of carp spermatozoa depends on a range of factors. They include the collection of high-quality sperm, conditions of transportation and storage of sperm (Tiersch and Green, 2011), equilibration conditions, choice of cryoprotectant medium, cooling/thawing regimes, and conditions for fertilization (Kopeika et al., 2007).

Resistance of common carp sperm to cryopreservation is determined mainly by initial sperm quality (Tiersch and Green, 2011). The quality of sperm of farmed fish can be affected by various components of spawner rearing, seasonal variability in temperature and photoperiod and may vary depending on the frequency of stripping (Levandusci and Cloud, 1988; Fauvel et al., 1999). It was thought that sperm quality declines at the end of the spawning season. However, the reasons for this decline should be studied in the future (Cejko et al., 2018). At the same time, it is clear that water salinity, pollution with chemical substances, and stress caused by breeder transportation to hatcheries negatively affect sperm quality (Kowalski and Cejko, 2019; Cejko et al., 2022) via deterioration of seminal plasma and sperm membrane composition, which in turn influence the sperm cryoresistance (Cabrita et al., 2010).

Recently, increased attention has been paid to the issue of protective properties of seminal plasma. Seminal plasma is a component of semen that plays an important role in sperm metabolism, function, survival, and motility (Judycká et al., 2021). It is a natural medium that has a unique composition that provides an optimal environment for the spermatozoa during the maturation and storage of spermatozoa in the sperm duct and has

protective properties (Shaliutina-Kolesova et al., 2019). That is why, the dilution of natural seminal fluids by artificial cryoprotective media has a potentially harmful effect on cryopreservation outcomes.

Quite common is a fact when, despite the use of carefully selected sperm samples according to sperm quality indicators, that are considered as predictors of successful cryopreservation (motility, viability, sperm concentration), inexplicable variability between cryoresistance of males was observed (Dietrich et al., 2017). According to some research, this inter-male variability can be explained also by differences in seminal plasma composition.

In 2017 Dietrich et al. analysed protein formulation of the seminal plasma of carp sperm and found a correlation between particular proteoforms of some proteins presented in the sample and its cryoresistance. Generally, a negative relationship was found between the presence of proteins reflecting the processes that may occur within the reproductive tract, such as infection or stress, and the cryoresistance of sperm. On the other hand, higher freezability was attributed to the higher representation of proteins involved in the maintenance of sperm membrane integrity and antioxidative protection (Dietrich et al., 2017). Identifying these special proteins would possibly serve as a screening for males with better freezing resistance, which will lead to improved artificial insemination and conservation techniques for carp (Dietrich et al., 2017). Seminal plasma fractions have been associated with protective properties and can restore the integrity of damaged sperm (Shaliutina-Kolesova et al., 2019).

In one of the experimental parts of the current thesis, seminal plasma was used as a diluent, allowing variation of sperm density during cryopreservation. Its use was attributable to the necessity of discriminating the impact of sperm concentration on the cryopreservation outcomes from influences arising from the changes in osmolarity or decrease in the concentration of protective components of seminal fluid.

Effect of the presence of non-motile spermatozoa during cryopreservation on cryopreservation outcomes

In general, until the limit is reached, a higher sperm to egg ratio results in a higher fertilizing efficacy in carp. However, in the case of using sperm with a very low motility percentage, another effect was observed in some fish species. Results published by several authors demonstrate that non-motile sperm can interact with motile sperm, that at

least partly responsible for the disproportionally low fertility rate (Levanduski and Cloud, 1988; Lubzenc et al., 1997). This fact is useful to consider as thawed sperm frequently contains a high percentage of non-motile spermatozoa. In some cases, when the number of dead sperm in the sample is many times greater than the number of live ones, an increase in the amount of these thawed semen per egg results in a reduction in fertility (Stoss and Holtz, 1981; Levanduski and Cloud, 1988). This effect is dependent on or altered by several factors. Firstly, inconsistent fertility comes out when semen samples contain less than 10 % motile sperm. Secondly, this reduced fertility only occurs when the non-motile sperm have prior or equal access to the egg. Thirdly, adding of a large quantity of motile sperm per egg can overcome the effect of non-motile sperm on fertility (Levanduski and Cloud, 1988).

The mechanism of this interaction needs further research. Levanduski and Cloud (1988) studying this problem on rainbow trout (*Oncorhynchus mykiss*) provided several possible explanations. They assumed that due to the congestion on the surface of the oocyte non-motile sperm physically partially obstruct the micropyle allowing restricted passage of motile sperm through this passageway. Another version of the reduced fertility is that non-motile sperm cells produce semen components that interfere with the ability of normal sperm to react to the chemotactic factors from the micropylar region and inhibit their relocation.

These interactions should be taken into account, especially in the case of examination of the method of cryopreservation laid in using more concentrated sperm samples where the losses caused by packing effect occurrence are neglected.

Evaluation of sperm cryopreservation outcomes

Another difficulty lies in the correct evaluation of outcomes. The main objective of sperm conservation as an applied area of science is the preservation of genetic material with the further procreation of new organisms. In the early stages, the usual method of assessing sperm quality consisted of mixing eggs and sperm and measuring fertilisation or hatch rate (Kime et al., 2001). But in this case, the experiments were complicated by bringing another variable - female gametes. Further in several studies, the positive correlation of post-thaw motility and fertilization capacity in fish, including common carp was confirmed (Magyari et al., 1996; Rurangwa et al., 2004). In fact, sperm motility is a prerequisite for successful fertilization, but other factors must also be taken into account (Warnecke and Pluta, 2003; Judycka et al., 2021). This made it possible to predict the

success of future fertilization using assessing sperm movement. Observation was carried out either directly on the microscope slide or via videotape. No matter what further assessment methods were used, they did not provide a complete picture of the movement, were subjective and time-consuming (Kime et al., 2001).

A major advance in the rapid and objective assessment of fish sperm motility has come from using computer-assisted sperm analysis (CASA) systems that were initially developed to examine sperm quality in mammals and birds and have only recently been applied with some adaptation to fish sperm. CASA assessment is of significant value in predicting the ability of sperm to achieve fertilisation and subsequently hatching since percent of motile sperm and sperm progressive velocity are the sole predictors of fertility (Kime et al., 2001; Rurangwa et al., 2001).

Cryopreservation techniques involve the addition of a cryoprotectant, freezing and thawing of sperm, which may result in some damage to the sperm and a decrease in its fertilizing ability. Sperm motility measurement has already been widely used to assess the most appropriate extender for sperm dilution and activation media. The use of CASA allows testing a range of cryoprotectant's effects on sperm and finding the most promising protectant and identifying cryoprotectants that seriously damage sperm even before freezing (Kime et al., 2001).

2.3. Sperm concentration in carp reproduction and cryopreservation

The concentration of spermatozoa in the milt is an important parameter for study. Knowledge of this characteristic allows an estimation of the whole spermatozoa population without considering the individual spermatozoon status (Rurangwa et al., 2004) (if it is required such individual characteristics of sperm may be provided by the estimation of the motility parameters). The term of sperm concentration is relevant in several moments, which are described below.

2.3.1. Initial concentration of sperm

Primarily concentration is an important parameter of native sperm, that provides knowledge about male quality (Rurangwa et al., 2004). Naturally, carp sperm is characterized by high sperm concentration that can widely vary according to the age of the fish, population, date of the spawning season and other factors (Lahnsteiner, 2000;

Cejko et al., 2018). According to the methodology „Hodnocení čerstvého spermatu ryb" (Linhart et al., 2011) published by the University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, normally the spermatozoa (spz) count varied between 10–25 × 10^9 spz mL^{-1}. However, a wider range can be found in different scientific papers, for example, 17–32 × 10^9 spz mL^{-1} (Kaspar et al., 2007) or 5.6–32.5 × 10^9 spz mL^{-1} (Lubzenc et al., 1997). Because most freezing protocols of fish sperm require a dilution of semen with an extender at different dilution rates (Yang and Tiersch, 2009), the final sperm concentration is variable (Judycka et al., 2018). For this reason, there is a need to standardize cryopreservation protocols by establishing a constant optimal sperm concentration in straws at a constant cryoprotectant concentration (Judycka et al., 2018).

2.3.2. Sperm samples concentration at artificial fish propagation

Determining the optimal sperm concentration is an important constituent for successful artificial insemination. Insufficient or excessive amount of sperm significantly reduces the results of fertilization in fish species (Dettlaff et al., 1993). In carp, a high concentration of sperm during fertilization does not have a noted negative effect, unlike, for example, during sturgeon artificial reproduction, where an excessive amount of sperm leads to considerable losses caused by polyspermy (Dettlaff et al., 1993). The ratio 200 000 spermatozoa per ova was tested in carp and any decrease in fertilization and hatching rates hadn't been observed compared to the normally used, 10 times lower concentration (Linhart et al., 2015). In hatchery practice, several millilitres (2 – 10) of good quality sperm (motility 9 – 100 %) per kg of eggs (800 000 eggs) are used in carp artificial reproduction, and hatchery water is added for fertilization in the amount of 0.5 L of hatchery water on 1 kg of eggs (Gela et al., 2009). The use of heterosperm mixed from 3 – 5 males is not an exception.

Experimental works conducted with manipulated sperm require low but defined densities of spermatozoa per egg to detect the best fertilizing ability of spermatozoa (Linhart et al., 2015). Therefore, other proportions are recommended as more testifying. The optimum proportion (by volume) was recommended by Linhart et al. (2008) as 1:500:1000 (sperm: egg: activation medium (water), respectively). For experimental purposes, 1 or 2 parts of water are added to 1 part of eggs (Magyary et al., 1996; Linhart et al., 2015). A much lower volume of water and a higher number of spermatozoa per volume of eggs can mask low viability and poor sperm quality, increasing the

experimental variability, and thereby affecting the precision of results (Linhart et al., 2008). Amounts of 1000 and 5000 spz per egg were described as appropriate for common carp (Linhart et al., 2015), but these findings should be further proved for other species.

The quantity of spermatozoa per egg during insemination in the case of using fresh sperm amounts 10 000 - 30 000 sperm for one egg (Rodina et al., 2010) but this amount is insufficient when using thawed sperm due to a typically lower percentage of motile sperm after thawing (Sotnikov et al., 2023). For this reason, in methodologic "Zmrazování spermatu kapra obecného (*Cyprinus carpio L.*) pro potřeby uchování genofondu v praktických podmínkách" (Rodina et al., 2010), it is recommended to increase this amount 10 times for thawed sperm, i.e. 100 000 – 300 000 sperm per egg to achieve good fertilization rate. Lubzenc et al. (1997) stated that 4200 – 5000 spermatozoa per egg are needed for successful fertilization with non-treated or treated with cryoprotectant DMSO sperm samples, while about 300 000 spermatozoa per egg are needed in the case of cryopreserved sperm.

An excess amount of thawed sperm used for in-vitro fertilisation trials can provide a high fertilization outcome. However, this approach is not appropriate to test the effect of different treatments on sperm quality, or to compare sperm from different males (Kime et al., 2001). A reduced ratio is recommended to be applied for testing sperm quality by studying fertilisation rate over a range of serially diluted sperm concentrations. The optimal sperm per egg ratio to use is then that at which fertilising ability has fallen by 20 – 30 % in comparison with native sperm, and any further decrease in sperm quality would cause a further decrease in fertilisation rate (Rurangwa et al., 1998; Kime et al., 2001). Magyary et al. (1996) compared different concentrations of thawed spermatozoa in carp and determined the optimum ratio as 100 000 – 150 000 spz per 1 egg in case of using optimal conditions of fertilization (dilution of sperm in modified Kurokura's "Extender 2" containing DMSO as cryoprotectant at 10% final concentration; the dilution rate was 1:9 (sperm: diluent); ratio of frozen sperm: egg: water was 1:20:20 in volume). Higher sperm to egg ratio decreased the fertilization rate, possibly due to the suspected toxic effects of DMSO (Magyary et al., 1996).

Although it has been shown that sperm concentration plays a crucial role in the development of practical and reliable protocols for sperm cryopreservation in many aquaculture species, the importance of sperm concentration is often overlooked and most studies of aquatic species regarding cryopreservation do not control or report this

parameter (Pataki et al., 2022). “Knowledge of sperm concentration would maximize the information that can be obtained from each sample, avoid the introduction of uncontrolled male-to-male variation, and allow for better analysis of results and the understanding of cryopreservation as a whole” (Tan et al., 2010).

2.3.3. Changing of sperm concentration related to the cryopreservation procedure

During the cryopreservation process concentration of sperm is important in the context of understanding a process occurring inside freezing samples. Following ice nucleation, a continuous network of ice propagates through the sample entrapping both cells and freezing concentrated solutes in a colloidal suspension between these ice crystals (Morris et al., 2012). If the cell concentration is very high, cell to cell contact can lead to mechanical cell compression, the so-called packing effect (Morris et al., 2012). Different studies confirm that more diluted sperm has a higher chance of escaping this mechanical damage (Sotnikov et al., 2023). Bernath et al. (2016) summarised the tendency that carp sperm tolerates higher sperm to extender dilution rates and for cryopreservation purposes should be diluted at ratios between 1:5 and 1:20 in a regular (sugar-based) composition.

Despite the detection of the higher motility of spermatozoa after cryopreservation in a more diluted solution, a high dilution ratio during cryopreservation of carp sperm however has some disadvantages. This technique is inefficient due to the necessity of raising the number of samples for successful insemination of oocytes. It leads to the necessity of increasing the cryostorage capacity the maintenance of which increases material costs (Nascimento et al., 2021; Sotnikov et al., 2023). Moreover, the substantial volume of cryomedia complicates the fertilization process and subsequent elimination of stickiness in common carp (Sotnikov et al., 2023). Since, as mentioned above, concentration is individual in nature, measurement of sperm density and adaptation of the dilution ratios are recommended for each broodstock and fish population (Lahnsteiner, 2000). Lahnsteiner, conducting experiments on freezing sperm from salmonid species in straws, concluded that the cell concentration in the extenders may not extend $2 – 3 \times 10^9$ cell mL^{-1} to prevent cell compression, which probably takes place in limited intracellular space.

The sperm concentration optimum for cryopreservation is species-specific, and boundary concentration (the maximum sperm concentration at which the packing effect is not detectable) cannot be predicted theoretically, for example, only judging on the size

of the spermatozoon in various species (Nascimento et al., 2021). This conclusion can be reached by analysing results on cryopreservation of sperm from different salmonid fish (Judycka et al., 2019b). In this experiment, rainbow trout and Atlantic salmon (*Salmo salar*) extremely differed in optimal sperm concentration during freezing, although both species have quite similar sperm dimensions (head and tail lengths). Sperm plasma membrane composition can also be considered as one of the important determinants of cryoresistance (Cabrita et al., 2010). The reason for this is the fact that membrane lipid composition, especially the cholesterol/phospholipid ratio and free fatty acid composition, influences permeability to water and other molecules, membrane fluidity and lipid phase transitions in the membrane bilayer (Holt, 2000). Horokhovatskyi et al. (2016) published a report focused on this subject. Their work confirmed the effect of phospholipid components of sperm plasma membranes on the cryoresistance of samples. Finally, the results of this study demonstrated that there is a positive correlation between lower total lipid content in sperm and higher post-thaw motility and that higher levels of unsaturated fatty acid are associated with higher levels of cryoresistance (Horokhovatskyi et al., 2016).

For carp, the precise required value of sperm concentration during the cryopreservation process has not been determined so far. At a low concentration of sperm in samples, a quite high percentage of survived cells is achieved after thawing, while the multiple increases in concentration lead to vitality percentage decrease due to biophysical aspects that occur during freezing. Nevertheless, the use of a more concentrated sample will probably be more profitable despite the low motility, which is compensated by higher overall quantity of surviving sperm in the sample.

Finding the optimal sperm concentration through discovering a compromise between these aspects is necessary in practical terms, especially in preventing the heterogeneity of cryopreservation outcomes.

2.3.4. Estimation of sperm concentration in fish species

Determination of sperm concentration is traditionally performed with a counting chamber or haemocytometer (e.g., Bürker-Türk, Neubauer, Neubauer improved, Thoma, Makler). This method has been widely applied for many years due to its affordability and is considered an accurate means of estimating cell concentration (Rurangwa et al., 2004; Tan et al., 2010; Yang et al., 2015). But some features and disadvantages lie in the need of manual counting. This method is impractical during the necessity of preparation of a

large number of samples for cryopreservation as it is time consuming, and this method can have limited application in case of the minuscule volumes of sperm samples from some small model fishes (Tan et al., 2010). At present, a counting chamber is not the only method of sperm concentration estimation.

Many flow cytometers, designed to test a number of individual cell functions, allow accurate calculation of cell concentrations (Yang et al., 2015). Automated fluorescent microscopy, exemplified by nucleocounter devices, presents an alternative for determining cell concentration (Judycka et al., 2019a). However, these devices are expensive, and many of them require continuous maintenance (Judycka et al., 2019a). The measurement of sperm absorbance with a spectrophotometer (and microspectrophotometer that has been developed specifically for determination of sperm concentration in small-sample sizes) has also been tested with good results on the sperm of several fish species (Takashima et al., 1984; Ciereszko and Dabrowski, 1993; Tan et al., 2010). Centrifugation to determine spermatocrit (%, the ratio of volume of white packed material to total volume of milt × 100) has been used in some species, in which sperm density was correlated with spermatocrit to rapidly determine sperm density. In some experiments using a Coulter counter as a replacement for a haemocytometer proved good results (Rurangwa et al., 2004). Finally, CASA systems also allow sperm concentration measurement (Pataki et al., 2022).

All the above methods have their specificities and require detailed refinement and verification for individual species samples. Possible problems may be caused by the presence of aggregations of spermatozoa, which can complicate an observation under the microscope during manual counting or other methods of sperm estimation. Small volumes of high-density milt can cause pipetting errors. The absence of a clearly defined interface between the packed sperm cells and the seminal fluid between the phases after centrifugation or the absence of correlation between sperm density and spermatocrit in some species can induce a false estimate (Ciereszko and Dabrowski, 1993; Rurangwa et al., 2004). System-to-system variations between the image processing units of CASA systems and its cost can be a barrier to the widespread use of CASA for concentration estimation (Lesani et al., 2020).

Each of the applicated methods exhibits its own effective concentration range for measurement, determined by its underlying counting mechanism.

Presently, the most frequently employed methods for determining sperm concentration include haemocytometry, the Makler counting chamber, and spectrophotometry (Yang et al., 2015).

Estimation of sperm concentration using spectrophotometry

The method of sperm concentration determination by a spectrophotometer is based on measuring the transmission of light through a known volume of sample, in which light absorption values at specific wavelengths are used to estimate cell concentration (Leclercq et al., 2012). If used correctly, it can be considered as a precise, efficient, and inexpensive method to estimate cell concentrations (Tan et al., 2010; Lesani et al., 2020).

Due to the inherent nature of spectrophotometry, the sensitivity of the method is influenced by the concentration of somatic cells and contaminants in the native semen sample that additionally absorb light and result in overestimating sperm concentration (Lesani et al., 2020). Thus, special carefulness should be devoted to pure sample preparation. For example, the presence of seminal plasma proteins remains one of the most serious possible interferences in using this method for mammalian semen (Radford and Herdan 1961; Ciereszko and Dabrowski, 1993). In fish, this problem is not so relevant due to the lower concentration of proteins in seminal plasma and the higher dilution ratio of sperm (Ciereszko and Dabrowski, 1993). Furthermore, according to Leclercq et al. (2012), separation of the seminal fluid by centrifugation to analyse the resuspended milt-centrifugate is an unnecessary step as it did not increase and even slightly decreased the accuracy of the estimated sperm concentration. Probably, this situation occurred because of mistakes introduced by the separation of the supernatant and the resuspension of the centrifugate.

Another critical step in the estimation of sperm concentration by using the optical absorbance principle is the accuracy of sampling the desired volume of milt for dilution. Particular attention should be paid to this act in the case of high-density sperm samples (Ciereszko and Dabrowski, 1993).

The optimal wavelength for the most accurate determination of sperm concentration is species-specific (Kumar et al., 2013). It is necessary to calibrate a spectrophotometer before the procedure of determining the concentration of a sample. This procedure requires the determination of the wavelength appropriate for measurement, which varies depending on the chemical and physical structure of the analyte and requires the comparison of a set of concentrations at multiple wavelengths within an absorbance

spectrum (Tan et al., 2010). The measurement of absorbance from serial dilutions with different concentrations enables the generation of a calibration curve and its equation, which can be used for future measurements of sperm concentration (Tan et al., 2010). Ensuring the reliability of measuring this method requires a periodically repeated verification of the calibration curve (Tan et al., 2010).

The spectrophotometric method for the measurement of cell concentrations is widely used in artificial insemination procedures and for cryopreservation purposes regarding semen of wild and captive animals and aquatic species (Ciereszko and Dabrowski, 1993; Tan et al., 2010; Leclercq et al., 2012). Cuevas-Uribe (2010) summarised information related to the issue of using spectrophotometry in sperm concentration estimation in fish. The application of this method to fish sperm can be dated back at least to 1971 (Billard et al. (1971) cited in Cuevas-Uribe, 2010). According to Cuevas-Uribe, to the year 2010, the direct relationship between sperm concentration and absorbance has been established in approximately 41 species of fish (but only one-third of the studies gave a description of the calibration curve equations or other spectrophotometric measurement protocols). Wavelengths between 260 and 660 nm have been used to determine sperm concentrations in fish. The most frequently used (20 %) wavelength was 505 nm. The sperm concentration of common carp milt was previously measured at a broad range of wavelength values: 410 nm (without detailed descriptions) (Takashima et al., 1984), 505 nm (Pataki et al., 2022), 610 nm (Dzyuba and Kopeika, 2002). A critical step is to find the most appropriate wavelength for cell concentration estimation in individual species samples, and this question will be discussed further in the result section of this thesis.

3. Materials and methods

The experimental work was carried out in several stages. The first part was devoted to the elaboration of a method of sperm concentration estimation in common carp using the measurement of the absorbance of sperm suspension. Subsequently, the importance of sperm concentration during freezing was studied. Two different experimental approaches were used to adjust the sperm concentration in samples before cryopreservation. In the first approach, the sperm was diluted with seminal plasma to vary sperm concentration before freezing, and in the second approach, the different sperm concentrations were created by using different dilution rates of sperm by cryoprotective medium. Finally, the impact of sperm concentration during freezing on post-thaw sperm motility and fertilizing ability was studied.

Statistical approaches used during the experimental work of the thesis are described where appropriate in the corresponding sub-chapters. Statistical treatments and plottings were performed by software: Microsoft Excel for Windows (Microsoft Corporation, USA), STATISTICA (TIBCO Software Inc., USA), and GraphPad Prism (Insight Partners, USA).

3.1. Sperm and egg collection

Manipulations with animals were performed according to the authorization for breeding and delivery of experimental animals (reference number: 64155/2020-MZE-18134) and the permission for the use of experimental animals (reference number: 68668/2020 MZE-18134 and 68763/2020-MZE-18134) issued to the Faculty of Fisheries and Protection of Waters, the University of South Bohemia by the Ministry of Agriculture of the Czech Republic.

Gametes of common carp (*Ciprinus carpio*) were obtained from carp breeders (age: 3 – 7 years old) of health conditions appropriate for fisheries practice, raised in aquaculture ponds and indoor facilities of the Research Institute of Fish Culture and Hydrobiology of the Faculty of Fisheries and Protection of Waters at the University of South Bohemia, Vodnany, Czech Republic. Males were kept in an indoor RAS system at constant optimal conditions, providing the possibility of induced spermiation throughout the year. Fertilization experiments were performed during the common carp natural spawning period according to recommendations for artificial propagation (Gela et al.,

2009). Before hormonal stimulation, females were kept in 8 m^3 hatchery tanks with a water flow rate of 0.2 L s^{-1} and water temperature adjusted to 20 °C. Carp breeders were induced hormonally for spermiation and ovulation.

Spermiation was stimulated by intramuscular injection of acetone-dried carp pituitary powder suspended in 0.9% (w/v) NaCl solution at doses of 1 mg × kg^{-1} of body weight 24 h before sperm collection. Fish were laid out on a wet towel and their urogenital papilla was dried. Semen was collected into 10 mL plastic syringes using abdominal massage. Special care was taken to prevent contamination with mucus, faeces, or water. Sperm samples were stored on ice (4 °C) under aerobic conditions for a period not exceeding 2 hours before freezing.

Females were injected with carp pituitary powder extract twice: the first dose of 0.3 mg × kg^{-1} 24 h before egg collection and the second dose of 2.7 mg × kg^{-1} 12 h after the first injection. Eggs were obtained by an abdominal massage directly into dry plastic bowls.

3.2. Elaboration of spectrophotometry-based method for sperm concentration estimation

To establish standard curves correlating absorbance with sperm concentration, sperm concentration in each sperm sample involved in the study was measured by Bürker haemocytometer (Paul Marienfeld GmbH & Co. KG, Germany) when sperm samples were diluted to a final ratio of 1:10 000 (sperm: 150 mM NaCl). Sperm counting was conducted under a microscope equipped with optical negative phase-contrast and ×10 magnification. Spermatozoa were counted across 12 squares, and each measurement was performed in duplicate. The average value from these duplicate measurements was used as a standard to establish standard curves for the determination of sperm concentration by measurement of absorbance in sperm suspension.

The following successive steps assessed the effectiveness of optical absorbance measurement for evaluating sperm concentration.

3.2.1. Determination of light wavelength appropriate for sperm concentration measurement

The initial step in spectrophotometric analysis involves determining the absorbance spectrum of a given compound to optimize sensitivity for measurements. Individual

absorbance spectra (across wavelengths ranging from 250 to 700 nm) of sperm from five males were recorded using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and subsequently presented using Microsoft Excel. A 1-μL sample volume was used during this test, and 150 mM NaCl solution employed to suspend the sperm samples was used as a reference blank. Between measurements, the instrument was cleaned with a dry Kim-wipe.

Four wavelengths were tested based on the obtained spectra to get standard curves correlating absorbance with sperm concentration. The selection of wavelengths was based on the following known properties of the cell suspension to absorb the light: 260 nm - the peak in the DNA absorption spectrum; 280 nm - the peak in the proteins absorption spectrum; 400 nm and 600 nm are wavelengths which are in the wavelength range used to generate standard curves correlating absorbance with sperm concentration in fishes (Ciereszko and Dabrowski 1993; Tan et al., 2010; Pataki et al., 2022).

Standard curves correlating absorbance at each of the selected wavelengths with sperm concentration were plotted based on measurements of sperm samples from three common carp males. Each sperm sample was diluted in the range of 1:200 – 1:20 to obtain nine sperm samples with differing sperm concentrations. These sperm samples were thrice subjected to absorbance measurement at selected wavelengths (260, 280, 400, and 600 nm). Based on the obtained absorbance data and sperm concentration measured by the Bürker haemocytometer, standard curves correlating absorbance at each selected wavelength with sperm concentration were plotted, and goodness of fit to the linear regression was estimated by the coefficient of determination (R^2) and visually validated by residual plots. Based on these considerations, the 600 nm wavelength was selected for future development of the methods.

3.2.2. Establishing standard curves correlating absorbance with sperm concentration

Standard curves (lines) for regression between optical absorbance at the wavelength 600 nm and sperm concentration in nine samples of common carp sperm, diluted as previously described, were potted. Descriptive statistics of these lines (slopes and elevations) with determination coefficients were calculated. ANCOVA analysis of slopes and elevations of these lines was performed to establish a pooled standard curve for validation purposes (Tan et al., 2010).

For the validation of the pooled standard line, sperm samples from 15 males were used. In each sample, sperm concentration was measured by the obtained standard line and by the Bürker haemocytometer. Based on these data, the regression line was plotted and its goodness of fit was estimated by R^2. Additionally, the estimation of the significance of the difference in the coefficient of variance of sperm concentration obtained by both methods was evaluated after ten measurements of one sperm sample followed by the estimation of the coefficient of variance (CV = (standard deviation / mean) × 100) and checking by Levene's test if variations are different.

3.3. Measurement of sperm motility parameters

Sperm motility parameters for each male were estimated by CASA as a quantitative assessment method of fish sperm quality. This method was applied to estimate sperm motility parameters in samples after sperm collection, before cryopreservation, and after thawing. Sperm motility was initiated by exposure of common carp sperm to an activation solution elaborated for carp, comprising 30 mM Tris, 45 mM NaCl, 5 mM KCl, pH 8.0 (Billard et al., 1995; Horvath et al., 2003; Linhart et al., 2003), and supplemented with 0.125% Pluronic F-127 (catalogue number P2443, Sigma-Aldrich) to prevent sperm stickiness to the glass slide. Sperm motility was initiated in the ISAS spermtrack-10 sperm counting chamber (PROISER, Spain) and video recorded for a duration of 40 seconds post-activation. Video recordings were done using a microscope with optical negative phase contrast at ×10 magnification (PROISER, Spain) and an IDS digital camera (IDS Imaging Development Systems GmbH, Germany). The video records were analysed by CASA plugin for image processing program ImageJ (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI), University of Wisconsin, USA) according to Purchase and Earle (2012) and Wilson-Leedy and Ingermann (2007). The percentage of motile cells (%), curvilinear velocity (VCL, μm s^{-1}), average path velocity (VAP, μm s^{-1}), straight-line velocity (VSL, μm s^{-1}), linearity of the path (LIN, calculated as VSL/VAP), oscillation of the track (WOB, calculated as VAP/VCL), and beat-cross frequency (BCF, Hz) were evaluated. Spermatozoa with a VCL below 20 μm s^{-1} were considered immotile and consequently excluded from the analysis. Spearman's rank correlation coefficient was used to analyse data obtained by CASA for all sperm samples used in the study. The VCL and LIN had low correlation coefficients ($r < 0.1$) and were chosen as general proxies of sperm motion.

The data on sperm motility percentage, VCL, and LIN from duplicate records (of each male in each experimental condition at each second of 10 – 40 s after motility activation) were averaged and used to plot polynomial regression lines (third-order) to visualize the changes during motility period. These lines have high R^2 values (ranging from 0.989 to 0.999 for sperm motility percentage, 0.985 to 0.997 for VCL, and 0.544 to 0.953 for LIN). The maximum differences in parameters on polynomial regression lines were observed at 10 s post activation. At this time, the average values of sperm motility percentage, VCL, and LIN for individual males were utilized to assess the significance of differences. Before analysis, the data underwent testing for normality and homogeneity of variances (by Kolmogorov-Smirnov and Levene's tests, respectively). While all studied parameters were normally distributed, they exhibited dissimilar dispersion values, and that is why they were subsequently analysed using the non-parametric Kruskal-Wallis test, followed by multiple comparisons of mean ranks for all groups.

3.4. Cryopreservation and thawing of sperm samples

The sperm cryopreservation was carried out in accordance with the already mentioned earlier methodology "Zmrazování spermatu kapra obecného (*Cyprinus carpio*) pro potřeby uchování genofondu v praktických podmínkách" (Rodina, 2010). Diluted sperm samples were packaged into 0.5 mL plastic straws (IMV Technologies, France). After 10 min of equilibration at 4 °C, the straws were placed on a 3 cm thick polystyrene raft floating on the surface of liquid nitrogen. Ten minutes later, the straws were plunged directly into liquid nitrogen. Then, the samples were warmed with agitation in a 40 °C water bath for 6 s and used immediately for motility control or fertilization.

3.4.1. Dilution of samples for cryopreservation

Cryopreservation of sperm samples from 34 males was carried out in total. Samples were subjected to sperm motility and concentration measurements. Sperm concentration in native (fresh, not tritted) samples was in the range of 22 – 26 × 10^9 spz mL^{-1}. Sperm samples showing at least 80 % motility were used for further studies. Two methods of sperm dilution before freezing were employed to vary sperm density during cryopreservation. Application of these methods, described below, was required to determine the possible cryoprotective effect of seminal fluid.

In the first approach, sperm samples from 21 males were individually treated. One aliquot from each sample was centrifugated for 10 min at 10 000 g, 4 °C, and obtained seminal fluid was used to dilute four other aliquots of sample from the same male to the concentration of 1, 2, 10, and 20 × 10^9 spz mL^{-1}. Subsequently, these diluted samples and native (not diluted, control) sperm samples were mixed 1:1 with a cryoprotective medium, resulting in 0.5, 1, 5, and 10 × 10^9 spz mL^{-1} and control (1/2 native concentration) and then frozen. The composition of the cryoprotective medium used in this approach is derived from the modified Kurokura's "Extender 2" (Horváth, 2003) and described in Table 1.

In the second approach, sperm concentration was varying by dilution with the cryoprotective medium. In this approach, sperm samples from 18 males were divided into three aliquots and diluted using only the cryoprotective medium at ratios 1:1, 1:3, and 1:9. The composition of cryoprotective medium for each dilution ratio was individually formulated according to Horváth et al. (2003), who used a 1:9 dilution rate in their experiments (Table 1). This method was used to maintain the consistent ionic composition and final cryoprotectant content in the samples during freezing. After this manipulation, sperm concentrations in samples were in ranges 11 – 13, 5.6 – 6.5, and 2.2 – 2.4 × 10^9 spz mL^{-1}.

Table 1. Composition of cryoprotective media (in mg) used at different sperm dilution ratios.* The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

For 100 mL final volume (in distilled water)	**Dilution rate**		
	1:9	**1:3**	**1:1**
NaCl	360	432	648
KCl	1000	1200	1800
$CaCl_2$	22	26,4	39,6
$MgCl_2$	8	9,6	14,4
$NaHCO_3$	20	24	36
DMSO	10	12	18

* The recipes of each solution were calculated to get the final concentration of components, corresponding to the original extender proposed by Horvath et al. (2003) for a dilution rate of 1:9

3.5. Test of sperm fertilizing ability

Sperm from five males with the best post-thaw motility parameters were selected from the samples frozen applying the second method of sperm dilution (without using seminal plasma for dilution) for implementation of fertilization tests. For the fertility test, oocytes from three females were mixed to minimize the female factor. To assess egg quality, fresh sperm obtained from six males (10 µL) was used as a control.

Portions of two grams from this egg batch (approximately 1600 eggs) were fertilized by sperm samples from each male frozen at various sperm concentrations. Two mL of activation medium with the required amount of semen sample was added to carp eggs. This amount was calculated taking into account very close values of sperm concentration in different sperm samples ($22 – 26 \times 10^9$ spz mL^{-1}), allowing the use of an average concentration of 24×10^9 spz mL^{-1} for calculations of sperm volume required for the test. The amount of sperm added is presented in Table 2. After gentle mixing for 1 minute, the eggs underwent washing with water. After washing, approximately 100 eggs from each experimental variant were transferred to Petri dishes, which were afterwards located in a closed water flow system, maintaining a constant temperature of 21 °C, and incubated until hatching. Each fertilization trial was conducted in duplicate.

Table 2. The volume of cryopreserved sperm used in the tests of sperm fertilizing ability. The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

Average spermatozoa number per egg	**Sperm: cryoprotective medium dilution rate**		
	1:1	**1:3**	**1:9**
4.5×10^5	60 µl	120 µl	300 µl
1.5×10^5	20 µl	40 µl	100 µl
0.75×10^5	10 µl	20 µl	50 µl
0.3×10^5	4 µl	8 µl	20 µl

The development rate of the eggs was determined 24 h post fertilization, calculated as the ratio of developing embryos to the total number of eggs in the Petri dish, multiplied by 100 %. The hatching rate was evaluated 96 h post fertilization, expressed as the ratio of hatched larvae to the total number of eggs in the Petri dish, multiplied by 100 %.

After testing for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, data on fertilization tests were treated by Two-way

ANOVA concidering sperm to egg ratio and sperm dilution ratio at freezing as factors. Because no significant interaction of factors and no significant effect of sperm dilution rate was detected, post-hoc comparisons were performed only for significant factor of sperm to egg ratio using the Tukey test (Wei et al., 2012).

4. Results

The data presented in this work has been partially published in the paper "High sperm concentration during cryopreservation decreases post-thaw motility percentage without compromising in vitro fertilization outcomes in common carp" in journal “Aquaculture” (Sotnikov et al., 2023); the applicant of this thesis directly participated in performing the study and is a co-author of the publication. The paper is included in the supplementary materials of the current thesis.

4.1. The elaboration of the method for estimation of sperm concentration using spectrophotometry

Absorption spectra of common carp sperm suspensions from five males are presented in Fig. 1. Absorbance values were systematically higher in the UV range, with an absorbance peak at 260 nm. The absorbance gradually decreased in the visible light spectrum part, getting a plateau near 600 nm.

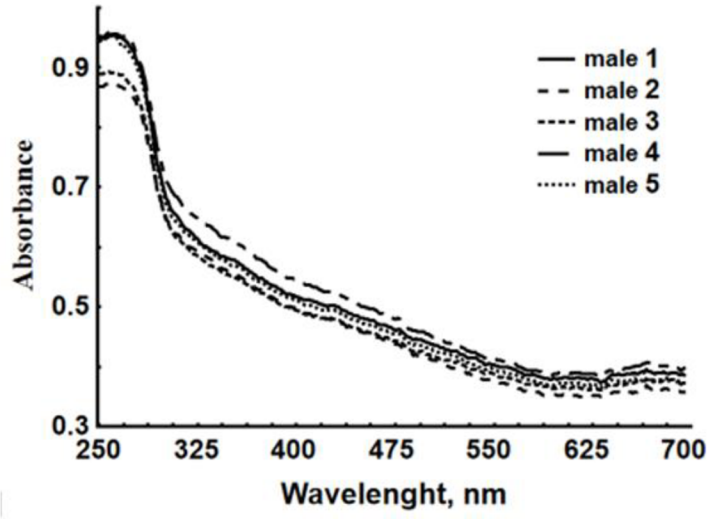

Figure 1. Absorbance spectra of common carp sperm suspensions. Examples from 5 different males are presented. The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

Wavelengths 260, 280, 400, and 600 nm were selected for subsequent study (the reasoning for the selection is presented in the Material and Methods section). The graph shows the regression lines of dependency between absorbance at different wavelengths and concentration of samples (Fig. 2). These regression lines were characterized by a

relatively high value of the coefficient of determination (range 0.908 – 0.940), suggesting that all of them are appropriate for the determination of sperm concentration by absorbance measurement at the selected wavelength.

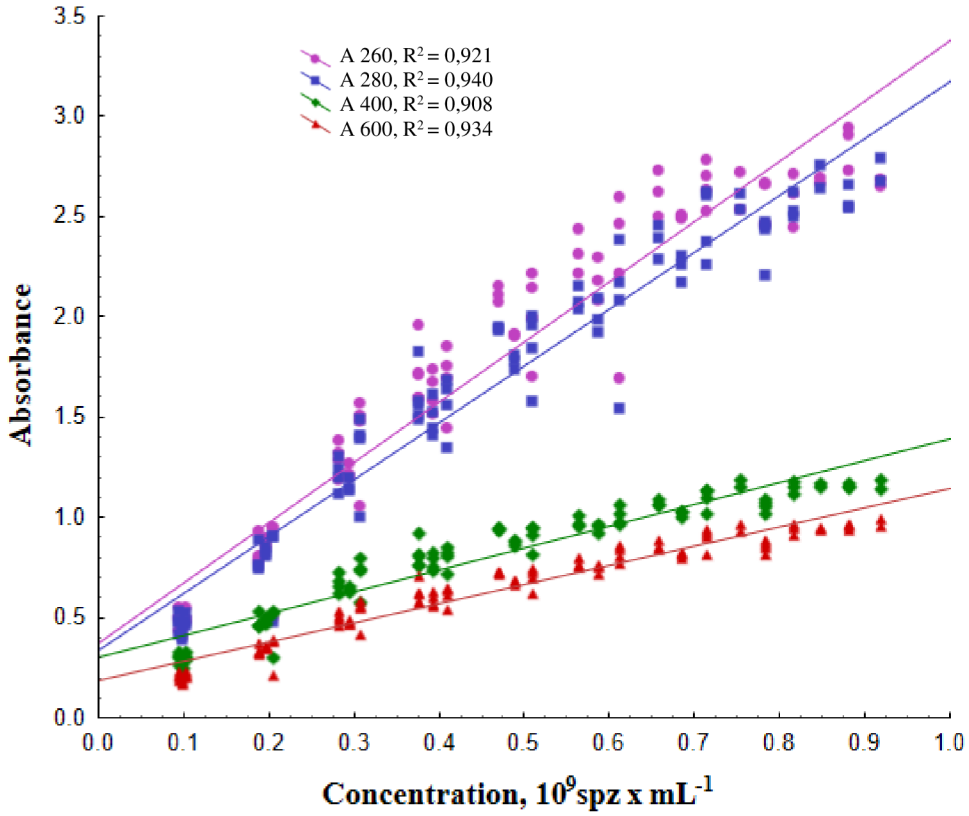

Figure 2. Regression lines for absorbance against sperm concentration at different wavelengths (260, 280, 400, and 600 nm), with corresponding coefficients of determination (R^2).

However, a residual plot applied as a diagnostic method to assess the goodness-of-fit of a regression model (Fig. 3) has shown the lowest scatter of absorbance data from the regression line at 600 nm (see red triangles, Fig. 3). The exact 600 nm wavelength was selected for generating a standard curve and calculations in the following experiments.

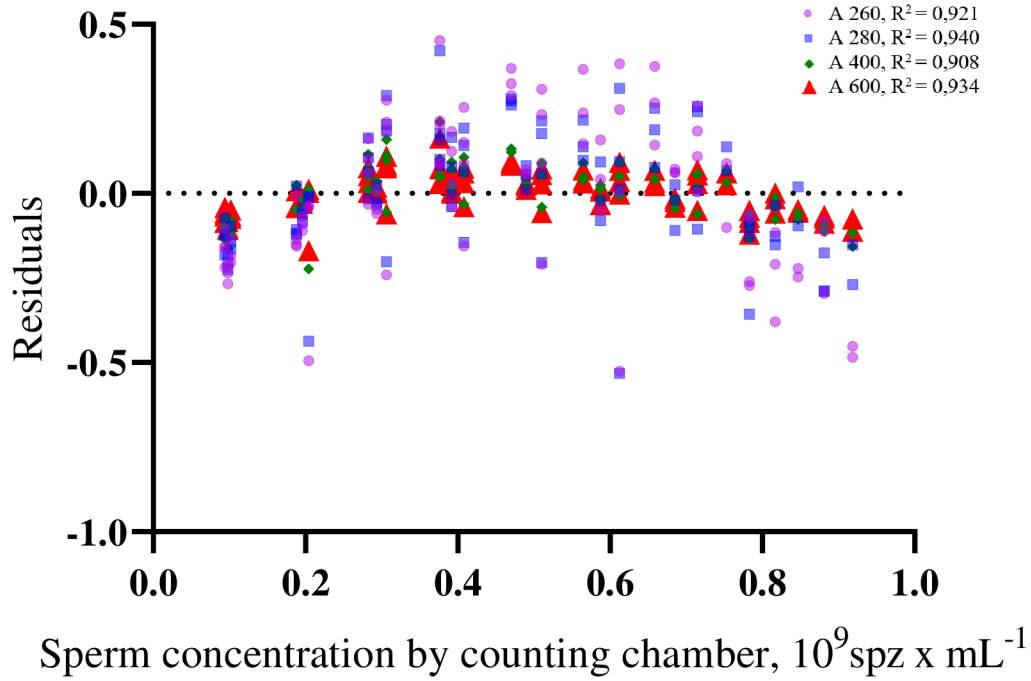

Figure 3. Residual plot for linear regressions of absorbance at different wavelengths (260, 280, 400, and 600 nm) versus sperm concentration evaluated by counting chamber.

For samples from nine males, serial dilution resulted in a significant linear correlation between absorbance and concentration (linear regression lines are presented in Fig. 4). The ANCOVA analysis detected no male-specific differences in slopes ($p = 0.90$) and intercepts ($p = 0.07$). The effective absorbance range for the standard curve was 0.2 – 0.8. Pooling the data from all samples, the equation between absorbance and sperm concentration was deduced: $Y = 0{,}84\ X - 0{,}09$ with an $R^2 = 0.95$, where X is the sperm concentration (10^9 spermatozoa in mL) and Y is the absorbance at 600 nm (Table 3).

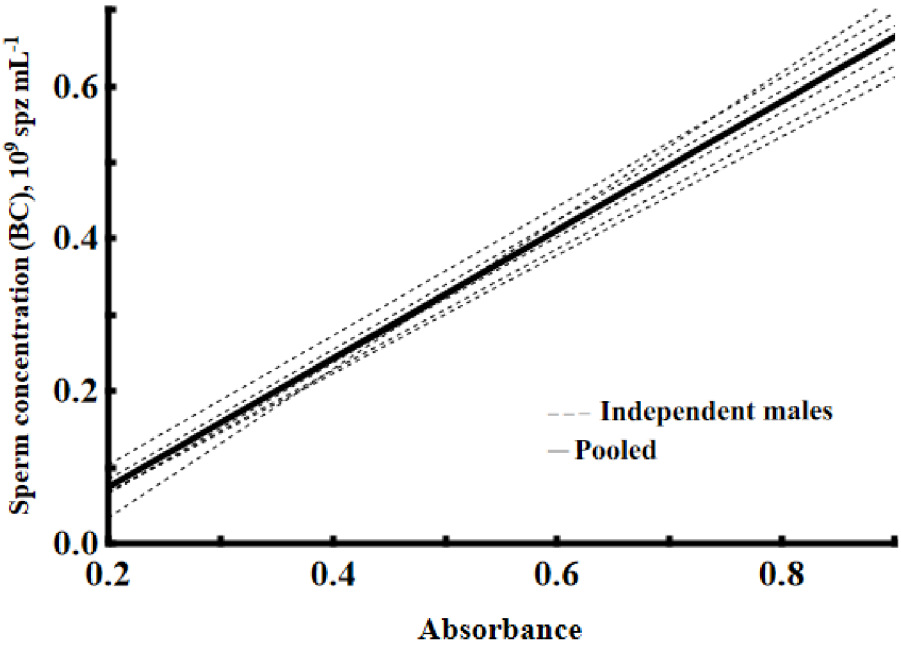

Figure 4. Linear regression lines of dependency between average sperm concentration for male measurement by Bürker chamber and absorbance at 600 nm. Nine lines were plotted from independent males data, and the bold line is a pooled regression line used further for the validation of the method. (BC) – sperm concentration evaluated by Bürker chamber. The coefficients of linear regression and coefficients of determination of regression lines are presented in Table 3. The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

Table 3. Coefficients of linear regression and coefficients of determination between absorbance and sperm concentration measured by Bürker chamber (n = 9). The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

	Male 1	Male 2	Male 3	Male 4	Male 5	Male 6	Male 7	Male 8	Male 9	Pooled
Slope	0.78	0.77	0.96	0.90	0.84	0.84	0.82	0.86	0.88	0.84
Y intercept	-0.08	-0.08	-0.15	-0.08	-0.10	-0.11	-0.09	-0.09	-0.12	-0.09
R^2	0.95	0.96	0.97	0.94	0.98	0.97	0.98	0.97	0.97	0.95

Sperm samples collected from another 15 males were used to validate the standard curve's applicability (Fig. 5). Methods of validation included correlation analysis of sperm concentration data obtained by both methods from these males and estimation of variances in sperm concentration measurement by both methods performed ten times for one sperm sample. A notably high coefficient of determination ($R^2 = 0.95$) was achieved

during validation (Fig. 5). No significant differences in variations of sperm concentration estimated ten times in the same sample by absorbance (CV = 6.84) or by the Bürker chamber (CV = 8.70) were found (Leven's test, p = 0.4871).

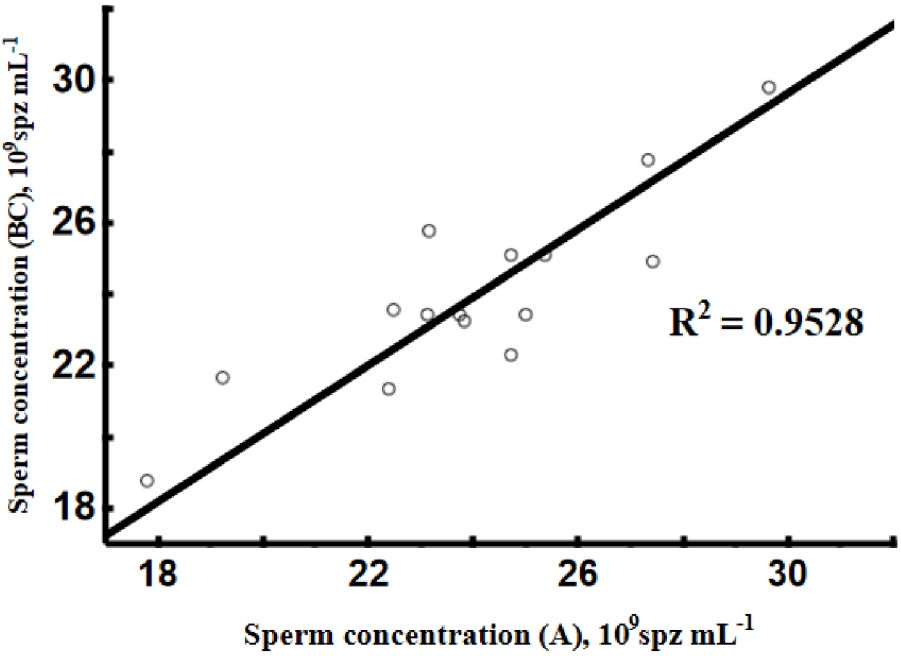

Figure 5. Regression line obtained during method validation using samples from 15 carp males. (BC), (A) – sperm concentration evaluated by Bürker chamber (BC) and absorbance (A) measurement, respectively. The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

4.2. Determination of the optimal concentration range for common carp sperm cryopreservation

4.2.1. Effect of sperm concentration before freezing on post-thaw sperm motility parameters

Motility percentage

Both approaches used for adjusting sperm concentration before freezing showed a similar downward trend in the percentage of motility post activation (Fig. 6 A). The most evident differences were found at 10 s post activation (the start of observation). Significant differences were found between groups (Kruskal-Wallis test, followed by multiple comparisons of mean ranks for all groups, $p < 0.05$). Motility percentage at 10 s

post activation in the control group was significantly higher than in all cases of thawed sperm (Fig. 6 B). The highest post-thaw sperm motility percentage from all experimental samples was observed at the lowest sperm concentration of 0.5 × 10^9 spz mL^{-1} (concentration adjusted by the first approach), and the lowest motility percentage was found for the highest concentration range of 11 – 13 × 10^9 spz mL^{-1} (concentration adjusted by the second approach). A significant decrease in motility percentage associated with high sperm concentration at freezing was found when both approaches of varying sperm concentration were applied. Motility percentage was significantly higher in samples with concentrations of 0.5 × 10^9 spz mL^{-1} (41 ± 3 %) compared to 5 × 10^9 spz mL^{-1} (27 ± 2 %) and 10 × 10^9 spz mL^{-1} (24 ± 7 %) for the first approach and between concentration ranges of 2.2 – 2.4 × 10^9 spz mL^{-1} (32 ± 2%) and 11 – 13 × 10^9 spz mL^{-1} (23 ± 3 %) for the second one.

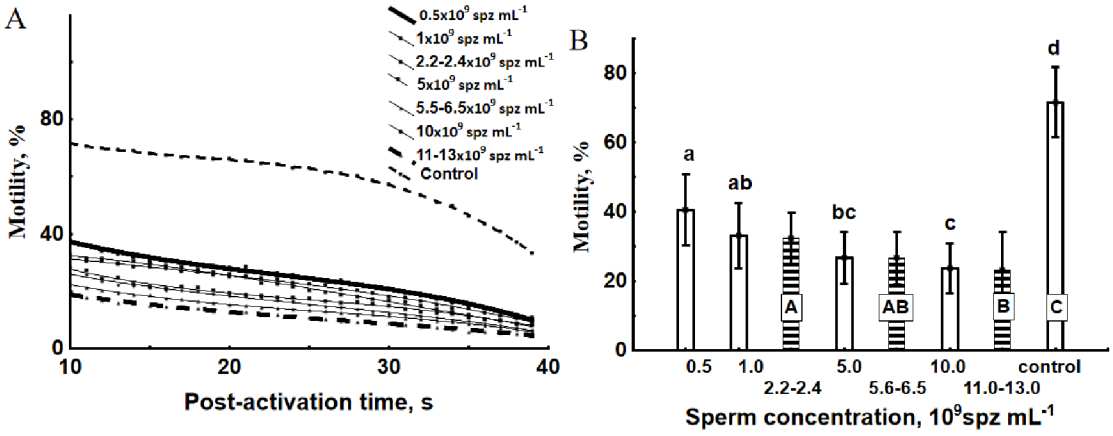

Figure 6. Sperm motility percentage in common carp sperm samples before and after cryopreservation. A) Polynomial regression lines of 3rd order of sperm motility percentage. B) Average post-thaw sperm motility percentage at 10 s post activation, mean ± SD. Data for the first approach of sperm concentration variation (i.e., constant dilution rate) are represented by white columns (values 0.5, 1, 5, and 10 × 10^9 spz mL^{-1}), values with different small letters differ significantly (Kruskal-Wallis test, followed by multiple comparisons of mean ranks for all groups, $p < 0.05$, $n = 18$). Data for the second approach of concentration variation (i.e., sperm dilution rate is different) are represented by dashed columns. Values with different capital letters are significantly different (Kruskal–Wallis test, followed by multiple comparisons of mean ranks for all groups, $p < 0.05$, $n = 16$). The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

Velocity and linearity

In all experimental cases, spermatozoa velocity decreased during the post-activation period, similarly to the motility percentage (Fig. 7 A). Significant differences were observed only between control and cryopreserved sperm samples, while no significant differences between cryopreserved groups were found (Kruskal-Wallis test, followed by multiple comparisons of mean ranks for all groups, $p < 0.05$) (Fig. 7 B).

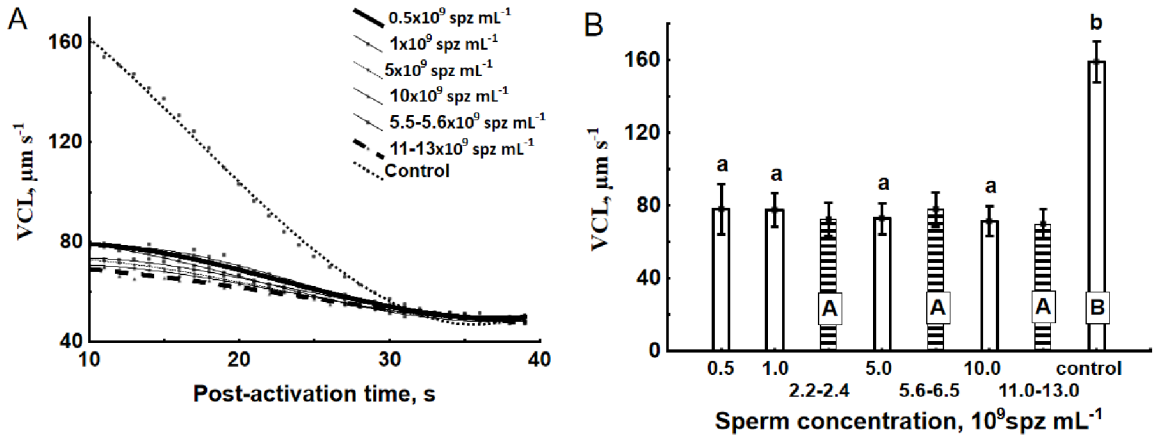

Figure 7. Sperm curvilinear velocity (VCL) in common carp sperm samples before and after cryopreservation. A) Polynomial regression lines of 3rd order of VCL. B) Average post-thaw VCL at 10 s post activation, mean ± SD. Data for the first approach of sperm concentration variation (i.e., constant dilution rate) are represented by white columns (values 0.5, 1, 5, and 10×10^9 spz mL^{-1}), values with different small letters differ significantly (Kruskal–Wallis test, followed by multiple comparisons of mean ranks for all groups, $p < 0.05$, $n = 18$). Data for the second approach of concentration variation (i.e., sperm dilution rate is different) are represented by dashed columns. Values with different capital letters are significantly different (Kruskal–Wallis test, followed by multiple comparisons of mean ranks for all groups, $p < 0.05$, $n = 16$). The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

The trend lines for LIN changes during the post-activation time for different groups were in quite a narrow range (min/max: 84 – 92 % among all experimental groups) with the maximum differences at 30 s post activation (Fig. 8). Significant differences in LIN at 30 s post activation were found only between control and post-thaw samples, while no significant differences were found within the post-thaw samples (Fig. 8).

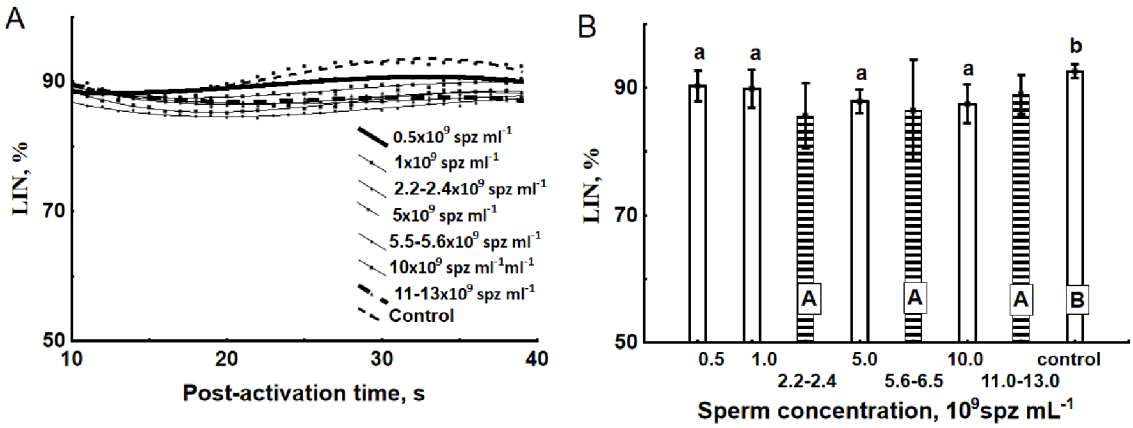

Figure 8. Path linearity (LIN) in common carp sperm samples before and after cryopreservation. A) Polynomial regression lines of 3rd order of LIN. B) Average post-thaw sperm LIN at 10 s post activation, mean ± SD. Data for the first approach of sperm concentration variation (i.e., constant dilution rate) are represented by white columns (values 0.5, 1, 5, and 10 × 10^9 spz mL^{-1}), values with different small letters differ significantly (Kruskal–Wallis test, followed by multiple comparisons of mean ranks for all groups, $p < 0.05$, $n = 18$). Data for the second approach of concentration variation (i.e., sperm dilution rate is different) are represented by dashed columns. Values with different capital letters are significantly different (Kruskal–Wallis test, followed by multiple comparisons of mean ranks for all groups, $p < 0.05$, $n = 16$). The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

4.2.2. Development and hatching rates after in vitro fertilization with cryopreserved semen

From data analysis it was observed that sperm/egg ratio has a significant effect on fertilization success (two-way ANOVA; $p = 0.007$ for development rate and $p = 0.008$ for hatching rate). In contrast, the effect of sperm dilution rate was insignificant ($p = 0.717$ for development rate and $p = 0.58$ for hatching rate), and the interaction between these two factors (sperm sample dilution rate and sperm/egg ratio) was also nonsignificant ($p=0.995$ for development rate and $p = 0.96$ for hatching rate).

That is the reason why the exclusion of the sample dilution rate from the next step of statistical analysis was made. Significantly better development (47 ± 3 %) and hatching rates (41 ± 4%) were observed at the highest studied sperm per egg ratio 450 000: 1. Significant differences were also found between groups fertilized at 30 000: 1 and 150 000: 1 sperm per egg ratio in terms of fertilization rate (16 ± 4% and 27 ± 2 %, respectively) and hatching rate (15 ± 2 % and 25 ± 2 %, respectively). From this experiment, it is obvious that the 450 000 sperm per egg ratio at application of sperm after cryopreservation is still insufficient to get a fertilization rate corresponding to that at the application of native (not cryopreserved) sperm (Fig. 9 A-B).

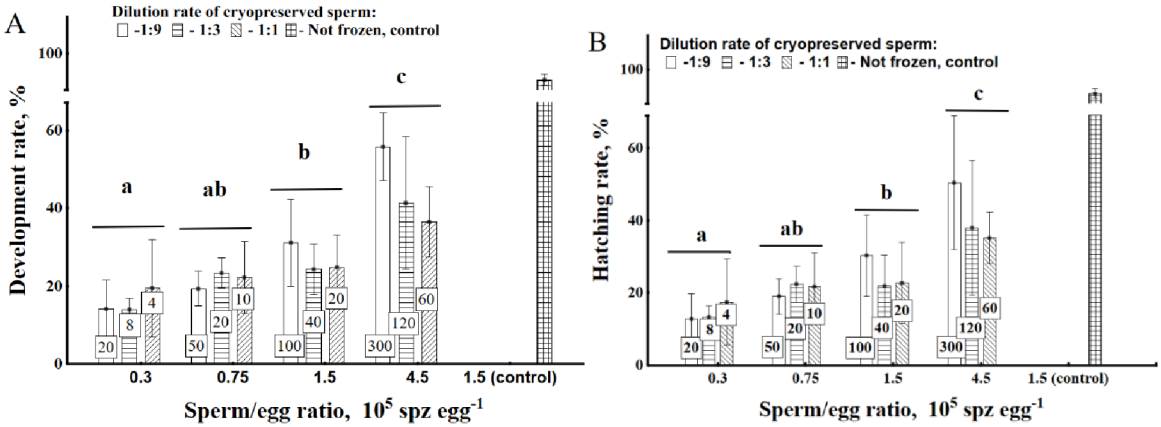

Figure 9. Development rates (A) and hatching rates (B) obtained after the application of sperm samples cryopreserved at different spermatozoon concentrations and different sperm/egg ratios. Sperm samples with the concentrations adjusted by the cryoprotective medium (the second approach) were used in experiments. The data are presented as mean ± SD. Values with different letters are significantly different ($p < 0.05$; Tukey post-hoc test). Values (in µl) in boxes represent the volume of sperm used for fertilization of 2 g eggs. Control is a value obtained after fertilization by fresh, not frozen sperm to see the quality of eggs and these data are not involved in statistical analysis. The data were presented in the publication by Sotnikov et al. (2023), with Stechkina as a co-author.

5. Discussion

This study confirmed the assumption that, due to close cell-to-cell interactions (Morris et al., 2012), more concentrated sperm samples after cryopreservation account for a smaller percentage of alive cells than in low-concentrated samples. However, in the case of common carp, this low percentage can be compensated by high sperm content in such samples, which is sufficient for successful fertilization, especially when using a high sperm per egg ratio during the fertilization process. That is why the elaborated in the current work method for concentration estimation in common carp sperm would be useful for the future application in practice. Generally, the objectives of the study were fulfilled during experiments, and the relationships between obtained data and current knowledge in the field are discussed below.

5.1. Spectrophotometric determination of sperm concentration

Osmolality of diluent used for sperm concentration measurement

The osmolality was measured for carp seminal fluid by several authors, and the value varies in the range of 276 – 392 mOsm kg^{-1} (Billard et al., 1995; Dzyuba et al., 2013). In the current work for the determination of sperm concentration, the dilution medium with an osmolality of 300 mOsm kg^{-1} was used as the closest to the average isotonic level in carp sperm. However, different reactions of spermatozoa from different males may still take place in this environment due to the individual sperm plasma composition. Exposure to a more hypotonic environment leads to swelling of the cells, and on the contrary, in a hypertonic medium, cells will shrink. These changes in cell volumes can be reflected in the absorbance value of samples and contribute enough to some heterogeneity of measurement outcomes.

Potentially, using another osmolality that would definitely change the cell volumes in the same way for different males could be more suitable for the spectrophotometry method of concentration estimation in carp semen. Alteration of environmental osmolality could differentially affect fish sperm, depending on species, because of differences in their osmotic- or ionic-dependent spermatozoa motility activation mode (Bondarenko et al., 2013). Carp exhibits a hypotonic mode of spermatozoa motility activation. Within 1 second, cells respond to environmental hypotonicity with activation of motility characterized by rearrangement of intracellular ion concentration, changes in

membrane structure, and spermatozoa volume increase (Dzyuba et al., 2013). A strong correlation was found between light absorbance alteration and carp spermatozoa head volume change (Bondarenko et al., 2013). Spermatozoa, which were increasing their volume due to the water transport in hypotonic solutions, showed decreased absorbance, and these changes had a dynamic character. Much less information is available about lower, compared to seminal plasma, osmolarity. Generally, a hypertonic environment reduces the cell volume and, in freshwater fishes, inhibits sperm motility.

Testing the influence of different osmolarity of diluents during absorption measurement may have a positive contribution to the accuracy of the method for estimating sperm concentration by spectrophotometry.

Wavelengths used for sperm concentration measurement

The 600 nm wavelength was selected in this work to make an absorbance dependence plot between absolute concentration measured by the Bürker chamber and optical absorbance. At this wavelength, the maximum sensitivity for measurements was observed. However, according to some assumptions, the wavelength 260/280 nm, where the light absorption by DNA molecules occurs, may be more suitable for this purpose. Further study is needed to confirm this assumption. On the other hand, an advanced type of spectrophotometer is necessary to study the UV absorption spectrum, and this factor can be limiting in the use of this wavelength in practice due to the expense of such devices.

Full spectrum analysis

In 2020, Lesani et al. combined a well-elaborated spectrophotometry method with machine learning and artificial neural networks. During the experiment, they evaluated human sperm concentration by establishing a correlation between the full absorption spectrum and sperm concentration, thereby eliminating the most critical step of a traditional optical absorbance approach - the need to search the most appropriate wavelength for accurate prediction of sperm concentration, which varies from species to species and/or depending on device setups. Sperm concentration from the full spectrum neural network (FSNN) model strongly correlated ($R^2 = 0.98$) with clinical measurements and demonstrated a prediction accuracy of 93 %, with over 15% improvement over the linear regression model.

At that time, the authors lacked sufficient number of sample s for better validation of a model and its clinical adoption. However, from published results, it was evident that

the FSNN model provides a rapid and powerful tool for quantifying sperm concentration, improving current spectrophotometry methods for semen analysis (Lesani et al., 2020).

It is not excluded that the full spectrum neural network model will be adopted (with some elaborations) to the fishery field, and the spectrophotometric method for sperm concentration estimation in fish will develop in this direction.

5.2. Determination of the optimal concentration range for sperm cryopreservation in common carp

Sperm concentration in samples is one of the key elements of the standardized cryopreservation procedure as an appropriate dilution of sperm is required to avoid the packing effect. The optimum concentration during the freezing process is species-specific and can be related to the spermatozoon dimension, the biochemical composition of the cell membrane, and sperm plasma (Morris et al., 2012). For some species, this value has already been found. The impact of sperm concentration in the samples during freezing on cryopreservation outcomes in fish was studied in species representatives from different taxa like Salmonidae (Lahnsteiner et al., 1996; Nynca et al., 2017; Judycka et al., 2018; Judycka et al., 2019a), Esocidae (Lahnsteiner, 2000), Percidae (Bernath et al., 2016), Acipenceridae (Nascimento et al., 2021) and Cyprinidae (Lahnsteiner et al., 2000). Our study provides a detailed description of the effect of sperm concentration on post-thaw outcomes in common carp.

The obtained data allowed us to conclude that the lower concentration during freezing has a positive correlation with post-thaw motility percentage, while a significant decrease in sperm motility percentage associated with an increase in sperm concentration at freezing appears in the range of 2.4 and 5.6 × 10^9 spz mL^{-1}. The value of sperm concentration at which a 50% decline in the post-thaw percentage of sperm motility caused by cell packing will appear is predicted to be 12.7 × 10^9 spz mL^{-1}.

The results suggest that the packing effect was responsible for the decrease in post-thaw motility percentage, while it did not significantly influence the kinetic parameters of spermatozoa (see Fig. 6 – 8). The observed in the present study negative effect of increased sperm concentration during cryopreservation on motility percentage corresponds to the same trend found for trout (Judycka et al., 2019a). A decrease in the velocity of spermatozoa after thawing compared with control value is a well-known phenomenon observed by different researchers (e.g., Lahnsteiner et al., 2000;

Boryshpolets et al., 2009; Dzyuba et al., 2010; Boryshpolets et al., 2017). The results of this thesis documented the same trend. No additional decrease in velocity caused by the packing effect was found in the current study (Sotnikov et al., 2023).

During the dilution of samples for cryopreservation, two different approaches were used in this study. Results on motility parameters after thawing (Fig. 7) demonstrated no significant differences between samples diluted by seminal plasma and cryoprotective medium and using cryoprotective medium only. The reduction in post-thaw sperm motility parameters and fertilizing ability were caused mainly by sperm concentration in samples. The additional positive effect of seminal plasma presence during cryopreservation on sperm cryoresistance (Lahnsteiner, 2007; Shaliutina-Kolesova et al., 2020) was not confirmed in this study (Sotnikov et al., 2023). The first approach, which incorporated the dilution of sperm with seminal plasma, is more complicated in realization in practice. Since the dilution of sperm samples with seminal plasma didn't influence outcomes, only samples diluted with the cryoprotective medium were used to fertilize eggs.

Embryo development and hatching rates after fertilization with cryopreserved semen

The preservation of fertilization ability is the most crucial goal of sperm cryopreservation as an applied method. Sperm assessment using the CASA system allows the rapid prediction of sperm's ability to fertilize oocytes (Kime et al., 2001; Rurangwa et al., 2001). However, the fertilization process is equally essential for cryopreservation outcomes studies. Whereas insemination in practice can be implemented with the highly concentrated samples, the experimental evaluation of the fertilizing ability of sperm samples may be performed by keeping a particular sperm per egg ratio, which allows a correct comparison of results obtained in different studies. An excessive number of spermatozoa could mask the impact of applied approaches, and that is why it should be specifically adjusted according to the purpose of fertilization trials. The optimal sperm to egg ratio is based on the purpose of the fertilization trial. Table 4 illustrates some data related to this issue. Experiments in this thesis were focused on determining the optimal concentration of sperm during freezing resulting in high outcomes and, at the same time took into account the problem of increasing cryostorage capacity.

Table 4. Sperm to egg ratio used for different application purposes.

Purpose	Minimum	Optimum	Maximum	Supplementary information	Source
	Sperm per egg ratio				
Hatchery practice; normal native sperm	10 000	20 000 – 30 000	Studied value: 200 000; without negative impact on fertilization and hatching processes	Native sperm is generally characterized by high initial motility percentage; sperm:eggs:water ratio is suggested as 2-10:1000:500 in volume	Gela et al., 2009; Linhart et al., 2015
Experimental work; manipulated sperm (not cryopreserved)	1 000	5 000	10 000	Sperm:eggs: water ratio is suggested as 1:500:500-1000 in volume	Linhart et al., 2015; Magyary et al., 1996; Linhart et al., 2008
Hatchery practice; thawed sperm	–	100 000 – 300 000	Is limited by sample availability	–	Rodina et al., 2010
Experimental work; cryopreserved sperm	–	100 000 – 150 000	–	DMSO as cryoprotectant (10%), dilution rate 1:9; sperm: egg: water ratio 1:20:20 in volume	Magyary et al., 1996; Levanduski and Cloud, 1988

In the current thesis, the averaged spermatozoa per egg ratios were 30 000, 75 000, 150 000, and 450 000 (Table 2). We obtained lower developing and hatching rates for cryopreserved sperm compared to values when control (fresh) sperm was used. As well,

the decrease in sperm motility was observed (20 – 40 % vs 80 – 100 % in fresh sperm). Although the final concentration of motile sperm in both cases was similar or exceeded the sperm per egg ratio routinely used in hatchery practice, this amount of spermatozoa per egg can be considered insufficient (probably due to the decreased post-thaw spermatozoa velocity), and that is why it should be increased to achieve the maximum fertilization rate.

6. Conclusions

Elaboration of a quick method for sperm concentration estimation in carp

The obtained data suggest that, for common carp, the method of optical absorbance can be applied in practice as a reliable and efficient alternative to cell concentration estimation by haemocytometer. The advantages of this method include time savings and a smaller sample volume. The use of 300 mOsmol solutions for sample dilution and measurements at the wavelength of 600 nm are suggested. The absorbance of sperm suspension during the determination of sperm concentration is proposed to be in the range of 0.1 – 0.9 for effective measurement applying this method.

Concentration of sperm during cryopreservation

Common carp spermatozoa can survive cryopreservation at high cell concentrations, and cryopreservation of samples at high density can be used for future development of the most effective carp cryoprotocols applicable for aquaculture.

7. References

Asturiano, J. F., Cabrita, E., Horváth, Á., 2017. Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. General and Comparative Endocrinology, 245, 69–76.

Balon, E.K., 1995. Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman gourmets to the swimming flowers. Aquaculture, 129 (1–4), 3-48.

Bernath, G., Bokor, Z., Zarski, D., Varkonyi, L., Hegyi, A., Staaszny, A., Urbanyi, B., Radoczi, J., Horvath, A., 2016. Commercial-scale out-of-season of Eurasian perch (*Perca fluvialis*) sperm and its application for fertilization. Animal Reproduction Science, 170, 170–177.

Billard, R., Breton B., Jalabert B., 1971. La production spermatogénétique chez la truite. Annales de Biologie Animale Biochimie Biophysique, 11, 199-212.

Boryshpolets, S., Dzyuba, B., Rodina, M., Li, P., Hulak, M., Gela, D., Linhart, O., 2009. Freeze-thawing as the factor of spontaneous activation of spermatozoa motility in common carp (*Cyprinus carpio* L.). Cryobiology, 59, 291–296.

Boryshpolets, S., Sochorova, D., Rodina, M., Linhart, O., Dzyuba, B., 2017. Cryopreservation of carp (*Cyprinus carpio* L.) sperm: impact of seeding and freezing rates on post-thaw outputs. Biopreservation and Biobanking, 15, 234–240.

Cabrita, E., Sarasquete, C., Martínez-Paramo, S., Robles, V., Beirao, J., Perez-Cerezales, S., Herra, M.P., 2010. Cryopreservation of fish sperm: applications and perspectives. Journal of Applied Ichthyology, 26, 623–635.

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.

Ciereszko, A., Dabrowski, K., 1993. Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique. Aquaculture, 109, 3-4.

Cejko, B.I., Sarosiek, B., Krejszeff, S., Kowalski, R.K., 2018. Multiple collections of common carp *Cyprinus carpio* L. semen during the reproductive period and its effects on sperm quality. Animal Reproduction Science, 188, 178-88.

Cejko, B. I., Żarski, D., Sarosiek, B., Dryl, K., Palińska-Żarska, K., Skorupa, W., Kowalski, R.K., 2022. Application of artificial seminal plasma to short-term storage of a large volume of common carp (*Cyprinus carpio*) sperm for two weeks under controlled conditions. Aquaculture, 546, 737385.

Cognie, F., Billard, R., Chao, N.H., 1989. La cryoconservation de la laitance de la carpe, *Cyprinus carpio*. Journal of Applied Ichthyology, 5, 165-176.

Cuevas-Uribe, R., 2011. A general approach for vitrification of fish sperm. Louisiana State University, Doctoral Dissertations, 3597.

Cugia, G., Centis, F., Del Zotto, G., Lucarini, A., Argazzi, E., Zini, G., Valentini, M., Bono, M., Picardi, F., Stramigioli, S., Cesarini W., Zamai, L., 2011. High Survival of Frozen Cells Irradiated with GAMMA Radiation. Radiation Protection Dosimetry, 143, 2–4, 237–240.

Dettlaff, T.A., Ginsburg, A.S., Schmalhausen, O.I., 1993. Sturgeon Fishes. Developmental Biology and Aquaculture. N. K. Koltsov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia, 65-66.

Dietrich, M.A. Irnazarow, I., Ciereszko A., 2017. Proteomic identification of seminal plasma proteins related to the freezability of carp semen. Journal of Proteomics 1-10.

Dzuba, B.B., Kopeika, E.F., 2002. Relationship between the changes in cellular volume of fish spermatozoa and their cryoresistance. Cryoletters. 23 (6), 353–360.

Dzyuba, B., Boryshpolets, S., Rodina, M., Gela, D., Linhart, O., 2010. Spontaneous activation of spermatozoa motility by routine freeze-thawing in different fish species. Journal of Applied Ichthyology, 26, 720–725.

Dzyuba, B., Cosson, J., Yamaner, G., Bondarenko, O., Rodina, M., Gela, D., Bondarenko V., Shaliutina A., Linhart, O., 2013. Hypotonic treatment prior to freezing improves cryoresistance of common carp (*Cyprinus carpio* L.) spermatozoa. Cryobiology, 66, 2, 192–194.

Fanson, B.G., Hale, R., Thiem, J.D., Lyon, J.P., Koehn, J.D., Bennett, A.F., Stuart, I., 2024. Assessing impacts of a notorious invader (common carp *Cyprinus carpio*) on Australia‘s aquatic ecosystems: Coupling abundance-impact relationships with a spatial biomass model. Biological Conservation, 2024, 110420.

FAO 2024. Czechia. Text by Adamek, Z. and Kouril, J. In: Fisheries and Aquaculture. Rome. [Cited Tuesday, March 19th 2024].

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., Suquet, M., 1999. Characteristics of captive seabass in relation to fertilization potential. Journal of Fish Biology, 54, 356369.

Flajšhans, M., Rodina, M., Linhart, O., Dzyuba, B., Boryshpolets, S., 2015. Experience with fish sperm cryobanking in the Czech Republic. Cryobiology, 71, 556.

Flajšhans, M., Linhart, O., Šlechtova, V., Šlechta, V., 1999. Genetic resources of commercially important fish species in the Czech Republic: present state and future stratégy. Aquaculture, 173, 471-483.

Fuller, B. J., 2004. Cryoprotectants: the essential antifreezes to protect life in the frozen state. Cryoletters, 25(6): 375-388.

Garman, E., 2003. Cool' Crystals: Macromolecular Cryocrystallography and Radiation Damage. Current Opinion in Structural Biology, 13(5): 545–551.

Gela D., Kocour M., Rodina M., Flajšhans M., Beránková P., Linhart O., 2009. Technologie řízené reprodukce kapra obecného *(Cyprinus carpio L.).* Jihočeská univerzita v Českých Budějovicích fakulta rybářství a ochrany vod. ISBN 978-80-85887-99-0.

Holt W.V., 2000. Basic aspects of frozen storage of semen. Animal Reproduction Sciense, 62, 3-22.

Horváth A., Miskolczi E., Urbányi B., 2003. Cryopreservation of common carp sperm. Aquatic Living Resources, 16(5), 457–460.

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.

Horváth, A., Miskolczi, E., Urbányi, B., 2003. Cryopreservation of common carp sperm. Aquatic Living Resources, 16, 5, 457 – 460.

Hulak, M., Kaspar, V., Kohlmann, K., Coward, K., Tešitel, J., Rodina, M., Gela, D., Kocour M., Linhart O., 2010. Microsatellite-based genetic diversity and differentiation of foreign common carp (*Cyprinus carpio*) strains farmed in the Czech Republic. Aquaculture, 298, 3–4, 194-201.

Jamieson, B.G.M., 1991. Fish Evolution and Systematics: Evidence from Spermatozoa. Cambridge, United Kingdom, 56-224.

Judycka, S., Nynca, J., Liszewska, E., Dobosz, S., Grudniewska, J., Ciereszko, A., 2018. Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa. Aquaculture, 486, 90–97.

Judycka, S., Zarski, D., Dietrich, M.A., Palinska- Zarska, K., Karol, H., Ciereszko, A., 2019a. Standardized cryopreservation protocol of European perch (*Perca fluviatilis*) semen allows to obtain high fertilization rates with the use of frozen/thawed semen. Aquaculture, 498, 208–216.

Judycka, S., Nynca, J., Ciereszko, A., 2019b. Opportunities and challenges related to the implementation of sperm cryopreservation into breeding of salmonid fishes. Theriogenology, 132, 12–21.

Judycka, S., Nynca, J., Hliwa, P., Ciereszko, A., 2021. Characteristics and Cryopreservation of Semen of Sex-Reversed Females of Salmonid Fish. International Journal of Molecular Sciences, 22 (2), 964.

Kaspar, V., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Kocour, M., Hadi Alavi M.A., Hulák M., Linhart, O., 2007. Equalizing sperm concentrations in a common carp (*Cyprinus carpio*) sperm pool does not affect variance in proportions of larvae sired in competition. Aquaculture, 272, 204–209.

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? In book: Current Frontiers in Cryobiology. CELLTRONIX and Sanford-Burnham Institute for Medical Research, San Diego, California, USA.

Kime, D.E., Van Look, K.J.W., McAllister, B.G., Huyskens, G., Rurangwa, E., Ollevier, F., 2001. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comparative biochemistry and physiology, 130 (4), 0–433.

Kohlmann, K., Gross, R., Murakaeva, A., Kersten, P., 2003. Genetic variability and structure of common carp (*Cyprinus carpio*) populations throughout the distribution range inferred

from allozyme, microsatellite and mitochondrial DNA markers. Aquatic Living Resources, 16, 421–431.

Kocour, M., Gela, D., Šlechtová ,V., Kopecká, J., Šlechta, V., Rodina, M., Flajšhans, M., 2008. Carp breeds of the Czech Republic. Catalogue of Carp Breeds (*Cyprinus caprio* L.) of the Countries of Central and Eastern Europe. Ministry of agriculture of the Russian Federation, ISBN 978-5-9974-0063-7. 14.

Kopeika, E., Kopeika, J., Zhang, T., 2007. Cryopreservation of Fish Sperm. Methods in Molecular Biology, 203–217.

Kossmann, H., 1973. Versuch zur Konservierung des Karpfenspermas (*Cyprinus carpio*). Archives fur Fischereiwissenschaft, 23: 125-128.

Kowalski, R.K., Cejko, B.I., 2019. Sperm quality in fish: determinants and affecting factors. Theriogenology, 135, 94–108.

Kumar, K.V., Reddy, B.R., Krishna, K.S., 2013. Comparison of different methods for assessing sperm concentration in infertility workup: A review. International Journal of Medicine and Medical Sciences, 5(9): 396-400.

Kurokura, H., Hirano R., Tomita M., Iwahashi M., 1984. Cryopreservation of carp sperm. Aquaculture, 37: 267-273.

Labbé, C., Robles V., Herraez M.P., 2013. Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. In: Allan, G., Burnell, G. (Eds.) Advances in Aquaculture Hatchery Technology. University College Cork, Ireland, 76-116.

Lahnsteiner, F., Patzner, R.A., Weismann, T., 1996. Semen cryopreservation of salmonid fishes: influence of handling parameters on the post-thaw fertilization rate. Aquaculture Research, 27, 659–671.

Lahnsteiner, F., 2000. Semen cryopreservation in the Salmonidae and in the Northern pike. Aquaculture Research, 31(3), 245–258.

Leclercq, E., Antoni, L., Bardon-Albaret, A., Anderson, C. R., Somerset, C. R., Saillant, E. A., 2012. Spectrophotometric determination of sperm concentration and short-term cold-storage of sperm in Atlantic croaker *Micropogonias undulatus* L. broodstock. Aquaculture Research, 45(8), 1283–1294.

Lesani, A., Kazemnejad, S., Zand, M.M., Azadi, M., Jafari, H., Mofrad, M.R.K., Nosrati, R., 2020. Quantification of human sperm concentration using machine learning-based spectrophotometry. Computers in Biology and Medicine, 104061.

Levanduski, M. J., Cloud, J.G., 1988. Rainbow Trout (*Salmo gairdneri*) Semen: Effect of Non-motile Sperm on Fertility. Aquaculture, 75 171-179.

Linhart, O., Alavi, S.M.H., Rodina, M., Gela, D., Cosson, J., 2008: Comparison of sperm velocity, motility and fertilizing ability between firstly and secondly activated spermatozoa of common carp (*Cyprinus carpio*). Journal of Applied Ichthyology, 24, 386–396.

Linhart, O., Rodina, M., Boryshpolets, S., 2011. Hodnocení čerstvého spermatu ryb. Fakulta rybářství a ochrany vod jihočeská univerzita v českých budějovicích. ISBN 978-80-87437-32-2.

Linhart, O., Rodina, M., Kašpar, V., 2015. Common carp (Cyprinus carpio Linneaus, 1758) male fertilization potency with secure number of spermatozoa per ova. Journal of Applied Ichthyology, 31, 169–173.

Lubzens, E., Daube, N., Pekarsky, I., Magnus, Y., Cohen, A., Yusefovich, F., Feigin, P., 1997. Carp (*Cyprinus carpio* L.) spermatozoa cryobanks - strategies in research and application. Aquaculture, 155, 13-30.

Magyary, I., Urbányi, B., Horváth, L., 1996. Cryopreservation of common carp (*Cyprinus carpio* L.) sperm II. Optimal conditions for fertilization. Journal of Applied Ichthyology, 12(2), 117–119.

Magyary, I., Urbányi, B., Horváth, Á., Dinnyés, A., 2011. Cryopreservation of Gametes and Embryos of Cyprinid Fishes. In: Cryopreservation in Aquatic Species, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana, 525-538.

Martínez-Páramo, S., Horváth, A., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Terrence, R.T., Cabrita, E., 2017. Cryobanking of Aquatic Species. Aquaculture, 472: 156–77.

Mattei, X., 1991. Spermatozoon ultrastructure and its systematic implications in fishes. Canadian Journal of Zoology, 69, 3038–3055.

Mazur P., 2004. Principles of Cryobiology. In Life in the Frozen State edited by Fuller, B.J., Lane N., Benson E.E. ISBN 0-415-24700-4. 3-66.

Moczarski, M., 1977. Deep freezing of carp (*Cyprinus carpio L.*) sperm. Bulletin of the Academy of Poland, Serial Biology, 24:187-190.

Morris, J,G., Acton, E., Murray, B.J., Fonseca, F., 2012. Freezing injury: the special case of the sperm cell. Cryobiology, 64, 71–80.

Napora-Rutkowski, L., Rakus, K., Nowak, Z., Szczygieł, J., 2017. Genetic diversity of common carp (*Cyprinus carpio L.*) strains breed in Poland based on microsatellite, AFLP, and mtDNA genotype data. Aquaculture, 473: 433-442.

Nascimento, J.P., Horokhovatskyi, Y., Kholodnyy, V., Rodina, M., Dzyuba, V., Stechkina, T., Boryshpolets S., Dzyuba, B., 2021. Optimization of sterlet sperm concentration for cryopreservation. Aquaculture, 540, 736682.

Nynca, J., Judycka, S., Liszewska, E., Dobosz, S., Ciereszko, A., 2017. Standardization of spermatozoa concentration for cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture, 477, 23–27.

Pataki, B., Horváth, A., Mészáros, G., Kitanović, N., Ács, A., Hegyi, Á., Molnár, J., Csorbai, B., Urbányi, B., 2022. Adjustment of common carp sperm concentration prior to cryopreservation: Does it matter? Aquaculture Reports, 24, 101109.

Pokorný, J., Flajšhans, M., Hartvich, P., Kvasnička, P., Pružina, I., 1995. Atlas kaprů chovaných v České republice. Atlas of Common Carps Bred in the Czech Republic. Victoria Publishing, Praha, 69 pp. in Czech, with English summary.

Rana, K., 1995. Cryopreservation of aquatic gametes and embryos: Recent advances and applications. In: 5th International Symposium on Reproductive Physiology of Fish, July 2-8, 1995, Austin, Texas (in press).

Radford, P., Herdan, G., 1961. A nephelometric method for estimating spermatozoan density of gel-free boar spermatozoa. Vet. Rec., 73: 759-76.

Rodina, M., Dzyuba, B., Boryshpolets, S., Linhart, O. 2010. Zmrazování spermatu kapra obecného (*Cyprinus carpio L.*) pro potřeby uchování genofondu v praktických podmínkách. Jihočeská univerzita v Českých Budějovicích, Fakulta rybářství a ochrany vod, Jihočeské výzkumné centrum akvakultury a biodiverzity hydrocenóz a Výzkumný

ústav rybářský a hydrobiologický, Zátiší 728/II, 389 25 Vodňany. ISBN 978-80-87437-07-0.

Rurangwa, E., Roelants, I., Huyskens, G., Ebrahimi, M., Kime, D.E., Ollevier, F., 1998. The minimum effective spermatozoa: egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilization ability in *Clarias gariepinus*. Journal of Fish Biology, 53, 402-413.

Rurangwa, E., Volckaert, F.A.M., Huyskens, G., Kime, D.E., Ollevier, F., 2001. Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilisation in African catfish (*Clarias gariepinus*). Theriogenology, 55, 751 – 769.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture, 234(1-4), 0–28.

Shaliutina-Kolesova, A., Xian, M., Nian, R., 2019. Antioxidant defence system in protein fractions of common carp (*Cyprinus carpio*) seminal plasma. Czech Journal of Animal Science, 64, 6, 265–271.

Sneed, K.E., Clemens M.D., 1956. Survival of fish sperm after freezing and storage at low temperatures. The Progressive Fish-Culturist 17: 99-103.

Sotnikov, A., Rodina, M., Stechkina, T., Benevente, C.F., Gela, D., Boryshpolets, S., Kholodnyy, V., Linhart, O., Dzyuba, B., 2023. High sperm concentration during cryopreservation decreases post-thaw motility percentage without compromising in vitro fertilization outcomes in common carp. Aquaculture, 562, 738746.

Stein, H., Bayrle H., 1978. Cryopreservation of the sperm of some freshwater teleosts. Annales de Biologie animale Biochimie Biophysique 18:1073-1076.

Stoss, J., Holtz, W., 1981. Cryopreservation of rainbow trout (*Salmo gairdneri*) sperm. 1. Effect of thawing solution, sperm density and interval between thawing and insemination. Aquaculture, 22: 97-104.

Takashima, F., Weil C., Billard R., Crim L., Fostier A., 1984. Stimulation of spermination by LHRH analogue in carp. Bulletin of the Japanese Society of Scientific Fisheries 50: 1323-1329.

Tan E., Yang H.H., Tiersch T.R., 2010. Determination of Sperm Concentration for Small-Bodied Biomedical Model Fishes by Use of Microspectrophotometry. Zebrafish, 7, 2.

Tiersch, T.R., 2008. Strategies for commercialization of cryopreserved fish semen. Revista Brasileira de Zootecnia, 37, 15-19.

Tiersch, T.R., Green, C.C., 2011. Cryopreservation in Aquatic Species, 2nd Edition. World Aquaculture Society. Baton Rouge, Louisiana, USA. ISBN: 978-1-888807-20-2 Cryopreservation of Gametes and Embryos of Cyprinid Fishes. 525-538.

Warnecke, D., Pluta, H-J. 2003. Motility and fertilizing capacity of frozen/thawed common carp (*Cyprinus carpio L.*) sperm using dimethyl-acetamide as the main cryoprotectant. Aquaculture, 215, 167 – 185.

Wei, J., Carroll, R.J., Harden, K.K., Wu, G., 2012. Comparisons of treatment means when factors do not interact in two-factorial studies. Amino Acids, 42, 2031–2035.

Woynarovich, A., Bueno, P.B., Altan, Ö., Jeney, Z., Reantaso, M., Xinhua, Y., Van Anrooy, R., 2011. Better Management Practices for Carp Production in Central and Eastern Europe, the Caucasus and Central Asia. FAO Fisheries and Aquaculture Technical Paper 566. Ankara, FAO.

Yang, H., Tiersch, T.R., 2009. Current status of sperm cryopreservation in biomedical research fish models: Zebrafish, medaka, and Xiphophorus. Comparative Biochemistry and Physiology, C, 149, 224–232.

Yang, H., Daly, J., Tiersch, T.R., 2015. Determination of sperm concentration using flow cytometry with simultaneous analysis of sperm plasma membrane integrity in zebrafish *Danio rerio*. Cytometry, A, 89(4), 350–356.

8. Abstract

Influence of sperm density on outcomes of cryopreservation in carp

The current work is devoted to the study of the influence of common carp (*Cyprinus carpio*) sperm concentration during freezing on cryopreservation outcomes. During the experiments, seven concentrations of sperm (from 0.5 to 11 – 13 × 10^9 spz mL^{-1}) were used. Two different approaches of sperm dilution (using seminal plasma or cryoprotective medium) were tested to directly determine the impact of cell concentration in solutions and simultaneously to test an additional positive effect of seminal plasma presence during cryopreservation on sperm cryoresistance.

This study confirmed the assumption that, due to close cell-to-cell interactions, more concentrated samples after thawing account for a smaller percentage of living cells than low-concentrated samples, while different densities didn't significantly affect other motility parameters of sperm, except velocity (VCL). However, the low motility is compensated in the case of using concentrated sperm by the initially large number of spermatozoa in these samples, and the sperm content in such samples is sufficient for successful fertilization, especially when using a high sperm per egg ratio during the fertilization process. In this case, a lower volume of sperm is required in comparison to low-concentrated samples, which is beneficial in terms of saving cryostorage space, chemicals, and work. Therefore, from the considerations of optimization, the use of highly concentrated sperm samples can be offered.

As a part of this work, an elaboration of the spectrophotometry method for the estimation of common carp sperm concentration was carried out. A strong correlation was obtained between absorbance and sperm concentration calculated using the Bürker counting chamber, and an equation of this relation was deduced. The results confirm the possibility of using this method as an alternative to the widely used method of counting sperm concentration with a haemocytometer, however, some further improvements of the method remain possible.

Keywords: cryopreservation, common carp (*Cyprinus carpio*), sperm concentration, sperm motility, fertilization outcomes

9. Abstrakt

Vliv hustoty spermií na výsledky kryokonzervace u kapra

Tato diplomová práce je věnována problematice vlivu koncentrace spermií kapra obecného (*Cyprinus carpio*) při zmrazování na výsledky kryokonzervace. Během experimentu bylo použito sedm koncentrací spermií (od 0,5 do 11 – 13 × 10^9 spz ml^{-1}) a byly testovány dvě různé metody ředění spermatu (pomocí seminální plazmy a pomocí kryoprotekčního média) pro určení přímého vlivu koncentrace buněk ve vzorcích a pro současné testovaní eventuálního pozitivního účinku přítomnosti semenné plazmy během kryokonzervace na kryorezistenci spermií.

Tato studie potvrdila předpoklad, že kvůli mezibuněčným interakcím tvoří koncentrovanější vzorky po rozmrazení menší procento živých buněk než vzorky zmražené při nižších koncentracích, přitom parametry pohyblivosti spermií mimo rychlosti pohybu (VCL) nebyly významně ovlivněny odlišnou koncentrací. Nízké přežití buněk v případě použití koncentrovaného spermatu je kompenzováno původně velkým počtem spermií ve vzorku. a obsah spermií v těchto vzorcích je dostatečný pro úspěšné oplození, zejména při použití vysokého poměru spermií na jikru během procesu fertilizace. V tomto případě menší objem spermií ve srovnání s nízko koncentrovanými vzorky může být použit, což je výhodné z hlediska úspory prostoru potřebného ke skladování kryobiologických kontejnerů, chemikálií a práce. Z hlediska optimalizace procesu kryokonzervace by mohlo být doporučeno použití vysoce koncentrovaných vzorků spermatu.

Součásti této práce bylo rovněž vypracování spektrofotometrické metody pro měření koncentrace ve vzorcích spermatu kapra obecného. Byla zjištěna silná korelace mezi absorbancí a koncentrací spermií vypočtenou pomocí Bürkerovy počítací komůrky a byla odvozena rovnice tohoto vztahu. Výsledkem je potvrzení možnosti využití této metody jako alternativy k široce používané metodě počítaní koncentrace spermií hemocytometrem, nicméně některá další vylepšení této metody nejsou vyloučena.

Klíčová slova: kryokonzervace, kapr obecný (*Cyprinus carpio*), koncentrace spermatu, pohyblivost spermií, výsledky oplození

10. Supplements

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Short communication

High sperm concentration during cryopreservation decreases post-thaw motility percentage without compromising in vitro fertilization outcomes in common carp

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ABSTRACT

To avoid mechanical compression of spermatozoa during the cryopreservation, a high dilution ratio with the cryoprotective medium is applied. Due to this, the sample volume increases, which entails increased cryobank capacity. Moreover, the high volume of cryomedias does not allow to fertilize of large volumes of eggs in practical artificial reproduction of common carp aquaculture. The current study demonstrated that reasonable lowering of dilution rates might still be effective for carp sperm cryobanking —elevation of spermatozoa concentration up to 13×10^9 spz mL^{-1} resulted in a significant decrease in post-thaw sperm motility percentage to 20% compared with 39% in 0.5×10^9 spz mL^{-1}. Nevertheless, no significant differences in sperm kinetic parameters (VCL and LIN) were found in this case. The fertilization outcome (embryo development and hatching rates) was similar after applying thawed sperm samples with "optimal" ($2.2–2.4 \times 10^9$ spz mL^{-1}) and "sub-optimal" ($11.0–13.0 \times 10^9$ spz mL^{-1}) concentrations (sperm/egg ratio at fertilization was in the range of $0.3–4.5 \times 10^5$ spz/egg). Thus, applying a low dilution rate such as one part of sperm to one of the cryoprotective mediums is favorable for decreasing cryo-storage space and the sperm volume needed to fertilize big egg numbers. The experiment also shows that the 4.5×10^5 spz/egg ratio is not sufficient for good fertilization, and it is necessary to use higher sperm concentrations per egg or improve the method of fertilization.

1. Introduction

Various species of the Cyprinidae family contribute to around 50% of worldwide finfish aquaculture production. The production of only common carp (*Cyprinus carpio*) accounts for 7.7% of the total fish production in 2018 in the world (FAO, 2020). As common carp has the longest history of domestication among aquaculture species (Balon, 1995), many common carp strains exist worldwide, and carp aquaculture in Europe exploits strain crossbreeding for sustainable market production (Janssen et al., 2017). Maintaining common carp strains in live gene banks is also crucial for aquaculture breeding programs (Gorda et al., 1995). The Czech Republic is a good example of a country where the conservation of carp strains is a centuries-old activity supported now by the government: Czech National Program for Conservation of Farm Animal Genetic Resources was established in 1996 (Flajshans et al., 1999). Currently, this Program is actively progressing using both in situ preservation of specific fish lines for breeding purposes and sperm cryobanking. The latter is essential to store semen from various local carp lines as a historical heritage of Czech fisherman's traditions and genetic material for selective breeding.

The mentioned above Program utilizes various freezing regimes, cryoprotective media, and cryo-containers (Boryshpolets et al., 2017; Dzyuba et al., 2013; Linhart et al., 2000) for carp sperm cryobanking, based on the earlier reports, showed high post-thaw motility and fertilizing ability of cryopreserved carp sperm (Horvath et al., 2007; Magyary et al., 1996a), Nevertheless, after >20 years of carp sperm

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cryobanking, some problems still exist, e.g., related to the highly heterogeneous cryopreservation outcomes (Flajshans et al., 2015). The sperm concentration during freezing is one of the parameters which can influence them. During freezing of cell suspension, the growing ice crystals push the cells into the narrow channels of concentrated saline, where mechanical cell compression occurs, the so-called packing effect (John Morris et al., 2012). More diluted sperm has a higher chance of escaping this mechanical damage. That is why screening for an optimal spermatozoa concentration for better cryopreservation was already done for some fish species essential for aquacultures, such as rainbow trout, brown trout, sea trout, brook trout, Atlantic salmon, rainbow and brook trout neomales (Judycka et al., 2019a), Arctic char, grayling (Lahnsteiner, 2000) and percids (Judycka et al., 2019b; Judycka et al., 2021), sturgeon (Nascimento et al., 2021).

Optimal concentration was also theoretically predicted for carp species in the range 2–3 $\times$ 10^9 spz mL^{-1}. To get this value, a relatively high sperm-cryoprotective medium dilution rate (1:5) should be applied (Lahnsteiner et al., 2000). Recently, a wide range of dilution rates 1:1–1:20 was studied (Bernath et al., 2016), confirming an advantage of a high dilution rate in carp sperm cryopreservation in terms of higher post-thaw motility. Nevertheless, seminal fluid in the common carp sperm showed some cryoprotective properties (Dietrich et al., 2017; Shaliutina-Kolesova et al., 2019), suggesting the advantage of its presence during freezing.

Furthermore, using a high dilution ratio during cryopreservation of carp sperm increases the cryostorage capacity required for storage. Thus, finding the balance between appropriate sperm concentration, dilution rate, and seminal fluid content would be beneficial for the elaboration of a standardized sperm cryopreservation protocol. Moreover, screening for the optimal sperm concentration value may help understand the reasons for the heterogeneity of cryopreservation outcomes.

Sperm concentration is one of the parameters which can be quickly measured and adjusted during the cryopreservation procedure. Using a counting chamber is one of the oldest and simplest methods for sperm concentration determination, but nowadays, more quick methods like flow cytometry and measurement of the absorbance of diluted sperm are also used (Fauvel et al., 2010; Tan et al., 2010; Yang et al., 2016). However, selecting the most appropriate method for the evaluation of sperm concentration and comparison of the effectiveness of different techniques are still essential (Dong et al., 2007; Tiersch, 2008).

The high volume of cryomedia complicates the process of fertilization and subsequent elimination of stickiness in common carp. Usually, several milliliters of sperm per kg of eggs (800,000 eggs) are used in carp artificial reproduction, and hatchery water is added for fertilization in a combination of 2 parts eggs and 1 part hatchery water (Linhart et al., 2003, 2015). Thus, at a low sperm concentration with a low percentage of motile sperm after thawing, we are not able to achieve the usual motile sperm concentrations in the fertilization volume of the egg activation medium, which is generally important for good fertilization (Ginzburg, 1972; Linhart et al., 2020).

Our study aimed to fill the gaps in understanding the importance of sperm concentration, seminal fluid content during common carp sperm cryobanking, and the application of thawed sperm for in vitro fertilization. We tested various sperm concentrations and content of the seminal fluid in the samples during freezing-thawing and measured their impact on post-thaw sperm motility and fertilizing ability.

2. Materials and methods

2.1. Ethical statement

Manipulations with animals were performed according to the authorization for breeding and delivery of experimental animals (Reference number: 56665/2016-MZE-17214 and 64,155/2020-MZE-18134) and the permission of the use of experimental animals (Reference number: 68763/2020-MZE-18134) issued to the Faculty of Fisheries and Protection of Waters, the University of South Bohemia by the Ministry of Agriculture of the Czech Republic.

2.2. Sperm and eggs collection

Experiments were performed during common carp *C. carpio* artificial propagation during the natural spawning period (May–June). Fish were collected from aquaculture ponds and transported to independent 8 m^3 hatchery tanks with a water flow rate of 0.2 L s^{-1} and with water temperature adjusted to 20 °C.

Males were injected with carp pituitary powder extract at 1 mg kg^{-1} 24 h before sperm collection. Sperm was collected by an abdominal massage directly into a 250 mL cell culture container avoiding contamination with urine, mucus, feces, or water. Sperm samples from different males were stored individually on the ice at 4 °C, not longer than 2 h before freezing. Females were injected with carp pituitary powder extract twice: at 0.3 mg kg^{-1}, 24 h before egg collection, and 2.7 mg kg^{-1} 12 h after the first injection. Eggs were obtained by an abdominal massage directly into dry plastic bowls.

2.3. Sperm motility analysis

After sperm collection, sperm motility parameters for each male were evaluated. Motility of sperm samples was initiated in activation solutions for carp consisting of 30 mM Tris, 45 mM NaCl, 5 mM KCl, pH 8.0 (Billard et al., 1995; Horvath et al., 2003; Linhart et al., 2003), and 0.125% Pluronic F-127 (catalog number P2443, Sigma-Aldrich) to avoid sperm sticking to the glass slide. Motility was recorded in the ISAS spermtrack-10 sperm counting chamber (PROISER, Spain) for 40 s after motility activation using a microscope with optical negative phase-contrast, x10 magnification (PROISER, Spain), and IDS digital camera (IDS Imaging Development Systems GmbH, Germany). The videos were analyzed using the CASA plugin for ImageJ (Purchase and Earle, 2012; Wilson-Leedy and Ingermann, 2007). Following parameters were evaluated: percent of motile cells (%), VCL (Curvilinear velocity, μm s^{-1}), VAP (Average path velocity, μm s^{-1}), VSL (Straight-line velocity, μm s^{-1}), LIN (Linearity of the path, VSL/VAP), WOB (Oscillation of the track, VAP/VCL), and BCF (beat-cross frequency, Hz). Only VCL and LIN were used to present the results (see Statistical analysis part).

2.4. Sperm concentration measurement

Sperm concentration was measured using two methods: by Bürker counting chamber and by optical absorbance measurement. This was done to validate the application of optical absorbance measurement for sperm concentration evaluation in common carp and metrological comparison between methods. In both cases, the samples were diluted in 0.9% (*w/v*) NaCl (physiological solution, PS) by 1:100 (sperm: PS). For the counting chamber (Paul Marienfeld GmbH & Co. KG, Germany), this diluted sperm (DS) was one more time diluted in the same ratio of 1:100 (DS:PS) to get a total dilution ratio of 1:10000 (sperm: PS). Sperm counting was performed under the microscope with optical negative phase-contrast and x10 magnification. The spermatozoa were counted in 12 squares, and each measurement was performed in duplicate.

The absorbance of sperm suspension at wavelengths from 250 to 780 nm was recorded from a single scan by a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Based on obtained spectra (supplementary materials, Fig. S1), 600 nm wavelength was selected to determine the dependency of absorbance from sperm concentration in suspension. Absorbance was analyzed thrice for each sample, and an average of these measurements was used in data analysis. To establish a standard curve between absorbance and sperm concentration calculated by the Bürker chamber, serial dilutions of samples from nine fish were used (Tan et al., 2010). Standard curves for regression between absorbance and sperm concentration measured by Bürker chamber for nine

males (3 repetitions for each of five concentrations) are presented in supplementary materials (Fig. S2A). Descriptive statistics of curves with coefficient determination coefficients in supplementary materials in Table S1. ANCOVA analysis detected no male-specific differences in slopes ($p = 0.90$) and intercepts ($p = 0.07$), which allows for getting pooled standard curve, which was used to validate the method. Sperm samples collected from another 15 males were used to validate the standard curve's applicability (Fig. S2B). Method validation was performed by correlation analysis of sperm concentration data obtained by two methods and by estimating the significance of the difference in the coefficient of variance by the Levene test. A very high coefficient of determination ($R^2 = 0.95$) was obtained during validation. No difference in coefficients of variation for sperm concentration in one sperm sample estimated 10 times by absorbance (6.84) or by Bürker chamber (8.70) was found ($p = 0.4871$). These data indicate that method of optical absorbance measurement in sperm suspension is quick and suitable for determining sperm concentration in common carp.

2.5. Sperm cryopreservation

Two different experimental approaches were used to adjust the sperm concentration in samples during cryopreservation:

2.5.1. Cell concentration adjusted by seminal fluid

By this approach, seminal fluid content was at its maximal level after 1:1 dilution of the sperm sample with a cryopreservation medium. Sperm samples from 21 males were individually treated. Sperm concentration was measured in each sample before being divided into six aliquots. One aliquot was centrifugated at 10^4 g, 4 °C, for 10 min, and obtained seminal fluid was used to dilute other aliquots from the same sample to the concentration of 1, 2, 10, and 20 × 10^9 spz mL^{-1}. Obtained this way, samples and native (not diluted) sperm samples were mixed 1:1 with a cryoprotective medium and then frozen. The composition of the cryoprotective medium used at each dilution rate is described in Table 1.

2.5.2. Cell concentration adjusted by the cryoprotective medium

By this approach, seminal fluid content varied as sperm samples were differentially diluted with a cryopreservation medium. Sperm samples from 18 males were divided into three aliquots and diluted with the cryoprotective medium at ratios 1:1, 1:3, and 1:9. After this manipulation, sperm concentration in samples was in ranges 11–13; 5.6–6.5, and 2.2–2.4 × 10^9 spz/ml. Cryoprotective media were adjusted individually for each dilution rate to keep the same ionic composition and final cryoprotectant content in samples during freezing (Table 1).

Diluted sperm samples were loaded into 0.5 mL plastic straws (IMV Technologies, France). After 10 min of incubation at 4 °C, the straws were placed on a 3 cm thick polystyrene raft floating on the surface of liquid nitrogen. Ten minutes after, the straws were plunged directly into liquid nitrogen. Then the samples were warmed in a 40 °C water bath for 6 s and passed immediately for motility control and/or fertilization.

Table 1
Composition of cryoprotective media used at different sperm dilution ratios.

For 100 mL final volume	Dilution rate		
	1:9*	1:3	1:1
NaCl, mg	360	432	648
KCl, mg	1000	1200	1800
$CaCl_2$, mg	22	26.4	39.6
$MgCl_2$, mg	8	9.6	14.4
$NaHCO_3$, mg	20	24	36
DMSO, mL	10	12	18

* Formulated according to (Horvath et al., 2003).

2.6. Fertilization test

The effect of various sperm concentrations during freezing on the fertilization capacity of cryopreserved sperm was examined during fertilization tests using samples from 5 males with the highest post-thaw motility parameters. The only second approach of sperm concentration adjustment was applied. Eggs from three females were mixed and used for the experiment. Aliquots of two grams of this mixed batch (approx. 1600 eggs) were fertilized individually by sperm samples of each combination male/sperm concentration at freezing. Native sperm concentration in samples was in the range of 22–26 × 10^9 spz mL^{-1}. The resultant sperm concentration in samples during freezing was different, so the volume of post-thaw samples added to eggs was adjusted (the values are shown in Table 2). The averaged ratio of spermatozoa per egg is presented in the results section.

An appropriate volume of cryopreserved sperm was added to 2 g of eggs together with 2 mL of activation medium. After 1 min of gently mixing, eggs were washed, and about 100 eggs were transferred to Petri dishes. Each fertilization was performed in duplicates. Fresh sperm mixed from six males (10 μL) was used to control eggs quality. Petri dishes with fertilized eggs were placed in the closed water flow system at a controlled temperature (21 °C) and incubated until hatching. The development rate of the eggs was calculated 24 h post-fertilization as a ratio of developing embryos to the total number of eggs in the Petri dish, multiplied by 100%. The hatching rate was examined 96 h post-fertilization as a ratio between the number of hatched larvae to the total number of eggs in the Petri dish, multiplied by 100%.

2.7. Statistical analysis

2.7.1. Sperm motility

Kinetic parameters obtained by CASA for all sperm samples used in the study were analyzed using Spearman's rank correlation coefficient. For simplifying data presentation, only parameters with a low correlation coefficient ($r < 0.1$) were selected as descriptors of sperm motility. These parameters were VCL (curvilinear velocity) and LIN (linearity). All spermatozoa with a VCL lower than 20 μm s^{-1} were considered immotile and were excluded from the analysis. The CASA data set extracted the sperm motility percentage, VCL, and LIN values for each male/experimental group/post-activation time. Data from duplicate records of each male in a particular state at each time point during observation, 10–40 s after activation of mobility, were averaged and used to plot regression lines.

Lines of third-order polynomial regression were used to visualize the changes in the parameters during the motility period. An average value of sperm motility percentage, VCL, and LIN for individual males was used to estimate the significance of difference at 10s post activation. At this time point, the maximum differences in parameters were observed on polynomial regression lines, which had very high R^2 (min-max ranges: 0.989–0.999, 0.985–0.997, and 0.544–0.953 respectively; Fig. 1A) and were used to describe general trends of sperm motility parameters during the post-activation time. Before analysis, the data were tested for normality and homogeneity of variances (Kolmogorov-Smirnov and Levene's tests, respectively). All studied parameters were

Table 2
The volume of cryopreserved sperm is used to get the average number of spermatozoa per egg during fertilization.

Average spermatozoa number per egg	Sperm: cryoprotective medium dilution rate		
	1:1	1:3	1:9
4.5 × 10^5	60 μL	120 μL	300 μL
1.5 × 10^5	20 μL	40 μL	100 μL
0.75 × 10^5	10 μL	20 μL	50 μL
0.3 × 10^5	4 μL	8 μL	20 μL

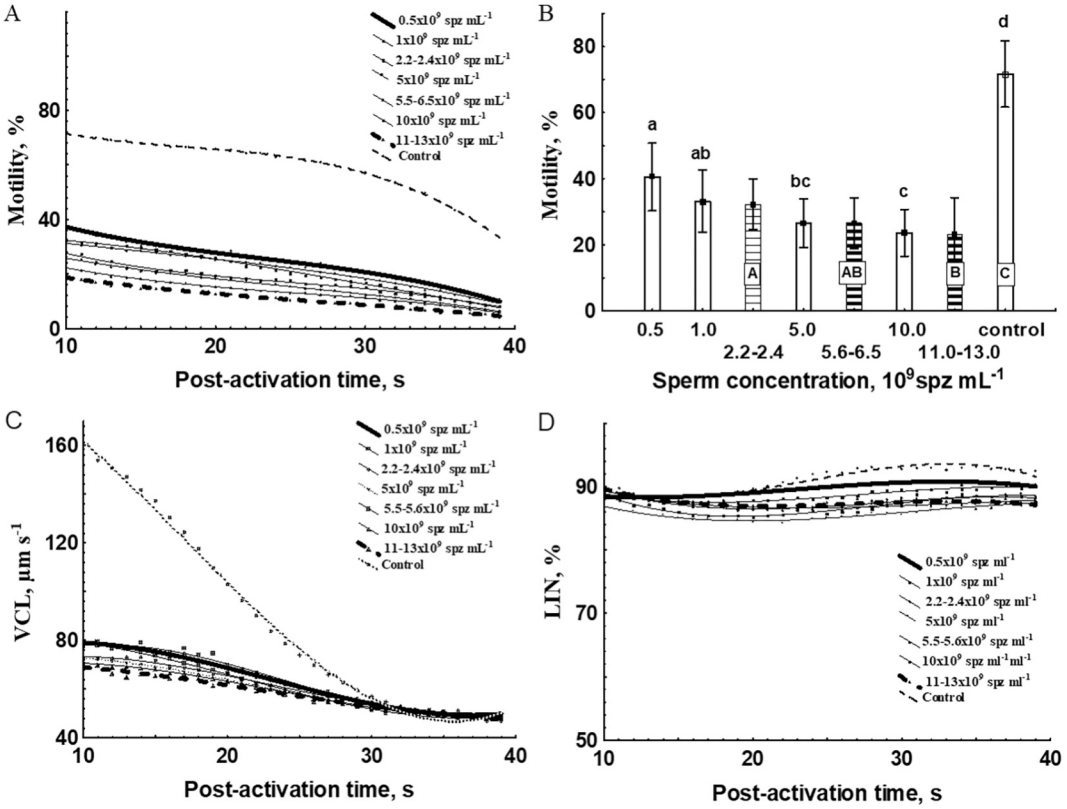

Fig. 1. Sperm motility parameters in common carp sperm samples before and after cryopreservation. A) Polynomial regression lines of 3rd order of sperm motility percentage. Dots represent averaged data at each second of observation time. B) Average post-thaw sperm motility percentage at 10 s post activation, mean ± SD. Data for the first approach of sperm concentration variation (i.e., constant dilution rate) are represented by white columns (values 0.5, 1, 5, and 10 × 10^9 spz mL^{-1}), values with different small letters differ significantly ($p < 0.05$; one-way ANOVA, Tukey post-hoc test, $n = 18$). Data for the second approach of concentration variation (i.e., sperm dilution rate is different) are represented by dashed columns. Values with different capital letters are significantly different ($p < 0.05$; one-way ANOVA, Tukey post-hoc test, $n = 16$). C) Curvilinear velocity (VCL) of carp spermatozoa before and after cryopreservation. Polynomial regression lines of 3rd order. Dots represent averaged data at each second of observation time. D) Path linearity (LIN) of carp spermatozoa before and after cryopreservation. Polynomial regression lines of 3rd order. Dots represent averaged data at each second of observation time. The line legends in A, C, and D show the spermatozoa concentration values in each experimental condition.

normally distributed but did not have similar dispersion values. The data were analyzed using the Kruskal–Wallis test, then multiple comparisons of mean ranks for all groups.

Averaged post-thaw sperm motility percentage for each sperm concentration in the sample was normalized by the highest post-thaw sperm motility percentage obtained for the lowest used concentration (0.5 × 10^9 spz mL^{-1}). This data was used to make a regression line to find sperm concentration in the sample causing the packing effect with a 50% decrease in post-thaw sperm motility percentage.

2.7.2. Development and hatching rates

Two-way ANOVA was performed to analyze the effect of the sperm to egg (sperm/egg) ratio on fertilization outcomes in sperm samples frozen at different sperm dilution rates. The data on development and hatching rates were tested for normality and homogeneity of variances (Kolmogorov-Smirnov and Levene's tests, respectively) and factors of sperm dilution(1:1, 1:3, and 1:9) and sperm: egg ratio (0.3, 0.75, 1.5, 4.5 × 10^5 spz egg^{-1}), and interaction of these two factors were analyzed by two-way ANOVA. Because no significant interaction of factors and no significant factor of sperm dilution rate was detected, post-hoc comparisons were performed only for significant factor of sperm:egg ratio using the Tukey test (Wei et al., 2012).

Calculations and plotting were performed using Statistica (version 13, TIBCO software Inc., 2017, Palo Alto, CA, USA) and a Microsoft Excel spreadsheet. All tested statistical hypotheses were rejected at $p < 0.05$.

3. Results

3.1. Effect of sperm concentration before freezing on post-thaw sperm motility parameters

The downward trend in the percentage of motility post-activation was the same for both approaches used for adjusting sperm concentration before freezing (Fig. 1A). The most pronounced differences were found at the start of observation (10 s post activation). These data were analyzed by one-way ANOVA, and significant differences between groups were found (one-way ANOVA; $p < 0.05$). Post-hoc analysis showed that control values were significantly higher than all cases of thawed sperm (Fig. 1B). The highest post-thaw sperm motility percentage was observed for the concentration of 0.5 × 10^9 spz mL^{-1} (concentration adjusted by the first approach), and the lowest was found for a concentration range of 11–13 × 10^9 spz mL^{-1} (concentration adjusted by approach two). The significance of changes in motility percentage was tested among the concentrations resulting from the same adjusting approach. Thus, the changes in motility percentage were significant between samples with concentrations of 0.5 × 10^9 spz mL^{-1} (41 ± 3%) and 5 × 10^9 spz mL^{-1} (27 ± 2%) for the first approach and between concentration ranges of 2.2–2.4 × 10^9 spz mL^{-1} (32 ± 2%) and 11–13 × 10^9 spz mL^{-1} (23 ± 3%) for the second one.

Normalized motility percentage data were used to get the regression line, linear regression equation y = 0.8916–0.0308x, with a coefficient of determination R^2 = 0.8111. From the equation, a sperm

concentration leading to a 50% decrease in post-thaw sperm motility percentage caused by the packing effect could be estimated at 12.7 × 10^9 spz mL^{-1}.

Similarly, in all experimental cases, spermatozoa velocity decreased during the post-activation period, similar to the motility percentage (Fig. 1C). Analysis by ANOVA of the data from 10 s post-activation revealed significant differences among groups with different concentrations ($p < 0.05$). Post-hoc analysis revealed significant differences only between control and cryopreserved sperm samples, while the velocities for post-thaw sperm samples did not differ significantly (supplementary materials Fig. S3).

The trend lines for LIN changes during the post-activation time for different groups were in quite a narrow range (min/max: 84–92% among all experimental groups). Maximum differences between them can be seen at 30 s post-activation (Fig. 1D). Nevertheless, significant differences in LIN at 30s post-activation were found only between control and post-thaw samples, while no significant differences were found between post-thaw samples (supplementary materials Fig. S4).

3.2. Development and hatching rates after in vitro fertilization with cryopreserved semen

We observed a significant effect of sperm/egg ratio on fertilization success (two-way ANOVA; $p = 0.007$ for development rate and $p = 0.008$ for hatching rate). In contrast, the effect of sperm sample dilution rate was insignificant ($p = 0.717$ for development rate and $p = 0.58$ for hatching rate), and the interaction between these two factors (sperm sample dilution rate and sperm/egg ratio) was also nonsignificant ($p = 0.995$ for development rate and $p = 0.96$ for hatching rate). That is why the sample dilution rate was excluded from the next step of statistical analysis. Significantly higher development (47 ± 3%) and hatching (41 ± 4%) rates were observed at a 4.5 × 10^5:1 sperm/egg ratio. Significant differences were also found between groups fertilized at 0.3 × 10^5:1 and 1.5 × 10^5:1 sperm/egg ratio in terms of fertilization rate (16 ± 4% and 27 ± 2%, respectively) and hatching rate (15 ± 2% and 25 ± 2%, respectively). The experiment also shows that the 4.5 × 10^5 sperm/egg ratio is insufficient for good fertilization. (Fig. 2 A-B).

4. Discussion

The necessity of the standardization of fish sperm cryopreservation protocols is apparent (Asturiano et al., 2017). For common carp, this is also true even though many studies have been performed in the field so far. These studies, in particular, deal with cryopreservation of carp spermatozoa using various compositions of cryoprotective medium (Horvath et al., 2003; Kopeika et al., 2007; Kurokura et al., 1984) or freezing/thawing protocols (Bernath et al., 2016; Kurokura et al., 1984; Linhart et al., 2000). The obtained data on post-thaw fertilization tests are highly variable, which means there is still a need to search for the proper cryopreservation methods considering specific sperm properties in certain groups of breeders. Sperm concentration in suspension is one of the key elements of the standardized cryopreservation procedure. Our study provides a detailed description of sperm concentration effect on post-thaw outcome in common carp.

4.1. Effect of sperm concentration on post-thaw sperm motility parameters

Studies performed in different fish species confirmed the importance of proper sperm concentration for successful cryopreservation. The optimum of this sperm concentration was species-specific and ranged 0.5–4 × 10^9 spz mL^{-1} for several salmoniformes species, northern pike, and sterlet (Judycka et al., 2019a; Lahnsteiner, 2000; Nascimento et al., 2021). For carp, this optimal value was not determined so far. Our study suggests that a significant decrease in sperm motility percentage associated with an increase in sperm concentration at freezing appears in the range of 2.4 and 5.6 × 10^9 spz mL^{-1}. The concentration of 12.7 × 10^9 spz mL^{-1} was calculated as the one, leading to a 50% decrease of post-thaw sperm motility percentage caused by the packing effect. It should be noted that this concentration in carp is essentially higher than one determined for sterlet (2.5–2.7 × 10^9 spz mL^{-1}) (Nascimento et al., 2021), which may be associated with bigger spermatozoa of the latter. In other words, species differences in optimal sperm concentration can be related to the spermatozoon dimension. Nevertheless, the biochemical composition of the cell membrane may additionally contribute to the packing effect (John Morris et al., 2012).

Naturally, carp sperm is characterized by high sperm concentration: e.g., 20 × 10^9 spz mL^{-1} reported by (Cejko et al., 2018) or 17–32 × 10^9 spz mL^{-1} (Kaspar et al., 2007). This implies a high dilution rate by the cryoprotective medium to avoid the packing effect during freeze-thawing. At the same time, the dilution of sperm is related to the decrease of seminal fluid content, which was supposed to have an additional cryoprotective effect (Lahnsteiner, 2007; Shaliutina-Kolesova et al., 2020). Nevertheless, our study did not confirm the latter: the decrease in the content of seminal fluid sperm caused by dilution did not cause any additional reduction in post-thaw sperm motility parameters and fertilizing ability.

The results suggested that the packing effect caused an additional

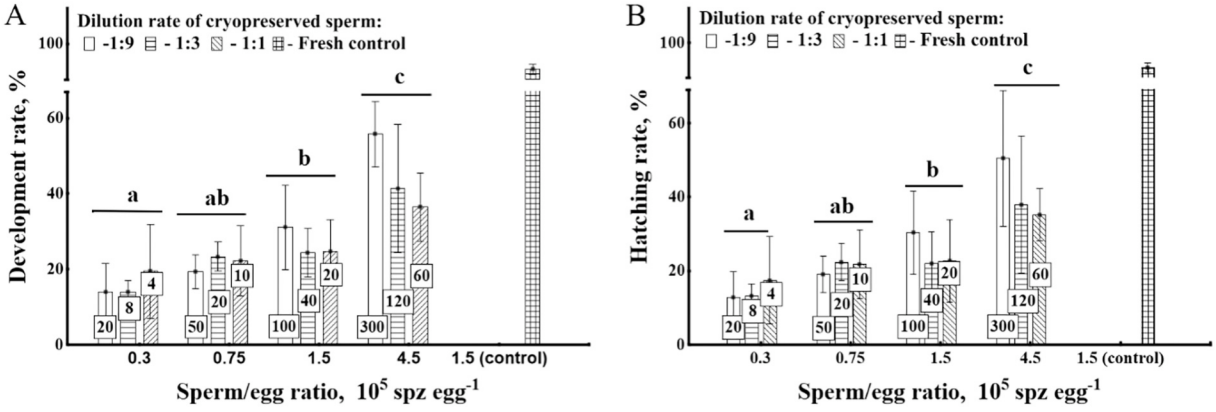

Fig. 2. Development rates (A) and hatching rates (B) obtained after fertilization with different sperm/egg ratios using sperm samples cryopreserved at different spermatozoon concentrations. Sperm samples with the concentrations adjusted by the cryoprotective medium (second approach) were used in experiments. The data are presented as mean ± SD. Values with different letters are significantly different ($p < 0.05$; Tukey post-hoc test). Values (in μl) in boxes represent the volume of sperm used for fertilization of 2 g eggs. "Fresh control" is a value obtained after fertilization by fresh, not frozen sperm to see the quality of eggs; data are not involved in statistical analysis.

drop-in post-thaw motility percentage, while it did not significantly influence the kinetic parameters of spermatozoa (see Fig. 1). As observed in the present study negative effect of increased sperm concentration on motility percentage corresponds well to the same trend found for trout (Judycka et al., 2019a). The general decrease of sperm velocity due to cryopreservation in carp is a well-known phenomenon (Boryshpolets et al., 2017; Boryshpolets et al., 2009; Dzyuba et al., 2010; Lahnsteiner et al., 2000), and our data on VCL decrease after cryopreservation are similar to already described ones. No additional drop in velocity caused by the packing effect was found in our present study. In contrast to our data, a significant increase in VCL due to decreased sperm concentration in cryosuspension was observed in common carp by Bernath et al. (2016). In addition, cryopreservation may reduce the concentration of cells in a sample because of ice formation, leading to spermatozoa destruction.

The absence of differences in VCL and LIN at the initial stage of motility and the appearance of difference in LIN just at late stages of motility in samples frozen at high sperm concentration suggests that an increase in the sperm concentration during freezing affects only the chances of cells to survive cryopreservation (expressed as motility percentage) but not the appearance of additional non-lethal sperm damages.

4.2. Embryo development and hatching rates after fertilization with cryopreserved semen

As the preservation of fertilization ability is the most crucial goal of sperm cryopreservation, the changes in motility percentage and kinetic characteristics arising from cryopreservation should be considered together with fertilizing capacity of spermatozoa. The experimental evaluation of fertilizing ability of sperm samples is typically performed by keeping a certain sperm/egg ratio, which allows a correct comparison of results obtained in different studies. Furthermore, this ratio is crucial for carp since even samples with extremely low post-thaw motility can be used to obtain progeny. For example, the fertilization rate of eggs fertilized by thawed sperm can be pretty close to one obtained with fresh sperm (up to 80%), mainly when an extremely high sperm/egg ratio is used (Kurokura et al., 1984). According to FAO, the optimal volume of carp sperm (securing the maximum fertilization rate of eggs) for fisheries practice is 10 mL sperm per kg of eggs (Horváth et al., 2015). This ratio can be transformed into spermatozoa per egg number of 3.3×10^5 sperm/egg (taking into account averages for carp egg mass 7.5×10^5 eggs kg^{-1} and sperm average sperm concentration 25×10^9 spz mL^{-1}, see Materials and Methods section). We obtained lower developing and hatching rates for cryopreserved sperm than fresh sperm. However, results show very well that the quantity of sperm per egg was probably insufficient. For fresh sperm, motility is at the level of 80–100%, while for thawed sperm, it has decreased to the level of 20–40%. In essence, this means that $0.9–1.8 \times 10^5$ motile spermatozoa were used for fertilization at the highest sperm/egg ratio of 4.5×10^5. This level was insufficient but still apparent due to the 100% fertilization level using fresh, non-frozen sperm.

Nevertheless, publications indicate the possibility of keeping the same fertilization rate for thawed and fresh sperm samples. In these studies, sperm/egg ratios of $1.8–2.4 \times 10^5$ sperm/egg (Linhart et al., 2000) and $1–1.5 \times 10^5$ sperm/egg (Magyary et al., 1996a, 1996b) were used, corresponding to FAO recommendation for fresh sperm application. In another study (Linhart et al., 2015), good fertilization of eggs is achieved with $2 \times 10^4–10^5$ fresh spermatozoa per egg in water for activation of gametes at a concentration of 1.6×10^7 spermatozoa per ml of water. This means that the concentration of spermatozoa in the activation medium can also play a role and must be considered during fertilization. Our study also compared most sperm/egg ratios to the FAO recommendation. The essential difference in fertilization rates between fresh and cryopreserved sperm can not be univocally explained. In particular, they may be associated with variability of sperm samples quality and correspondently cryoresistance (Flajšhans et al., 2015). In addition to cryodamage, nonuniform outcomes of in vitro fertilization trials in common carp may be associated with the technics used to mix sperm with eggs and apply specific sperm motility activating media (Magyary et al., 1996a). In particular, Magyary et al. (1996b) stated the preferability of water as an activation medium instead of specific ionic buffers. We can not confirm this statement since, in our study, better fertilization outcomes were obtained after applying AM (developed by Billard et al. (1995)) compared to water.

In the current study, we demonstrated that even though the spermatozoa frozen at high sperm concentration had a lower motility percentage than samples frozen at low sperm concentration, they still have a necessary number of live spermatozoa needed for successive fertilization. Moreover, it is evident that to get an appropriate sperm/egg ratio when post-thaw sperm is used, a lower volume of a sample of high sperm concentration is needed. Thus, the optimal cryopreservation protocol should meet the balance between the acceptable drop in post-thaw sperm motility due to high cell concentration and rational decrease of cryostorage space, as well as reducing of cryopreserved semen volume required for fisheries practice.

5. Conclusions

A significant decrease in the percentage of post-thaw sperm motility due to high sperm concentration does not limit the applicability of cryopreserved sperm for artificial fertilization. In addition, an increase in sperm concentration decreases the volume of samples needed for storage. That, in turn, reduces the costs of sperm cryobanking and simplifies the aquaculture-oriented application of cryopreserved sperm.

Carp spermatozoa can survive cryopreservation at higher cell concentrations than spermatozoa of other fish species. This phenomenon can be hypothetically explained by species specificity of cell size and the ability of spermatozoa to sustain mechanical squeezing, but the precise background of that requires future study. Undoubtedly, the possibility of increasing cryopreservation outcomes of high concentrated carp sperm by applying specific cryoprotective media and/or freezing regime is of interest in developing the most effective carp sperm cryopreservation technology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738746.

References

Asturiano, J.F., Cabrita, E., Horvath, A., 2017. Progress, challenges and perspectives on fish gamete cryopreservation: a mini-review. Gen. Comp. Endocrinol. 245, 69–76. https://doi.org/10.1016/j.ygcen.2016.06.019.

Balon, E.K., 1995. Origin and domestication of the wild carp, *Cyprinus carpio* - from Roman gourmets to the swimming flowers. Aquaculture 129, 3–48. https://doi.org/10.1016/0044-8486(94)00227-F.

Bernath, G., Zarski, D., Kasa, E., Staszny, A., Varkonyi, L., Kollar, T., Horvath, A., 2016. Improvement of common carp (*Cyprinus carpio*) sperm cryopreservation using a programable freezer. Gen. Comp. Endocrinol. 237, 78–88. https://doi.org/10.1016/j.ygcen.2016.08.013.

Billard, R., Cosson, J., Perchec, G., Linhart, O., 1995. Biology of sperm and artificial reproduction in carp. Aquaculture 129, 95–112. https://doi.org/10.1016/0044-8486(94)00231-C.

Boryshpolets, S., Dzyuba, B., Rodina, M., Li, P., Hulak, M., Gela, D., Linhart, O., 2009. Freeze-thawing as the factor of spontaneous activation of spermatozoa motility in common carp (*Cyprinus carpio* L.). Cryobiology 59, 291–296. https://doi.org/10.1016/j.cryobiol.2009.08.005.

Boryshpolets, S., Sochorova, D., Rodina, M., Linhart, O., Dzyuba, B., 2017. Cryopreservation of carp (*Cyprinus carpio* L.) sperm: impact of seeding and freezing rates on post-thaw outputs. Biopreserv. Biobank. 15, 234–240. https://doi.org/10.1089/bio.2016.0065.

Cejko, B.I., Sarosiek, B., Krejszeff, S., Kowalski, R.K., 2018. Multiple collections of common carp *Cyprinus carpio L.* semen during the reproductive period and its effects on sperm quality. Anim. Reprod. Sci. 188, 178–188. https://doi.org/10.1016/j.anireprosci.2017.12.002.

Dietrich, M.A., Irnazarow, I., Ciereszko, A., 2017. Proteomic identification of seminal plasma proteins related to the freezability of carp semen. J. Proteome 162, 52–61. https://doi.org/10.1016/j.jprot.2017.04.015.

Dong, Q., Huang, C., Tiersch, T.R., 2007. Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. Cryobiology 54, 87–98. https://doi.org/10.1016/j.cryobiol.2006.11.007.

Dzyuba, B., Boryshpolets, S., Rodina, M., Gela, D., Linhart, O., 2010. Spontaneous activation of spermatozoa motility by routine freeze-thawing in different fish species. J. Appl. Ichthyol. 26, 720–725. https://doi.org/10.1111/j.1439-0426.2010.01553.x.

Dzyuba, B., Cosson, J., Yamaner, G., Bondarenko, O., Rodina, M., Gela, D., Linhart, O., 2013. Hypotonic treatment prior to freezing improves cryoresistance of common carp (*Cyprinus carpio* L.) spermatozoa. Cryobiology 66, 192–194. https://doi.org/10.1016/j.cryobiol.2012.12.003.

FAO, 2020. The State of World Fisheries and Aquaculture 2020. FAO, Rome. https://doi.org/10.4060/ca9229en.

Fauvel, C., Suquet, M., Cosson, J., 2010. Evaluation of fish sperm quality. J. Appl. Ichthyol. 26, 636–643. https://doi.org/10.1111/j.1439-0426.2010.01529.x.

Flajshans, M., Linhart, O., Slechtova, V., Slechta, V., 1999. Genetic resources of commercially important fish species in the Czech Republic: present state and future strategy. Aquaculture 173, 471–483. https://doi.org/10.1016/S0044-8486(98)00477-3.

Flajshans, M., Rodina, M., Linhart, O., Dzyuba, B., Boryshpolets, S., 2015. Experience with fish sperm cryobanking in the Czech Republic. Cryobiology 71, 556. https://doi.org/10.1016/j.cryobiol.2015.10.080.

Flajšhans, M., Gela, D., Kocour, M., Rodina, M., Kašpar, V., Linhart, O., Chytka, V., 2015. Amur mirror carp, a recently certified breed of common carp in the Czech Republic. In: 3rd International Conference on Common Carp. FROV JU Vodňany, Vodňany, Czech Republic, pp. 21–23.

Ginzburg, A.S., 1972. Fertilization in fishes and the problem of polyspermy = Oplodotvorenie u ryb i problema polispermii. Israel Prog. Sci. Trans., Jerusalem. https://doi.org/10.5962/bhl.title.46320.

Gorda, S., Bakos, J., Liska, J., Kakuk, C., 1995. Live gene bank of common carp strains at the fish-culture-research-institute, Szarvas. Aquaculture 129, 199–202. https://doi.org/10.1016/0044-8486(94)00248-M.

Horvath, A., Miskolczi, E., Urbanyi, B., 2003. Cryopreservation of common carp sperm. Aquat. Living Resour. 16, 457–460. https://doi.org/10.1016/s0990-7440(03)00084-6.

Horvath, A., Miskolczi, E., Mihalffy, S., Osz, K., Szabo, K., Urbanyi, B., 2007. Cryopreservation of common carp (*Cyprinus carpio*) sperm in 1.2 and 5 ml straws and occurrence of haploids among larvae produced with cryopreserved sperm. Cryobiology 54, 251–257. https://doi.org/10.1016/j.cryobiol.2007.02.003.

Horváth, L., Tamás, G., Coche, A.G., Kovács, É., Woynarovich, A., 2015. Training Manual on the Artificial Propagation of Carps Second Revised, edition ed. FAO, Budapest.

Janssen, K., Chavanne, H., Berentsen, P., Komen, H., 2017. Impact of selective breeding on European aquaculture. Aquaculture 472, 8–16. https://doi.org/10.1016/j.aquaculture.2016.03.012.

John Morris, G., Acton, E., Murray, B.J., Fonseca, F., 2012. Freezing injury: the special case of the sperm cell. Cryobiology 64, 71–80. https://doi.org/10.1016/j.cryobiol.2011.12.002.

Judycka, S., Nynca, J., Ciereszko, A., 2019a. Opportunities and challenges related to the implementation of sperm cryopreservation into breeding of salmonid fishes. Theriogenology 132, 12–21. https://doi.org/10.1016/j.theriogenology.2019.03.022.

Judycka, S., Żarski, D., Dietrich, M.A., Palińska-Żarska, K., Karol, H., Ciereszko, A., 2019b. Standardized cryopreservation protocol of European perch (*Perca fluviatilis*) semen allows to obtain high fertilization rates with the use of frozen/thawed semen. Aquaculture 498, 208–216. https://doi.org/10.1016/j.aquaculture.2018.08.059.

Judycka, S., Dietrich, M.A., Żarski, D., Karol, H., Palińska-Żarska, K., Błażejewski, M., Ciereszko, A., 2021. Towards standardization of the cryopreservation procedure of cultured pikeperch (*Sander lucioperca*) semen. Aquaculture 538. https://doi.org/10.1016/j.aquaculture.2021.736539.

Kaspar, V., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Kocour, M., Linhart, O., 2007. Equalizing sperm concentrations in a common carp (*Cyprinus carpio*) sperm pool does not affect variance in proportions of larvae sired in competition. Aquaculture 272, 204–209. https://doi.org/10.1016/j.aquaculture.2007.08.019.

Kopeika, E., Kopeika, J., Zhang, T., 2007. Cryopreservation of fish sperm. In: Day, J.G., Stacey, G.N. (Eds.), Cryopreservation and Freeze-Drying Protocols. Humana Press. https://doi.org/10.1007/978-1-59745-362-2_14.

Kurokura, H., Hirano, R., Tomita, M., Iwahashi, M., 1984. Cryopreservation of carp sperm. Aquaculture 37, 267–273. https://doi.org/10.1016/0044-8486(84)90159-5.

Lahnsteiner, F., 2000. Semen cryopreservation in the Salmonidae and in the Northern pike. Aquac. Res. 31, 245–258. https://doi.org/10.1046/j.1365-2109.2000.00452.x.

Lahnsteiner, F., 2007. Characterization of seminal plasma proteins stabilizing the sperm viability in rainbow trout (*Oncorhynchus mykiss*). Anim. Reprod. Sci. 97, 151–164. https://doi.org/10.1016/j.anireprosci.2006.01.003.

Lahnsteiner, F., Berger, B., Horvath, A., Urbanyi, B., Weismann, T., 2000. Cryopreservation of spermatozoa in cyprinid fishes. Theriogenology 54, 1477–1498. https://doi.org/10.1016/S0093-691X(00)00469-6.

Linhart, O., Rodina, M., Cosson, J., 2000. Cryopreservation of sperm in common carp *Cyprinus carpio*: sperm motility and hatching success of embryos. Cryobiology 41, 241–250. https://doi.org/10.1006/cryo.2000.2284.

Linhart, O., Rodina, M., Gela, D., Kocour, M., Rodriguez, M., 2003. Improvement of common carp artificial reproduction using enzyme for elimination of egg stickiness. Aquat. Living Resour. 16, 450–456. https://doi.org/10.1016/S0990-7440(03)00083-4.

Linhart, O., Rodina, M., Kaspar, V., 2015. Common carp (*Cyprinus carpio* Linneaus, 1758) male fertilization potency with secure number of spermatozoa per ova. J. Appl. Ichthyol. 31, 169–173. https://doi.org/10.1111/jai.12736.

Linhart, O., Cheng, Y., Xin, M.M., Rodina, M., Tuckova, V., Shelton, W.L., Kaspar, V., 2020. Standardization of egg activation and fertilization in sterlet (*Acipenser ruthenus*). Aquac. Rep. 17, 100381 https://doi.org/10.1016/j.aqrep.2020.100381.

Magyary, I., Urbányi, B., Horváth, L., 1996a. Cryopreservation of common carp (*Cyprinus carpio* L.) sperm I. the importance of oxygen supply. J. Appl. Ichthyol. 12, 113–115. https://doi.org/10.1111/j.1439-0426.1996.tb00072.x.

Magyary, I., Urbányi, B., Horváth, L., 1996b. Cryopreservation of common carp (*Cyprinus carpio* L.) sperm II. Optimal conditions for fertilization. J. Appl. Ichthyol. 12, 117–119. https://doi.org/10.1111/j.1439-0426.1996.tb00073.x.

Nascimento, J.P., Horokhovatskyi, Y., Kholodnyy, V., Rodina, M., Dzyuba, V., Stechkina, T., Dzyuba, B., 2021. Optimization of sterlet sperm concentration for cryopreservation. Aquaculture 540. https://doi.org/10.1016/j.aquaculture.2021.736682.

Purchase, C.F., Earle, P.T., 2012. Modifications to the IMAGEJ computer assisted sperm analysis plugin greatly improve efficiency and fundamentally alter the scope of attainable data. J. Appl. Ichthyol. 28, 1013–1016. https://doi.org/10.1111/jai.12070.

Shaliutina-Kolesova, A., Xian, M., Nian, R., 2019. Antioxidant defence system in protein fractions of common carp (*Cyprinus carpio*) seminal plasma. Czech J. Anim. Sci. 64, 265–271. https://doi.org/10.17221/187/2018-cjas.

Shaliutina-Kolesova, A., Ashtiani, S., Xian, M., Nian, R., 2020. Seminal plasma fractions can protect common carp (*Cyprinus carpio*) sperm during cryopreservation. Fish Physiol. Biochem. 46, 1461–1468. https://doi.org/10.1007/s10695-020-00805-9.

Tan, E., Yang, H., Tiersch, T.R., 2010. Determination of sperm concentration for small-bodied biomedical model fishes by use of microspectrophotometry. Zebrafish 7, 233–240. https://doi.org/10.1089/zeb.2010.0655.

Tiersch, T.R., 2008. Strategies for commercialization of cryopreserved fish semen. Rev. Bras. Zootec. 37, 15–19. https://doi.org/10.1590/s1516-35982008001300003.

Wei, J., Carroll, R.J., Harden, K.K., Wu, G., 2012. Comparisons of treatment means when factors do not interact in two-factorial studies. Amino Acids 42, 2031–2035. https://doi.org/10.1007/s00726-011-0924-0.

Wilson-Leedy, J.G., Ingermann, R.L., 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67, 661–672. https://doi.org/10.1016/j.theriogenology.2006.10.003.

Yang, H., Daly, J., Tiersch, T.R., 2016. Determination of sperm concentration using flow cytometry with simultaneous analysis of sperm plasma membrane integrity in zebrafish *Danio rerio*. Cytometry A. 89, 350–356. https://doi.org/10.1002/cyto.a.22796.