# UNIVERSITY OF SOUTH BOHEMIA
# IN ČESKÉ BUDĚJOVICE

**FACULTY OF FISHERIES AND PROTECTION OF WATERS**



# Use common carp (*Cyprinus carpio* L.) sperm to study the cryoinjuries induced by cryopreservation

## Použití spermií kapra obecného (*Cyprimus carpio* L.) ke studiu poškození vyvolaných zmrazováním spermií


**Ping Li**



Czech Republic, Vodňany, 2011

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

In Vodňany 30th April, 2011 …….

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**Content**

# Chapter 1

# General Introduction

The era of reproductive cryobiology began in 1949 with the discovery of glycerol as a potent cryoprotectant (Polge et al., 1949). In general, low temperature preservation is a pretreatment of experimental materials, followed by a short-term storage at 4 ºC, while cryopreservation involves a short or long-term storage of living cells or treated materials using liquid nitrogen as a coolant. Cells must survive freezing and thawing at such low temperature for any cryopreservation method to be successful. Cryopreservation of sperm has been well established for many years in many fish species. Most studies have been conducted on species that are of commercial importance. Cryopreservation offers several benefits as follow.

- Cryopreservation is considered as one component in an effective strategy to save endangered species by facilitating the storage of their gametes in a gene bank.
- Stocks can be protected from being totally eliminated due to sudden disease outbreak and natural disasters.
- A stable supply of sperm for optimal utilization in hatchery production and laboratory experiments can be ensured.
- Stocks can be easily transported among hatcheries.
- Selective breeding can be further improved. Stocks can be maintained more economically and experimental materials for advanced studies, such as gene transfer, can be made more accessible.

Conventional methods of cryopreservation, such as the use of a styrofoam box filled with liquid nitrogen or a programmable freezer, rely on comparatively slow, controlled cooling during early ice formation. All of them have been proven suitable for sperm cryopreservation. The essence of cryopreservation is to effect cell dehydration and concentration of the cytosol in a controlled and minimally injurious manner so that ice crystallization in the cytosol is precluded or minimized during quenching in liquid nitrogen. There are several major cryoinjuries related to freezing and thawing processes during cryopreservation within the temperature ranges of generalized cryopreservation procedures. Most of these cryoinjuries take place over the temperature range between 0 and -40 ºC due to two major causes: heat removal and application of cryoprotectants. Other causes of cryoinjuries includes pH fluctuation, cold shock, ice crystal formation, osmometric effect, and cryoprotectant toxicity (Chao and Liao, 2001). Apart from lethal changes produced by cryoinjury in a general way, there may be sub-lethal changes in spermatozoa that take place at a molecular level, such as loss or modification of proteins and DNA fragmentation etc., impacting on their cellular function, impairing fertilizing capacity and embryo survival.

## 1. General procedure

### 1.1. Sperm collection and quality assessment

During sperm collection, one has to pay attention to the following items in order to maintain sperm quality: (1) to collect the sperm without contamination of feces, blood or scales; (2) to maintance the sperm during collection at the best condition by providing air or oxygen for respiration; and (3) to maintain temperature of collected sperm at 0–4 ºC during collection in the field and during transportation. Subsequently the sperm viability in terms of spermatozoa motility can be assessed using a microscope and a motility analyzer.

### 1.2. Equilibration with extender and cryoprotectant

Extenders and cryoprotectants have been well studied because cryopreservation is difficult without them. In addition to seminal fluid and the universal cryoprotectant, dimethyl

sulphoxide (DMSO), other single cryoprotectants, combined cryoprotectants, cryoprotectants with egg yolk or sucrose have been used for cryopreservation of fish gametes. The vast majority of penetrating cryoprotectants are simple, low molecular weight chemicals of high water solubility. The most commonly used for fish sperm cryopreservation are ethylene glycol (EG), glycerol, methanol, propylene glycol (PG), and dimethylacetamide (DMA). It seems that there is a pronounced species-specificity in extender requirements.

### 1.3. Freezing protocol varies with species

It appears that cooling rate in the freezing phase has a very wide optimal range in the cryopreservation. Different containers have varied cooling rates. In most cases, straws and cryovials are used to hold the sperm mixture (Barbato et al., 1988). The choice between pellet and straw and size of straw may be determined by the amount of sperm to be cryopreserved, the duration of cryopreservation, and the ease during thawing. A disadvantage of this method, however, is that the freezing rates could not be determined because the sizes of the pellets are not uniform. Still another choice is a programmable freezer. The simplest acceptable device is a styrofoam box fitted with a net set at 2 to 5 cm above the liquid nitrogen. The sample straws are placed on the net when the vapor in the closed box reaches a temperature range of -90 ºC to -100 ºC.

### 1.4. Thawing and fertilization

Preserved sperm is warmed directly from cryogenic temperature to the ambient or higher water temperature at the time of fertilization. The water temperature during the spawning season is usually ideal for sperm revitalization. Cryopreserved sperm in straws can be plunged directly in water of sufficient volume at ambient temperature, which offers the optimal warming rate possible. Cryopreserved pellets are mixed in appropriate kind and volume of thawing solution at temperature higher than ambient temperature to reach an optimal final temperature.

At the post-thaw phase, sperm that survived cryopreservation is ready for artificial fertilization. The following cautions should be taken. (1) Determination of sperm motility as a guide to estimate their fertilization success. (2) Shortening the time between thawing and fertilization. Sperm strength and duration of motility are reduced markedly in most of the experimental species, if cryopreserved sperm are not used immediately after thawing. Therefore, fertilization should be done immediately after sperm are thawed to ensure its viability. (3) Dilution ratio. Artificial fertilization rate using either cryopreserved sperm or fresh sperm is a function of spermatozoa concentrations. Since not all spermatozoa survive or maintain their fertility after cryopreservation, the density of thawed sperm is preferably higher than that of fresh sperm to maintain the ideal fertilization result.

## 2. Cryoinjuries

### 2.1. pH fluctuation

Most biological salts are eutectic at a temperature range of 0 to -55 ºC. During freezing and thawing, the buffering function of these salts is destroyed and the pH of the biological solution changes. This pH fluctuation caused by freezing or addition of cryoprotectants may result in temporary or permanent cryoinjuries (Privalov et al., 1986; Griko et al., 1988).

### 2.2. Cold shock

Cold shock is caused by the change in membrane lipids from the liquid phase to the solid phase in the freezing process from 10 to -16 ºC. Low temperature alone may not be sufficient for the phase change. At this temperature range, however, ice crystals spontaneously form, dehydrating the lipids and inducing change from lamellar liquid to the phase transition stage. Cold shock etiology involves damage to the cellular membranes and alteration in metabolic function, probably caused by changes in the arrangement of membrane constituents (Parks, 1997).

### 2.3. Ice crystal formation

During rapid cooling, intracellular ice formation occurs over a broad range of subfreezing temperatures in a diverse array of biological cells (Mazur, 1977; Steponkus, 1984). Both ice crystal formation and solute concentration contribute to cell damage in the freezing process. If solute concentration alone caused cell damage, a very rapid rate of cooling would be ideal for the freezing process, since there would be insufficient time for water to leave the cells. In contrast, if intracellular ice damage were the sole contributory factor to cell damage, a very slow rate of cooling would be most ideal, because this would provide the maximum time for water to leave the cells. Because both solute concentration and intracellular ice formation contribute to cell damage, an ideal cooling rate should be neither too fast nor too slow. Characterization of the temperature distribution at which intracellular ice formation occurs is of primary importance in predicting the probability of intracellular ice formation during freezing (Pitt et al., 1991).

The thawing of cells has to be performed correctly because the warming rate influences the survival of cells. Thawing rates comparable with the freezing rates that induce intracellular ice formation could lead to re-crystallization of small ice crystals resulting in lethal injury. Too slow warming may be lethal depending on cell type, e.g. through over-long exposure to the hypotonic stress that occurs at thawing. At this stage, possible defects in cell volume regulation may be critical (Mazur, 1984).

### 2.4. Osmometric effect

Cell permeability plays a major role in cryoinjury. Less water is removed from cells that are less permeable in the freezing process. Cells that are more permeable demonstrate a greater tolerance to the cooling and freezing process, but they may become dehydrated. In an aqueous suspension of living cells, ice crystals are formed first in the solution surrounding the cells, resulting in increasing concentrations of solutes outside the cells. Because of the difference in osmotic pressure inside and outside the cells, water continues to move out of the cells as long as the imbalance in salt concentration remains. The cells dry up as freezing proceeds extracellularly, with the rate and extent of this process depending upon the rate of cooling and permeability of the cells. The rational design of a cryopreservation protocol requires characterization of both static and dynamic osmometric behavior of the cells to be cryopreserved. This information can be used to stimulate the volumetric responses and the extent of supercooling of the cells during the freeze/thaw process (Mazur, 1963).

### 2.5. Eutectic Formation

Eutectic formation, i.e. the process of crystallization of the unfrozen fraction of the intracellular or extracellular medium, has thus far been relatively poorly studied in biological systems. Recently, quantitative information on direct cell injury due to eutectic formation has

been reported (Han and Bischof, 2004). The authors propose two major mechanisms of cell cryoinjury by eutectic formation: (1) mechanical cell damage to the cell membrane due to extracellular eutectic formation and (2) disruption of cytoplasmic structures by intracellular eutectic formation.

### 2.6. Cryoprotectant toxicity

Low toxicity and high water solubility are essential considerations for a chemical to be cryoprotective. Cryoprotectants should be non or minimally toxic, able to penetrate cell membranes easily, and able to bind either with electrolytes (to increase concentration in the freezing process) or with water molecules (to delay freezing). The apparent toxicity of cryoprotectants is dependent on type and concentration, the equilibration time, and the temperature during loading. Spermatozoa from different species require different protocols for successful cryopreservation because of inherited particularities in cell shape, cell volume, organelles size, and composition. Cryoprotectants are essential, but their toxicity may cause gamete mortality in pretreatment and post-thawing. It is necessary to determine the point of equilibrium between cryoprotective efficiency of cryoprotectants and the toxicity tolerance of the cell types to be cryopreserved.

### 2.7. Sub-lethal changes

*Changes of membrane integrity*

Changes in plasma or mitochontrial membrane structure and integrity appear to be an important component associated with reduced fertility of frozen-thawed spermatozoa (Sion et al., 2004). The phospholipid composition of the plasma membrane is asymmetric in most mature mammalian cells, with phosphatidylcholine and sphingomyelin concentrated on the outside of the plasma membrane and phosphatidylserine and phosphatidylethanolamine concentrated in the inner leaflet (Bevers et al., 1999). However, following cryopreservation in spermatozoa, there is a scrambling of the plasma membrane phospholipids, resulting in net externalization of phosphatidylserine and phosphatidylethanolamine (Anzar et al., 2002). The change in the plasma membrane selective permeability induces the loss of many components (e.g. ATP and proteins) from the sperm cell resulting in reduced metabolic activities (Zilli et al., 2005).

*Generation of ROS*

Both cold shock and freezing damages are associated with reactive oxygen species (ROS) and oxidative stress generation (Beconi et al. 1991; Alvarez and Storey, 1992). Among ROS-induced damages to sperm there are those mediated by oxidative reaction of spermatozoal phospholipid-bound polyunsaturated fatty acids, leading to lipid peroxidation. The effects of lipid peroxidation include adenosine triphosphate depletion with irreversible loss of sperm motility, a reduction of sperm-egg fusion and DNA damage (Aitken et al. 1998).

*Damage to DNA*

Damage of spermatozoa DNA has been observed in mammalian species (Baumber et al., 2003; Singh et al., 2003) as well as fish species (Labbe et al., 2001. Zilli et al., 2003). In fact the early events in embryonic development are controlled by maternally inherited information

alone (Braude et al., 1988) and, therefore the effects of the DNA damage would not be apparent at this stage. Genome alterations could affect late embryonic development and survival of larvae (Suquet et al., 1998).

*Damage of proteins*

Sperm protein alterations as direct consequentce of cryopreservation have been reported mainly in mammals, such as a loss of transmembrane proteins in bull sperm (Ollero et al., 1998), or decrease in sperm-bound bovine seminal plasma (BSP) protein (Nauc and Manjunath, 2000), and decrease of superoxide dismutase activity, and levels of reduced glutathione (Bilodeau et al. 2000). Moreover, osmotically induced shrinking or swelling is known to activate ion channels, presumably by phosphorylation or dephosphorylation, whence major intracellular ions are allowed to leave or to enter the cell and subsequent water transport takes place (Petrunkina et al., 2005; 2007).

## 3. Cryoability

So far, the various studies suggest that spermatozoa of different species have specific cryobiological properties and various degrees of sensitivity to experimental manipulation, cold shock (lipid phase transitions), freezing and osmotic tolerance (Woods et al., 2004). Moreover, high degrees of variability with respect to different individuals within a given species have been observed (Holt, 2000). Whereas species-specific differences in the molecular structure of the plasma membrane could explain differences in osmotic tolerance limits, hydraulic conductivity, cryoprotectant permeability and effects of different extenders on these biophysical properties, the causes of differences between individuals and especially of the heterogeneity within individual ejaculates from a particular male remain unclear.

## 4. Sperm cryopreservation in common carp

Sperm cryopreservation has been carried out successfully in more than 200 freshwater species, mainly salmonids (Lahnsteiner, 2000), and 40 marine species (Rana1995; Suquet et al., 2000). The importance of storage of viable gametes and embryos of cultured aquatic organisms, especially of teleosts, has been recognized for several years (McAndrew et al., 1993). The common carp (*Cyprinus carpio*) is one of the most important farmed freshwater fish species. So far, several successful methods have been reported for the cryopreservation of common carp sperm, which allow to establish numerous frozen gene banks (Lubzens et al., 1997; Linhart et al., 2000). Most of the experimental work in this field has been focused on trials to find optimal saline solutions for activation or for conservation, cryoprotective agents, thawing solutions, and freezing and thawing rates for cyprinid sperm.

**The aim of this thesis was:**

1) To review the current knowledges about protein composition of seminal plasma and spermatozoa in chondrostean and teleost fish species and to correlate those proteins to sperm functions (motility and fertility); and to discuss the protein studies in terms of cryopreservation.
2) To characterize the protein components of common carp sperm and to verify whether the cryopreservation procedure, applied to common carp sperm, affected the protein profiles of sperm involved in the control of sperm functions by proteomics approaches.
3) To examine different cryopreservation procedures with respect to osmotic and oxidative

stress in common carp spermatozoa.

4) To examine the effects of cryopreservation on the protein phosphorylation of common carp sperm with different extenders. Considering the relevance of protein phosphorylation for spermatozoa movement, the phosphorylated/dephosphorylated proteins induced by sperm motility activation were also examined.
5) To select a fraction of common carp spermatozoa that best survived a conventional freeze/thaw procedure, by centrifugation of frozen/thawed sperm through a Percoll gradient.

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# Chapter 2

# Sperm proteins in teleostean and chondrostean (sturgeon) fishes

Li, P., Hulak, M., Linhart, O., 2009. Sperm proteins in teleostean and chondrostean (sturgeon) fishes. Fish Physiology and Biochemistry 35 (4), 567–581.

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Záhlaví Liché stránky

Sperm proteins in teleostean and chondrostean (sturgeon) fishes

Záhlaví Sudé stránky Chapter 2

# Chapter 3

## Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, C*yprinus carpio* L.

Li, P., Hulak, M., Koubek, P., Sulc, M., Dzyuba, B., Boryshpolets, S., Rodina, M., Gela, D., Manaskova-Postlerova, P., Peknicova, J., Linhart., O., 2010. Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, *Cyprinus carpio* L. Theriogenology 74 (3), 413–423.

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Záhlaví liché stránky

**Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, C*yprinus carpio* L.**

Záhlaví sudé

Chapter 3

# Chapter 4

## Evaluating the impacts of osmotic and oxidative stress on common carp (*Cyprinus carpio*, L.) sperm caused by cryopreservation techniques

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

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Záhlaví liché stránky

**Evaluating the impacts of osmotic and oxidative stress on common carp (*Cyprinus carpio*, L.) sperm caused by cryopreservation techniques**

Záhlaví sudé
Chapter 4

# Chapter 5

## Percoll gradient separation of cryopreserved common carp spermatozoa to obtain a fraction with higher motility, velocity and membrane integrity

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

Sem vložit pdf Ping_chapter 5

Záhlaví liché stránky
Percoll gradient separation of cryopreserved common carp spermatozoa to obtain a fraction with higher motility, velocity and membrane integrity

Zápatí sudé
Chapter 5

## Chapter 6

## General Discussion
## English Summary
## Czech Summary
## Acknowlodgements
## List of Publications
## Training and Supervision Plan during Study
## Curriculum Vitae

# General Discussion

The use of sperm cryopreservation in aquatic species raises concerns about damages to spermatozoa during freezing and thawing. As a result, the development of an effective sperm cryopreservation protocol has been recommended to industry as a method for improving breeding programmes. While some studies reported issues of altered cell membrane integrity and damages of cellular organs (Cabrita et al., 1998) others were concentrated on the loss of DNA integrity in sperm cells during the cryopreservation procedure (Labbe et al., 2001). In comparison with mammalian sperm, little is known about the damage mechanism of fish sperm due to cryopreservation.

**Protein composition of fish sperm**

Sperm proteins of fish have developed adaptations due to the changes in the reproductive environment. Analysis of the composition and functions of these proteins provides new insights about sperm motility and fertilizing abilities, thereby creating possibilities for improving artifical reproduction and germplasm resource conservation technologies (e.g. cryopreservation). The swimming and fertilising capabilities of spermatozoa are under the control of various proteins, such as membrane proteins, axoneme proteins and metabolism proteins as well. Ion channels, including those for $Ca^{2+}$, $K^{+}$ and $Na^{+}$, represent main group of proteins in the membrane of fish spermatozoa. After the extracellular signals are received by specific ion channels or receptors in the sperm cells, intracellular signals are triggered through pathways closely arranged in sperm axoneme, leading to efficient activation of motility (Inaba et al., 2003). During sperm motility and immotile storage, fish spermatozoa produce energy through metabolic processes that are species-specific (Christen et al., 1987; Mansour et al., 2003). A study of enzymes involved in these metabolic processes would provide valuable information towards an explanation of metabolism strategies in fish. In addition, according to several authors (Jaffe, 1990; Evans and Kopf, 1998; Swann and Parrington, 1999), there should be some proteins in the spermatozoa surface for binding to the egg, despite the presence of egg micropyles in fish. However, which kind of proteins and their functions are not fully understood.

The main role of seminal plasma is to create an optimal environment to maintain spermatozoa in the quiescent state. This optimal environment consists of stable levels of osmolality, ionic concentrations, pH and proteins for protection of spermatozoa. Disturbances in the composition of seminal plasma will impair its protective functions and, consequently, lead to a decrease in sperm quality (Ciereszko, 2008).

**Effects of cryopreservation on sperm proteins**

Not only spermatozoa motility and fertilization ability have been the focus of studies on the usefulness of sperm proteins as estimators of sperm quality, other studies were focused on parameters of seminal plasma and spermatozoa after cryopreservation (Piros et al., 2002; Zilli et al., 2005). The latter showed that cryopreservation, in addition to causing the release of spermatozoa constituents into the seminal plasma, also initiates the degradation of spermatozoa proteins (Cabrita et al., 2001; Lahnsteiner et al., 2004; Desrosiers et al., 2006; Satorre et al., 2007).

In our study with sperm of common carp, *Cyprinus carpio* (Li et al., 2010a), spermatozoa velocity, motility, fertilization and hatching abilities of carp sperm were obviously decreased after cryopreservation. In terms of protein composition, we observed that a decrease in protein amount in spermatozoa following cryopreservation was mainly the

consequence of leakage of proteins from spermatozoa into the extracellular medium. Moreover, we did not find any differences in the protein profiles of frozen and thawed seminal plasma and fresh seminal plasma, indicating that the major effect of cryopreservation was damage to the spermatozoa cell rather than a change of protein composition. However, protein degradation seems to be responsible for reduction in protein amount. Furthermore, eleven of 14 selected protein spots were identified. The identified proteins comprised 3 membrane proteins, 6 cytoplasmic enzymes, and 2 extracellular proteins (seminal plasma proteins).

Among them, membrane proteins matched three protein families, anexins, cofilins, and N-ethylmaleimide-sensitive fusion attachment proteins (SNAPs). Annexin A4 is an epithelial isoform with cell functions suggested to be involved in membrane trafficking and membrane organization within cells through regulating membrane permeability and altering membrane fluidity (Warren et al., 2003). A reduction in annexin A4 in cryopreserved spermatozoa, as observed in the present study, could influence the stability of spermatozoa membrane structure, thereby leading to leakage of other membrane proteins as well as cytoplasmic proteins. Cofilins are a family of actin-binding proteins that promote rapid turnover of the actin cytoskeleton, which has a central role in cell movements (Bamburg, 1999). Cofilin 2 was detected in the spermatozoa of common carp in our study, and therefore we hypothesized that the cofilin 2 most likely plays an important role in spermatozoa movement. That could explain the large loss of cofilin 2 in cryopreserved spermatozoa and could be one of several reasons for poor spermatozoa motility after cryopreservation. Tomes et al. (2004) observed that α-SNAP localized in the acrosome of human spermatozoa and confirmed that it exhibited a direct role in the acrosome reaction, which is a prerequisite for fertilization. Therefore, in our study, the decreasing amount of α-SNAP could be the cause of the significantly decreased fertilization rate of spermatozoa after cryopreservation.

S-Adenosylhomocysteine hydrolase is a functionally conserved and ubiquitously expressed cytoplasmic enzyme, and is mainly involved in intermediate metabolism (Turner et al., 1997). Therefore, the reduction in spermatozoa velocity and motility could be a consequence of lower amount of S-Adenosylhomocysteine hydrolase. In addition, Si:dkey-180p18.9, Ldhb, Pgk1, Taldo1, and Esterase D/formylglutathione hydrolase proteins are considered to belong to the enzymes present in cytoplasm, such as oxidoreductase, kinase, transferase, hydrolase.

The most unusual finding was transferrin variants C and F in spermatozoa samples after cryopreservation. Most information on transferrin in seminal plasma is from mammalian studies, but recently, transferrin was also observed in common carp seminal plasma (Wojtczak et al., 2005; 2007). In mammalian body fluids, transferrin is regarded as a major antioxidant protein, acting by reducing the concentration of free ferrous ion that catalyzes the formation of reactive oxygen species (Chauhan et al., 2004). In the present study, transferrin variant C and F were identified, indicating that the transferrin present in cryopreserved spermatozoa samples was originally in seminal plasma. We suggest that the transferrin may be associated with the spermatozoa membrane and protect spermatozoa against oxidative damage during freeze-thaw stress.

**Effects of osmotic and oxidative stress on sperm caused by cryopreservation**

The response of cells to osmotic pressure during cryopreservation is complex, depending on colligative and permeability properties as well as effects on other membrane parameters and cell viability (Petrunkina, 2007; Peñaranda et al., 2009). The shrinkage and swelling of cells is limited by their physical capacity to withstand such changes. Damage will occur beyond certain levels of shrinkage or swelling (Petrunkina, 2007). In this study (Li et al.,

2010b), we found that spermatozoa membrane integrity was not influenced by equilibration time, but spermatozoa motility (percentage and velocity) from sperm equilibrated in dimethyl sulfoxide (DMSO) extender significantly decreased and was negatively correlated to equilibration time. We hypothesized that common carp spermatozoa are extremely permeable to EG. Hence, equilibrium can be achieved rapidly without large volume excursions, possibly facilitating cell survival during addition and withdrawal of ethylene glycol (EG). This was also confirmed by saline buffer (SB) triggering motility in spermatozoa exposed to DMSO extender but not in spermatozoa exposed to EG extender.

In mammals the effect of reactive oxygen species (ROS) on spermatozoa is well characterized: it may cause lipid peroxidation of spermatozoa membranes; damage of midpiece, axonemal structure, and DNA; malfunctions of capacitation and acrosomal reaction; loss of motility; and infertility (Gagnon et al., 1991; Sikka, 2001). Similar to mammalian spermatozoa, fish spermatozoa contain high levels of polyunsaturated fatty acids (PUFA), which are particularly susceptible to ROS-induced lipid peroxidation (Vernet et al., 2004). However, the precise mechanism of ROS induction in fish spermatozoa is not known. In our study, the increase in thiobarbituric-acid-reactive substance (TBARS) levels was observed in spermatozoa only after thawing, indicating that the ROS generation occurred mainly because of the freeze/thaw process rather than the effect of equilibration. As with TBARS, the freeze/thaw process can lead to oxidative stress in spermatozoa through excessive generation of carbonyl groups (CP).

The antioxidant systems in mammalian sperm have been well studied (Alvarez and Storey, 1989; Zini et al., 1993); however, little knowledge is available about them in fish sperm (Lahnsteiner et al., 2010). In our study (Li et al., 2010b), we measured three antioxidant enzyme activities in common carp spermatozoa: superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx). SOD activity was not activated or inhibited by either cryoprotectant or the freeze/thaw process. However, GR activity was chiefly inhibited by DMSO extender rather than by freezing and thawing. Inhibition of GR activity was only observed in spermatozoa incubated in EG extender for 30 min. In this instance, the GR activity was mainly influenced by CPA effects, and DMSO was more effective than EG. In contrast to GR, GPx activity was activated in the groups that were incubated in DMSO extender without freezing, and following freezing and thawing with both DMSO and EG extender. Therefore, our results suggested that GPx provided the most effective protection against cold shock and oxidative damages during the cryopreservation process. Moreover, the differences in GR and GPx activity in carp sperm after cryopreservation with DMSO and EG extender indicated that DMSO induced more oxidative stress than did EG, which may subsequently influence the fertilizing ability of carp spermatozoa.

**Percoll gradient separation of cryopreserved sperm**

It is clear that the development of methods to identify, prior to freezing, those spermatozoa that are potentially able to sustain freezing and thawing procedure without losing fertilizing ability is of utmost importance. The present study demonstrated that Percoll gradient centrifugation effectively selected and separated fragment of intact spermatozoa with higher motility from cryopreserved fish sperm (Li et al., 2010c). The observed high percentage of spermatozoa with intact membranes following Percoll separation may improve final fertilization abilities. Recent research in mammalian species has allowed the identification of a number of indicators for differences in freezeability among species and among individuals of the same species (Giraud et al., 2000; Nunez-Martinez et al., 2007). Differences in the biochemical characteristics of the sperm membrane and specifically the

relative content and ratio of membrane phospholipids and cholesterol have been claimed to be the main factors to explain inter-species differences in the sensitivity to cryoinjury (Darin-Bennet and White, 1977). Similar results have also been confirmed in fish sperm (Drokin et al., 1989; Drokin, 1993). Recently, the presence of apoptotic markers such as the externalization of phosphatydilserine and the permeability of the sperm membrane have been identified as markers of freezeability (Pena et al., 2006). Therefore, approaches such as the selection of "good freezers," either as individual fish or as well-defined spermiation portions, could have a tremendous impact on the success of cryopreservation. In this respect, the results obtained in the present study may be of practical interest, as the technique used is simple and suitable for the further detection of valuable markers as predictors of potential freezeability.

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# English Summary

Use common carp (*Cyprinus carpio* L.) sperm to study the cryoinjuries induced by cryopreservation

Ping Li

Damage to spermatozoa during cryopreservation is regarded as a major obstacle to the expansion of sperm storage technology. Therefore, in depth knowledge of cryoinjuries ocurring in fish sperm during cryopreservation, can provide new insights in sperm cryopreservation and improve artifical reproduction and germplasm resource conservation technologies.

Chapter 2 described composition of proteins in fish sperm. Seminal plasma proteins are involved in protection of spermatozoa during storage in the reproductive system, whereas all spermatozoa proteins contribute to swimming and fertilising abilities of sperm.

In Chapter 3, proteomics methods were used to characterize the protein components of common carp sperm and to verify whether the cryopreservation procedure, applied to common carp sperm, affected the protein profiles of spermatozoa involved in the control of sperm functions. Additionally, the protein profiles in the associated seminal plasma were also investigated. Fourteen protein spots were significantly altered following cryopreservation. Eleven of these were identified: three as specific membrane proteins (N-ethylmaleimide-sensitive fusion protein attachment protein alpha, cofilin 2, and annexin A4) involved in membrane trafficking, organization, and cell movement; six as cytoplasmic enzymes (S-Adenosylhomocysteine hydrolase, Si:dkey-180p18.9 protein, lactate dehydrogenase B, phosphoglycerate kinase 1, transaldolase 1, and esterase D/formylglutathione hydrolase) involved in cell metabolism, oxidoreductase activity, and signal transduction; and two as transferrin variant C and F. In conclusion, 2DE gel-based proteomic analyses revealed quantitative alterations in protein expression in cryopreserved spermatozoa. The observed downregulation of spermatozoa membrane proteins and cytoplasmic enzymes, together with the accumulation of transferrin in cryopreserved spermatozoa, could be the reason for the observed decrease in spermatozoa velocity, motility, and lower fertilization success as well as in ova hatching rate.

In Chapter 4, we examined osmotic and oxidative effects on common carp spermatozoa motility; membrane integrity; levels of thiobarbituric-acid-reactive substance (TBARS) and carbonyl groups (CP); and the activity of superoxide dismutase (SOD), glutathione reductase, and glutathione peroxidase (GPx). Sperm was diluted in dimethyl sulfoxide (DMSO) and ethylene glycolbased (EG) extenders, followed by equilibration, freezing, and thawing. Equilibration in DMSO extender resulted in a significant reduction of spermatozoa motility, but motility was induced in those spermatozoa following dilution with saline buffer, which usually inhibits undiluted spermatozoa motility. Spermatozoa velocity and membrane integrity decreased with both extenders following freezing and thawing. No significant difference in levels of TBARS or CP, or in SOD activity, was seen in samples equilibrated with either extender. The freeze/thaw process induced significantly higher levels of TBARS, CP, and GPx activity, but did not affect the level of SOD. Glutathione reductase activity was inhibited in samples exposed to DMSO extender. The results of this study provide new information about effects on osmotic stress of DMSO and EG in common carp spermatozoa, as well as the oxidative damage induced by cryoprotective agent equilibration and freeze/thaw processes. The results, together with reports of mammal studies, confirmed that the rapid removal of DMSO from spermatozoa resulted in a marked decline in spermatozoa motility, whereas EG was associated with less osmotic damage subsequent to rapid removal. Both CPA

effect and the freeze/thaw process can induce oxidative stress in spermatozoa, and DMSO showed more adverse effects than EG with respect to GR and GPx activity. In addition, the antioxidant response of spermatozoa is mainly due to the capacity of GPx to counteract ROS stress of spermatozoa and minimize cryopreservation damages. All data suggest that an alternative CPA, such as EG, should be considered for carp spermatozoa.

Furthermore, we attempted to select a fraction of common carp spermatozoa that best survived a conventional freeze/thaw procedure, by centrifugation of frozen/thawed sperm through a Percoll gradient (45% and 90%) (in Chapter 5). The effect of this separation method on spermatozoa viability was evaluated by calculating the proportion of motile spermatozoa, their velocity, and their membrane integrity. The proportion of motile spermatozoa (65.81 ± 5.19%), their velocity (77.58 ± 31.07 μm/sec), and membrane integrity (83.66 ± 4.38% intact) were significantly higher in separated sperm than in whole samples (motility 23.36 ± 2.98%, velocity 55.55 ± 19.03 μm/sec, and membrane integrity 57.92 ± 4.65%). Our results demonstrated that the Percoll gradient separation method was effective for removal of nonmotile spermatozoa and for removing spermatozoa with damaged membranes in common carp sperm exposed to the effects of freezing and thawing. Use of this technique may provide a simple way to harvest the best quality spermatozoa for other biotechnological procedures such as detection of biomarkers for freezeability of fish sperm.

# Czech Summary

Použití spermií kapra obecného (*Cyprinus carpio* L.) ke studiu poškození vyvolaných zmrazováním spermií

Ping Li

Poškození spermií v průběhu zmrazování je považováno za hlavní překážku pro rozšíření technologie uchování spermií. Proto můžou nové znalosti o poškození buněk, vyskytujících se v průběhu zmrazování spermií ryb, poskytnout nové pohledy na technologii zmrazování spermií ryb a zlepšení umělé reprodukce, stejně jako technologie ochrany genových zdrojů.

Kapitola 2 přináší literární přehled proteinové kompozice spermií u kostnatých a chrupavčitých ryb. Kapitola dále přináší detailní přehled dosavadních poznatků o proteinové kompozici spermií a seminální plazmy ve vztahu k fertilizační schopnosti a funkci proteinů v reprodukčním systému ryb.

Kapitola 3 přináší popis experimentů zaměřených na charakterizaci proteinového profilu zmrazeného spermatu (spermie a seminální plazma) kapra obecného s využitím metod komparativní proteomiky. V experimentech jsme zjistili, že, čtrnáct proteinových spotů bylo signifikantně změněno v průběhu zmrazování. Jedenáct z nich bylo posléze identifikováno: tři z nich jako specifické membránové proteiny (N-ethylmaleimide, cofilin 2, a annexin A4) podílející se na trans-membránovém transportu, organizaci a pohybu buňky, šest jako cytoplasmatické enzymy (S-Adenosylhomocysteine hydroláz, Si: dkey-180p18.9 protein, laktátdehydrogenázy B, phosphoglycerate kinázy 1, transaldolase 1, a esterázy D / formylglutathione hydroláz) zapojených do buněčného metabolismu, oxidoredukční činnosti a přenosu signálu a dva jako varianty transferinu C a F. Závěrem lze říci, že provedené proteomické analýzy prokázaly, kvantitativní změny v expresi proteinů u zmrazených spermií. Pozorované down-regulace bílkovin spermií membrány a cytoplazmatických enzymů, spolu s akumulací transferinu v zmrazených spermiích, by mohly vysvětlovat pozorovaný pokles rychlosti spermií, procento pohyblivosti a nižší fertilizační schopnosti spermií kapra obecného.

V kapitole 4 jsme zkoumali osmotický stres a oxidační účinky v průběhu zmrazování spermatu kapra obecného a jejich vliv na pohyblivost spermií kapra, membránovou integritu, úroveň thiobarbiturovou-acid-reaktivní látky (TBARS) a karbonylové skupiny (CP) a činnost superoxiddismutázy (SOD), glutathion reduktázy a glutathionperoxidázy (GPx). Osmotický a oxidační stres byl u spermatu kapra obecného indukován použitím dvou kryoprotektiv, a to dimethylsulfoxidu (DMSO) a ethylenglykolu s následnou ekvilibrací, a procesy zmrazení a rozmrazování. Ekvilibrace spermatu s použitím DMSO vedla k výraznému snížení pohyblivosti spermií. Navíc oba typy použitých extenderů měly negativní vliv na rychlost pohybu spermií a membránovou integritu po zmražení a rozmražení spermatu. Použití obou typů extenderů nemělo průkazný vliv na koncentraci TBARS nebo CP, nebo na činnost, SOD. Na straně druhé, procesy zmrazování a rozmrazování indukovaly vyšší koncentraci TBARS, CP a aktivitu GP, ale neměly průkazný vliv na hladinu SOD. Navíc aktivita glutathion reduktázy byla inhibována ve vzorcích vystavených extenderu DMSO. Závěrem můžeme konstatovat, že dosažené výsledky prokázaly, že ethylen glycol je lepším kryoprotektantem pro zmrazování spermatu kapra obecného z důvodu průkazně nižší indukce osmotického a oxidačního stresu v průběhu zmrazování spermatu.

Dále jsme se pokusili aplikovat separační gradientovou metodu s cílem výběru frakce spermií kapra, které nejlépe přežil podmínky zmrazování. Za tímto účelem byla u zmrazeného spermatu kapra obecného aplikována metoda gradientové separace v prostředí Percollu (45% a 90%) (kapitola 5). Účinek této metody separace byl hodnocen na základě výpočtu podílu

pohyblivých spermií, jejich rychlosti, a membránové integrity. Podíl pohyblivých spermií (65,81 ± 5,19 %), jejich rychlost (77,58 ± 31,07 um/sec) a membránové integrity (83,66 ± 4,38 %, neporušené) byly výrazně vyšší než v oddělených spermiích v celém vzorku (motilita 23,36 ± 2,98 %, rychlost pohybu 55,55 ± 19,03 um/sec a membránová integrita 57,92 ± 4,65 %). Naše výsledky ukazují, že Percoll separační metoda byla účinná pro odstranění nepohyblivých spermií a pro odstranění spermií s poškozenou membránou. Závěrem můžeme konstatovat, že uvedená metoda může poskytnout jednoduchý a účinný způsob separace zmrazených spermií kapra obecného a taktéž může nalézt široké uplatnění v biotechnologických aplikacích, například detekce různých typů biomarkerů ve výzkumu spermií ryb.

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8) 046/2010/Z – Reproduction and genetics of selected model species of teleostean and chondrostean fish (2010–2012, leader Prof. Dipl.-Ing. Otomar Linhart, DSc.)
9) 072/2008/P – Ice-age endurance: difference expressions of sperm proteins before and after cryopreservation in teleost fishes (2009, leader Ping Li, MSc.)

# List of publications

## Peer reviewed journals


**Li, P.**, Rodina, M., Hulak, M., Gela, D., Psenicka, M., Li, Z.H., Linhart, O., 2011. Physico-chemical properties and protein profiles of sperm from three freshwater chondrostean species: a comparative study among Siberian sturgeon (*Acipenser baerii*), sterlet (*Acipenser ruthenus*) and paddlefish (*Polyodon spathula*). Journal of Applied Ichthyology 27 (2), 673–677.

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Li, Z.H., Zlabek, V., **Li, P.**, Grabic, R., Velisek, J., Machova, J., Randak, T., 2011. Acute toxicity of carbamazepine to juvenile rainbow trout (*Oncorhynchus mykiss*): effects on antioxidant responses, hematological parameters and hepatic EROD. Ecotoxicology and Environmental Safety 74 (3), 319–327.

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

**Li, P.**, Hulak, M., Koubek, P., Sulc, M., Dzyuba, B., Boryshpolets, S., Rodina, M., Gela, D., Manaskova-Postlerova, P., Peknicova, J., Linhart, O., 2010. Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, *Cyprinus carpio* L. Theriogenology 74 (3), 413–423.

**Li, P.**, Hulak, M., Rodina, M., Sulc, M., Li, Z.H., Linhart, O., 2010. Comparative protein profiles: potential molecular markers from spermatozoa of Acipenseriformes (Chondrostei, Pisces). Comparative Biochemistry and Physiology: Part D: Genomics and Proteomics 5 (4), 302–307.

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

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long-term exposure to propiconazole. Ecotoxicology and Environmental Safety 73 (6), 1391–1396.

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**Li, P.**, Hulak, M., Linhart, O., 2009. Sperm proteins in teleostean and chondrostean (sturgeon) fishes. Fish Physiology and Biochemistry 35 (4), 567–581.

Li, Z.H., **Li, P.**, Randak, T., 2009. Effect of a human pharmaceutical carbamazepine on antioxidant responses in brain of a model teleost *in vitro*: An efficient approach to biomonitoring. Journal of Applied Toxicology 30 (7), 644–648.

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**Li, P.**, Wei, Q., Liu, L., 2008. DNA integrity of Polyodon spathula cryopreserved sperm. Journal of Applied Ichthyology 24 (2), 121–125.

Wei, Q., **Li, P.**, Psenicka, M., Alavi, S.M.H., Shen, L., Liu, J., Peknicova, J., Linhart, O., 2007. Ultrastructure and morphology of sperm in Chinese sturgeon (*Acipenser sinensis* Gray 1835) using scanning and transmission electron microscopy. Theriogenology 67 (7), 1269–1278.

**Proceedings and abstracts**

Boryshpolets, S., Dzyuba, B., Rodina, M., **Li, P.**, Hulak, M., Gela, D., Linhart, O., 2010. Can we avoid the spontaneous sperm motility activation arisen from freeze–thawing in carp? In: Book of abstracts from International symposium – 47 Annual Meeting of Society for Cryobiology, 17–20 July 2010, Bristol, UK. (oral presentation)

**Li, P.**, Dzyuba, B., Hulak, M., Rodina, M., Linhart, O., 2010. Attempt in separation of cryopreserved fish spermatozoa to obtain a fraction with higher motility, velocity and membrane integrity by Percoll gradient. In: Book of abstracts from International symposium – Satellite Symposium: Reproductive Biology of Aquatic Organisms -researches from the tropical to temperate zone-, 30 June 2010, Okinawa, Japan, p. 28. (poster presentation)

**Li, P.**, Hulak, M., Rodina, M., Linhart, O., 2010. Species-specific protein patterns from spermatozoa of endangered Acipenseriformes: potential candidate molecules for species identification. In: Book of abstracts from International symposium – 11th International Symposium on Spermatology, 24–29 June 2010, Okinawa, Japan, p. 44. (poster presentation)

Linhart, O., Alavi, S.M.H., Psenicka, M., Rodina, M., Kaspar, V., **Li, P.**, Hulak, M., Boryshpolets, S., Dzuba, S., Gela, D., Flajshans, M., Peknicova, J., Cosson, J., Ciereszko, A., 2010. Structure, motility and ability to fertilization of sperm of chondrostean fish species, a review. In: Book of abstracts from International symposium – 11th International Symposium on Spermatology, 24–29 June 2010, Okinawa, Japan, p. 21. (oral presentation)

**Li, P.**, Hulak, M., Koubek, P., Sulc, M., Dzyuba, B., Boryshpolets, S., Rodina, M., Gela, D., Manaskova-Postlerova, P., Peknicova, J., Linhart, O., 2009. Proteomics differentiations during cryopreservation in sperm of common carp, *Cyprinus carpio* L. In: Book of abstracts from International symposium – Second International Workshop on Biology of Fish Gametes, 9–11 September 2009, Valencia, Spain, p. 132. (oral presentation)

**Li, P.**, Rodina, M., Hulak, M., Gela, D., Psenicka, M., Linhart, O., 2009. Physico-chemical properties and protein profiles of sperm from three freshwater chondrostean species: a comparative study among Siberian sturgeon (*Acipenser baerii*), sterlet (*Acipenser*

*ruthenus*) and paddlefish (*Polyodon spathula*). In: Book of abstracts from International symposium – The 6th International Symposium on Sturgeon, 25–30 October 2009, Wuhan, China, pp. 33–35. (oral presentation)

Linhart, O., Alavi, S.M.H., Psenicka, M., Rodina, M., Kaspar, V., **Li, P.**, Hulak, M., Boryshpolets, S., Dzuba, B., Gela, D., Flajshans, M., Peknicova, J., Cosson, J., Ciereszko, A., 2009. Structure, motility, fertilization and cryopreservation of sperm of chondrostean fish species, a review. In: Book of abstracts from International symposium – The 6th International Symposium on Sturgeon, 25–30 October 2009, Wuhan, China, pp. 40–42. (oral presentation)

# Training and Supervision Plan during Study

| | |
|---|---|
| **Name** | Ping Li |
| **Research department** | 2007–2009 – Department of Fish Genetics and Breeding of RIFCH<br>2009–2011 – Laboratory of Mollecular, Cellular and Quantitative Genetics of FFPW |
| **Daily supervisor** | Prof. Dipl.-Ing. Otomar Linhart, DSc. |
| **Supervisor** | Prof. Dipl.-Ing. Otomar Linhart, DSc. |
| **Period** | October 2007 until September 2011 |

| **Ph.D. courses** | **Year** |
|---|---|
| Fish reproduction | 2008 |
| Pond aquaculture | 2008 |
| Applied hydrobiology | 2008 |
| Ichthyology and systematics of fish | 2009 |
| English language | 2009 |

| **Scientific seminars** | **Year** |
|---|---|
| Seminar days of RIFCH and FFPW | 2008<br>2009<br>2010<br>2011 |

| **International conferences** | **Year** |
|---|---|
| 11th International Symposium on Spermatology, 24–29 June 2010, Okinawa, Japan, p. 44. (poster presentation) | 2010 |
| Satellite Symposium: Reproductive Biology of Aquatic Organisms -researches from the tropical to temperate zone-, 30 June 2010, Okinawa, Japan, p. 28. (poster presentation) | 2010 |
| Second International Workshop on Biology of Fish Gametes, 9–11 September 2009, Valencia, Spain, p. 132. (oral presentation) | 2009 |
| The 6th International Symposium on Sturgeon, 25–30 October 2009, Wuhan, China, pp. 33–35. (oral presentation) | 2009 |

| **Foreign stays during Ph.D. study at FFPW and RIFCH** | year |
|---|---|
| Prof. Kazuo Inaba, Tsukuba University, Shimoda, Japan (2 months, proteomics techonology ) | 2008 |
| Prof. Qiwei Wei, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Sciences, China (1 month, sperm cryopreservation) | 2009 |

# Curriculum Vitae

**Personal information**

| | |
|---|---|
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**Present position**

Ph.D. student at the University of South Bohemia in České Budějovice (USB), Faculty of Fisheries and Protection of Waters (FFPW, *www.frov.jcu.cz*), Research Institute of Fish Culture and Hydrobiology (RIFCH), Laboratory of Molecular, Cellular and Quantitative Genetics, Vodňany, Czech Republic

**Education**

2004–2007 – MSc. – Huazhong Agriculture University, China
2007 – present – Ph.D. study – USB, FFPW, RIFCH, Laboratory of Mollecular, Cellular and Quantitative Genetics

**Responsible leader of projects**

072/2008/P – Ice-age endurance: difference expressions of sperm proteins before and after cryopreservation in teleost fishes (2009, leader Ping Li, MSc.)
003/2010/Z – Effect of cryopreservation on phosphorylation state of sperm proteins of common carp, *Cyprinus carpio* L. (2010, leader Ping Li, MSc.)

**Foreign stays during Ph.D. study at FFPW and RIFCH**

2008 – lab. of Prof. Kazuo Inaba, Tsukuba University, Shimoda, Japan (proteomics techonology)

**Ph.D. courses**

- Fish reproduction
- Applied hydrobiology
- Ichthyology and systematics of fish
- Pond aquaculture
- English language