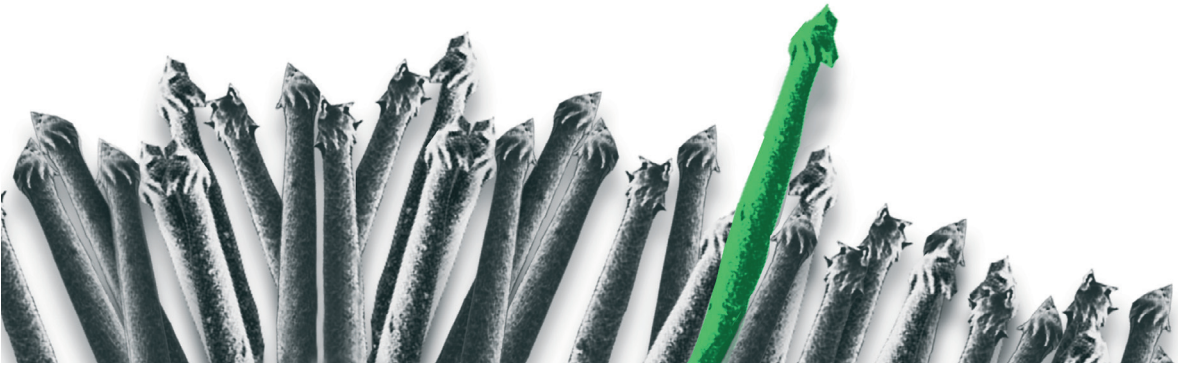
Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

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

Fish sperm motility parameters and total proteins profiles in seminal plasma during *in vivo* and *in vitro* storage

Parametry motility spermií ryb a celkové proteinové profily semenné plazmy během *in vivo* a *in vitro* uchování



Anna Shaliutina

Vodňany, Czech Republic, 2013

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Ph.D. thesis, USB, FFPW, RIFCH, Vodňany, 2013, 96 pages, with the summary in English and Czech.

I dedicate this work to my friend
Martin Hulák
who passed away too early

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. IMPORTANCE OF SPERM STORAGE STUDY

In vivo storage is defined as a physiological process for keeping spermatozoa in the reproductive duct after their release from cysts. In different fish species, during the annual cycle as well as the optimal period of *in vivo* storage, spermatogenesis varies significantly. For example, spermatozoa in the male gonads of carp are present throughout the year (Koldras et al., 1990), a feature which contrasts with trout where spermatozoa are present only during autumn or winter, the reproductive period (Billard et al., 1982). Apart from period of storage, physiological functions of the male reproductive accessory organ play an important role in sustaining sperm quality. The most prominent differences in the structure of reproductive system can be observed between teleostean and chondrostean. In the majority of teleostean fish, males release sperm into the aquatic environment through the sperm duct and neither testicular fold nor sperm ducts are in close contact with the kidneys (Fig. 1) (Blum, 1985). In contrast, chondrostean excretory system possess specific anatomical features, e.g. efferent ducts come to direct contact with kidney (Fig. 1), and testicular sperm is supposed to be diluted with urine, the latter being hypotonic in comparison with blood plasma (Krayushkina and Semenova, 2006).

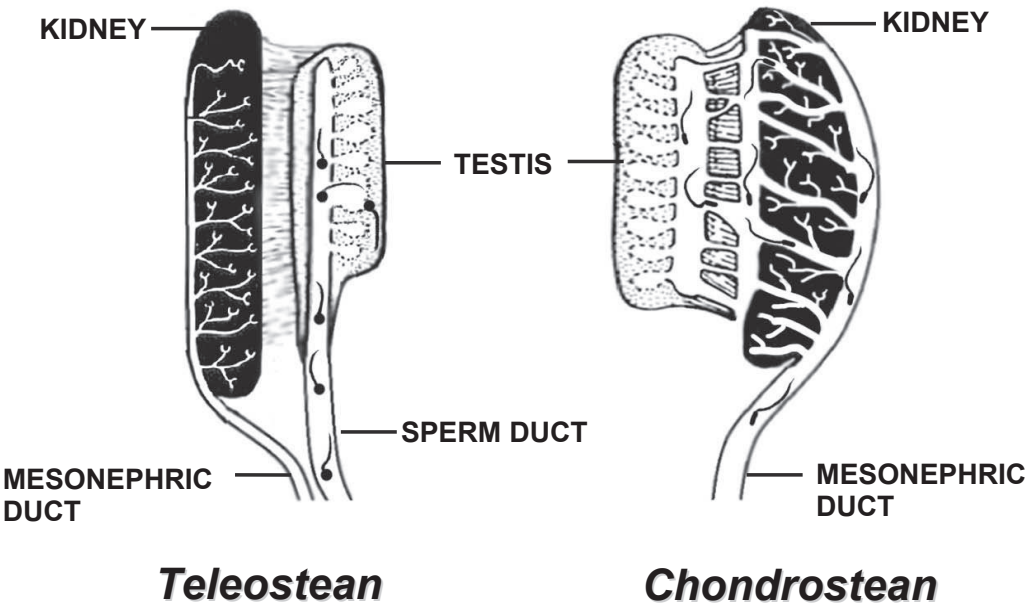

Figure 1. *Schematic diagrams showing the structural organization of the male reproductive system in fishes (Hoar, 1969).*

Quite probably, such dilution process by urine leads to specific characteristics of sturgeon semen and influence sperm properties during their *in vivo* storage. Among these characteristics, low osmolality, protein content in seminal plasma and quite low spermatozoa concentration in semen (Piros et al., 2002) should be emphasized. However, the existence of such milt dilution process by urine remains still partly speculative and needs to be experimentally confirmed. Moreover, the physiological involvement of this process in sperm maturation cannot be excluded. Motility activation of mature sturgeon spermatozoa is known to be inhibited by low concentration of potassium ions, such inhibition being abolished by addition of calcium ions (Cosson, 2004) and by environmental osmolality (Gallis et al., 1991). Consequently, these observations suggest that studies of the alterations in composition of media surrounding sperm should lead to better understanding of the processes taking place in sperm duct during *in vivo* sperm storage. On the other hand, investigations of changes in sperm quality after different periods of preservation in reproductive system can give additional knowledge essential for application of *in vitro* storage procedures.

In contrast to *in vivo*, *in vitro* storage of semen is a non-physiological process, practiced in many animal species to maintain male gametes in a functional state. In fish culture and farming, various methods of *in vitro* sperm storage are frequently used. For example, short-term storage is the most common technique. Sperm is maintained at 4 ºC either with or without aeration for several days before fertilization ability decreases (Billard et al., 2004). Moreover, short-term storage is a simple and inexpensive procedure often needed to deal with logistics of large-scale hatchery operations. In comparison with this approach of storage, cryopreservation was described as a powerful tool that allows sperm to be stored indefinitely. According to Lahnsteiner (2000), cryopreservation of fish sperm has been well established in many finfish species. This method offers several benefits such as male stock protection from being totally eliminated due to sudden diseases outbreak, natural disaster, or accidents such as oil spills. Additionally, cryopreservation includes stable supply of sperm for: 1) optimal utilization in hatchery production, easy sperm transportation among hatcheries, improvement in selective breeding whereby stock can be maintained more economically and effectively, and 2) experimental material for advanced studies, such as gene transfer. Despite a wide range of advantages of sperm storage procedures, our knowledge is not good enough to understand their impact on sperm physiological characteristics.

In the present study, we focus on certain biochemical and functional parameters of sperm under conditions of *in vivo* and *in vitro* storage in some chondrostean and teleostean fish species.

1.2. SPERMATOZOA INDEXES DURING *IN VIVO* AND *IN VITRO* STORAGE

Motility is regarded as one of the most essential parameters related to sperm quality, and decrease in spermatozoa movement performance under *in vivo* or *in vitro* conditions is a common reason for observation of reduced sperm fertility. Additional parameters, such as spermatozoa concentration, DNA integrity and level of oxidative damage, are also important indicators of sperm fertilizing potential. Therefore, in this section more detailed information about different sperm indexes during *in vivo* and *in vitro* storage is considered.

1.2.1. Spermatozoa motility

It has been reported that in most of the fish species studied so far, spermatozoa are immotile in the testis and seminal plasma (Stoss, 1983; Billard, 1986), in contrast to the situation in reptiles or mammals (Krasznai et al., 1995). Motility of fish sperm occurs after they are released into surrounding aqueous environment during natural reproduction or into diluents during artificial reproduction (Darszon et al., 1999; Alavi and Cosson, 2006; Cosson et al., 2008a,b). It is clear that the motility of sperm is induced due to hypo- and hyperosmotic pressure signal in fresh water and marine fish species, respectively (Morisawa et al., 1983). However in some fish species (e.g. from salmonids or sturgeon), it is known that spermatozoa activation occurs in wide range of environment osmolality (from hypotonic to hypertonic conditions). Thus, osmolality itself seems to have not regulatory role in sperm motility activation. In contrast to osmolality, specific ion concentrations and pH are necessary under any osmotic conditions (Alavi and Cosson, 2006). In these species, sperm motility is suppressed by a millimolar concentration of extracellular K^+ in seminal plasma, and a decrease in extracellular K^+, upon spawning, triggers the signaling for initiation of sperm motility (Morisawa and Suzuki, 1980).

Changes in the fish sperm motility characteristics occur both naturally over the breeding season (*in vivo* storage) and during *in vitro* storage of spermatozoa. It was shown that in fish species such as rainbow trout (Munkittrick and Moccia, 1987), carp and tilapia (Kruger et al., 1984) potentiality for sperm motility increased from the beginning to the middle of the spawning season and declined thereafter. Researches on several other fish species have reported either a decreasing trend in spermatozoa motility or stability as the reproductive season progressed (Suquet et al., 2005; Babiak et al., 2006). Studies dealing with the effects of cryopreservation and short-term storage on spermatozoa parameters shows a significant decline in motility of sperm after prolonged *in vitro* storage in both mammals and fish species; however, there is not a general consensus about it. Although basic information on fish spermatozoa motility is available (Cosson et al., 2000; Alavi and Cosson, 2005), knowledge about the biochemical mechanisms responsible for spermatozoa parameters changes during *in vivo* and *in vitro* storage are still required. The present study aimed to examine the effects of short-, long term *in vitro* sperm preservation as well as *in vivo* storage on the percentage of motile sperm and velocity in models of chondrostean and teleostean fish species.

1.2.2. Spermatozoa concentration

A large variation in the spermatozoa concentration (measured as ratio of spermatozoa number per semen volume) was noticed among the fish species (Lubzens et al., 1997; Hulak et al., 2008b; Babiak et al., 2006). At the same time, other studies indicated that spermatozoa concentration could also be affected by the duration of sperm storage in the reproductive tract. Preliminary observations have suggested that alteration in spermatozoa concentration could be the result of increased production of seminal plasma, which would dilute sperm and consequently lead to lowering spermatozoa concentration (Shaliutina et al., 2012). Moreover, spermatozoa concentration could be affected by environmental conditions (Linhart et al., 2000). The variations in spermatozoa concentration during *in vivo* storage still need more exact description for further investigation.

1.2.3. Generation of reactive oxygen species

The production of reactive oxygen species (ROS) is a normal physiological process but an imbalance between ROS generation and scavenging activities is detrimental to sperm (Sharma and Agarwal, 1996). Previous studies demonstrated that mammalian spermatozoa membranes are rich in polyunsaturated fatty acids (PUFAs) and are sensitive to oxygen-induced damage mediated by lipid peroxidation, and, thus are sensitive to ROS attack. This attack results in decreased sperm motility, presumably by several reasons such as: a rapid loss of intracellular ATP, axonemal damage, increased mid-piece sperm morphological defects (Sikka, 1996; Bansal et al., 2007). Whereas a significant negative correlation between ROS and fertilization rate has been found (Agarwal et al., 2005), controlled generation of ROS has shown to be essential for the development of sperm capacitation and hyperactivation (de Lamirande and Gagnon, 1993).

Similar to mammalian spermatozoa, fish spermatozoa also contain high level of PUFA, which is particularly susceptible to oxidative damage (Trenzado et al., 2006). An increase in ROS has been linked to abnormal or damaged spermatozoa (Gazo et al., 2013). For example, duroquinone induced ROS in carp spermatozoa that caused DNA damage in the sperm and subsequently impaired reproductive success (Zhou et al., 2006). In Russian sturgeon and Siberian sturgeon the cause of the loss of spermatozoa motility and velocity during short-term *in vitro* storage was oxidative stress that significantly interrupted cellular metabolism (e.g. oxidative phosphorylation) of spermatozoa, leading to subsequent decline of motility parameters (Shaliutina et al., 2013). It was also reported that the imbalance between ROS and sperm antioxidant activity was a major cause of spermatozoa damage during cryopreservation (Li et al., 2010; Wang et al., 1997). Moreover, the generation of ROS was considered as one of the main causes of cryo-damage, especially regarding DNA injury (Baumber et al., 2003; Li et al., 2007; Thomson et al., 2009). However, despite all these findings, the precise role of ROS production in fish spermatozoa remains unknown.

1.2.4. Antioxidant action

Spermatozoa are protected from oxidative damage by various antioxidants and antioxidant enzymes, which are present in the seminal plasma or in spermatozoa itself. Antioxidants are the agents, which break the oxidative chain reaction, thereby, reduce the oxidative stress. When ROS overwhelm the cellular antioxidant defense system, whether through an increase in ROS levels or a decrease in the cellular antioxidant capacity, oxidative stress occurs (Lahnsteiner et al., 2010). In fish sperm, an antioxidant system consist in enzymatic and non-enzymatic components (Liu et al., 1995; Ciereszko et al., 2000; Lahnsteiner et al., 2010) which have important meanings in maintaining the semen viability during *in vivo* storage. It was shown that substances such as ascorbic acid (Ciereszko and Dabrowski, 1995; Metwally and Fouad, 2009), uric acid (Ciereszko et al., 1999), and α-tocopherol (Martínez-Páramo et al., 2012) are considered as important non-enzymatic components. While superoxide dismutase, glutathione reductase, catalase and glutathione peroxidase (Li et al., 2010; Martínez-Páramo et al., 2012) constitute enzymatic antioxidant component of fish sperm.

Under *in vitro* storage conditions, the efficiency of the semen antioxidant systems is low and not efficient to protect spermatozoa from reactive oxygen species

(Lahnsteiner et al., 2010). Therefore, in some previous studies, it was demonstrated that addition of molecules with antioxidant capacity to the freezing media provides the effective protection against cold shock and reduces the oxidative damage during *in vitro* sperm storage (Cabrita et al., 2011). However, the effect of each antioxidant is species-specific and the positive effects found in some species may not be true in others.

In the current work we bring into focus the level of antioxidant enzymatic activity in sperm during the short-term *in vitro* storage.

1.2.5. Damage to DNA

Damage of spermatozoa DNA has been observed in mammalian species (Baumber et al., 2003) as well as fish species (Labbe et al., 2001; Zilli et al., 2003). Most of these reported data indicate that DNA damage is correlated with a reduction in fertilization ability, with development failure after fertilization and offspring problems (Kopeika et al., 2004; Evenson and Wixon, 2006).

DNA damage could be caused in spermatozoa by many different factors, such as aberrant chromatin packaging during spermatogenesis (Boissonneault, 2002; McLay and Hugh, 2003), excessive reactive oxygen species (ROS) production and decrease of seminal antioxidants (Pérez-Cerezales et al., 2009). Effect of *in vitro* sperm storage on spermatozoa DNA integrity has been also investigated previously (Labbe et al., 2001; Cabrita et al., 2005). Despite these numerous studies, the identification and characterization of DNA damage in fish spermatozoa is not well understood. Therefore, in the present study, one experiment was conducted to investigate the effect of short-term *in vitro* storage on spermatozoa DNA integrity.

1.3. SEMINAL PLASMA CHARACTERISTICS

1.3.1. Role of seminal plasma during sperm storage

In most fishes, the spermatogenesis is of cystic type. Cysts are the basic functional units of the spermatogenic epithelium formed by Sertoli cells surrounding and nursing synchronously developing spermatogenic germ cell clones. At final stage of spermatogenesis, so called spermiogenesis or spermiation, the spermatozoa are released into the efferent duct system, where they are subjected to the maturation phase. During this phase, the testicular epithelium produces the seminal plasma, which appears as a multi component fluid surrounding spermatozoa. The main role of seminal plasma is to create an optimal environmental for storage of spermatozoa. The condition of storage should protect sperm fertilizing ability, and maintain methabolism to preserve viability and energetic recourses for sperm motility activation (Piironen, 1994). In fish with internal fertilization, constituents of seminal plasma may support spermatozoa during their movement to ova (Yao and Crim, 1995). Moreover, it is also argued that seminal plasma, together with ovarian fluid, have beneficial functions for spermatozoa during external fertilization by creating a favorable micro-enviroment for sperm movement (Billard, 1986). Seminal plasma in most teleost fishes is a secretory product of the testes and of the spermatic duct (Loir et al., 1990). This is due to the fact that teleost fishes, with some exception, have no accessory gland. Disturbances

in composition of seminal plasma may lead to impairment of spermatozoa storage and therefore decrease sperm quality. In turn, this can be the result of decreasing suitability for sperm *in vitro* storage.

Accumulating evidence shows that seminal plasma is a complex mixture containing a wide variety of components that can affect the survival and motility of sperm during different storage conditions. Hence, in the present study, special attention was given to the effect of seminal plasma on spermatozoa functions with regard to *in vivo* storage.

1.3.2. Seminal plasma osmolality

Numerous studies on mammals reported that seminal plasma osmolality influences sperm motility parameters, regulates acrosome reaction and fertilizing ability acquisition (Rossato et al., 1996). In contrast to mammals, there is a significant lack of research investigating the role of seminal plasma osmolality on fish sperm functions. It is known that the osmolality of seminal plasma is mainly responsible for prevention of sperm motility in fish sperm ducts (Billard, 1986). The seminal plasma osmolality is lower in Acipenseridae than in Cyprinidae and Salmonidae (Alavi and Cosson, 2006). Moreover, it is worth remarking that the osmolality is higher in marine than in freshwater fish seminal plasma. Like other components of seminal plasma, the osmolality can vary between individuals, and this is correlated with the ''thinning'' (hydration) phenomenon of the semen (Morisawa et al., 1979). Additionally, the variations in seminal plasma osmolality observed in the literature can be due to hormonal induction of spermiation outside the natural reproductive season (Redondo-Müller et al., 1991). Overall, in fish, most of studies demonstrate that variation in seminal plasma osmolality can be a response to changes in secretory activity in the fish sperm duct during the spawning period (*in vivo* storage) (Li et al., 2009), but in contrast, it can also be used as a predictive indicator of sperm contamination by urine (Linhart et al., 2003; Hulak et al., 2008a). However, knowledge about the changes of osmolality seminal plasma under *in vivo* storage conditions is still weak.

1.3.3. Proteins composition of seminal plasma

Nowadays it is generally agreed that seminal plasma components protect mature sperm viability during storage (Ciereszko, 2008). Proteins in seminal plasma are involved in the protection of spermatozoa during their storage in the reproductive system. The protection includes maintaining spermatozoa in the quiescent state by means of sperm motility-immobilizing proteins in some species, supporting adequate levels of sperm nutrients for sperm metabolism, controlling and regulating the final maturation processes and protecting sperm against damage caused by proteolytic or oxidative attacks (Ciereszko et al., 1999). Billard (1983) found that the sperm dilution rate could have an influence on the fertilizing ability of salmonid spermatozoa and suggested that proteins possibly present in seminal plasma may play a role in the sperm protection mechanisms. Moreover, it have been demonstrated that after fractionation of seminal plasma proteins, several fractions were found to be the most effective for saving sperm longevity (Lahnsteiner, 2007). In ram, the efficiency of adding of seminal plasma proteins to the medium before cold shock demonstrated the prevention of membrane damage by inhibiting protein tyrosine phosphorylation (Perez-Pe et al.,

2002) and maintaining of antioxidant enzymes activities and their distribution on the sperm surface (Marti et al., 2008), leading to a viable sperm population (Perez-Pe et al., 2001). However, several studies on cryo-damage in fish spermatozoa have shown that the degradation of seminal plasma proteins could be partially responsible for the observed decrease in sperm motility duration and the lower hatching rate of eggs fertilized with cryopreserved sperm (Zilli and Vilella, 2012).

Mammalian semen contains numerous proteolytic enzymes, e.g. cysteine-, serine- and metallo-proteases, which are involved in many physiological functions in semen. Some of them are involved in coagulation and liquefaction of semen and proteolysis of seminal proteins (Wilson et al., 1993). Proteolytic and anti-proteinase activities have also been found in the seminal plasma of several species of teleostean and chondrostean fish (Tab. 1).

While multiple forms of proteolytic enzymes exist in seminal plasma of teleosts and differ among fish families and species, the exact role of these enzymes remains to be poorly known. It was also suggested that transferrins and lipoproteins found in fish seminal plasma participate to the protection of spermatozoa during storage in the spermatic duct, together with proteinase inhibitors (Ciereszko, 2008). Some metabolic enzymes have also been found in seminal plasma; these were presumed to be resulted from leaking out of the spermatozoa (Lahnsteiner et al., 1998). All these data show that seminal plasma potentially appears as a fluid comprising a series of substances, which protect sperm in a species-specific manner. It remains to be better elucidated if some metabolic processes in seminal plasma are involved in the production of seminal plasma components with protective properties during *in vivo* and *in vitro* sperm storage.

Taken together, in the current study the different methodological approaches for investigation of sperm functions and protein patterns of seminal plasma during *in vivo* and *in vitro* storage were applied and done with the following objectives:

AIMS:

1. To evaluate sperm production characteristics and spermatozoa motility parameters in teleostean and chondrostean species during *in vivo* storage.
2. To describe the effects of *in vivo* storage on protein composition of seminal plasma in models of chondrostean and teleostean fish species.
3. To examine the effect of *in vitro* storage on parameters of sperm motility, DNA damage and oxidative stress in sperm of chondrostean fish species.
4. To investigate if different *in vivo* storage time could influence sperm cryoresistance and post-thaw fertilizing ability in chondrostean fish species.

Table 1. *Proteins in seminal plasma of fish (modified after Li et al., 2009).*

Proteins	Species	Functions	References
SPP120 (sperm plasma glycoprotein 120)	*O. niloticus*	A sperm immobilising factor, interacting with spermatozoa; a good potential candidate as a sperm quality parameter	Mochida et al., 1999, 2002
Ubiquitins	*O. niloticus*	Can regulate spermatogenesis by activation of proenzymes and prohormones, stimulation of sperm motility and metabolism and removal of immature and damaged spermatozoa at the end of spawning	Osaki et al.,1999; Cierezko, 2008
Metalloproteinase; serine proteases	Salmonid; Cyprinid; Percid		Kowalski et al., 2003;
Aminopeptidase; chymotrypsin	*O. mykiss; C. carpio*		Breton et al., 1974
Miltpain	*O. keta*		Kawabata and Ichishima, 1997
Acid phosphatase; Alkaline phosphatase; β-D-glucuronidase	Salmonid; Cyprinid	responsible for elimination of degenerating spermatozoa at the end of spawning	Lahnsteiner et al., 1993, 1994, 1998
Inhibitor II α1-Antiproteinase	*O. mykiss C. carpio*	May participate in protection of spermatozoa from proteolytic attack	Mak et al., 2004; Huang et al., 1995a,b; Wojtczak et al., 2007
Transferrin	*C. carpio*	May protect spermatozoa against microbes, oxidative and heavy metal toxicity	Wojtczak et al., 2005, 2007
Lipoproteins	*O. mykiss*	Interaction with sperm plasma membranes to maintain optimal lipid composition during storage in the spermatic duct	Loir et al., 1990
Malate dehydrogenase; lactate dehydrogenase (LDH); aspartate aminotransferase; adenosine triphosphatase	Salmonid; Cyprinid; *E. lucius; P.flavescens*	Enzymes involved in metabolism	Lahnsteiner et al., 1993, 1994, 1998

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CHAPTER 2

EVALUATION OF SPERMIATION INDICES WITH MULTIPLE SPERM COLLECTIONS IN ENDANGERED STERLET (*ACIPENSER RUTHENUS*)

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Evaluation of Spermiation Indices with Multiple Sperm Collections in Endangered Sterlet (*Acipenser ruthenus*)

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Contents

This study investigated the effects of multiple collections of sperm on endangered sterlet (*Acipenser ruthenus*) sperm functional parameters [spermatozoa motility and curvilinear velocity (VCL)] as well as on protein concentration and osmolality of seminal plasma. The average sperm volume and mean spermatozoa concentration per male were significantly altered with multiple collections. On the other hand, no significant effect of multiple collections on protein concentration of seminal plasma was observed. In all experimental groups, moderate impact of sequential collection on osmolality ($p < 0.05$) of seminal plasma was observed. Ninety to 100% of motile spermatozoa were observed at 15 s after activation, with an average VCL of 181.12 ± 19.10 μm/s. After 90 s, average VCL decreased to 130 ± 26 μm/s. Motility was maintained for up to 4 min. The maximum percentage of motile spermatozoa was observed after the third collection of sperm. The spermatozoa VCL increased significantly with subsequent collections. The results of this study provide new data on the effects of multiple collections on quantitative and qualitative parameters of sperm in sterlet. The data confirmed that the sequential stripping has no negative effect on the percentage of motility and spermatozoa velocity. This should be beneficial for the development of sterlet aquaculture programs.

Introduction

Because of the commercial value of its flesh and the use of its roe for caviar, the demand for sturgeon has been intense and has resulted in severe depletion of wild sturgeon stocks worldwide (Birstein et al. 1997). The negative impact of commercial exploitation of sturgeon has been exacerbated by its low reproduction rate. The females of most of the commercially valuable sturgeon species do not reach reproductive maturity until 15–20 years of age [e.g. beluga (*Huso huso*)]. One approach to assuring their survival and to provide sturgeon for conservation and as a source of flesh and caviar is aquaculture.

Since 1971, artificial propagation and rearing of sturgeon has been implemented along with the general trend to development of fish culture in several European countries (Chebanov and Billard 2001). A major problem in the development of sturgeon culture is the lack of domestic broodstock and dependence of fish farmers on capture of wild broodstock, which is not only unreliable but severely restricted by government regulations. In addition to the limited availability of sexually mature females, a significant challenge is the requirement for mature males capable of producing sufficient amounts of sperm when needed. The stripping of sturgeon males more than once during hatchery operations is a common practice. So far, the physiological consequences of multiple sperm collection were studied by several authors [e.g. Podushka (2003), Alavi et al. (2004), Linhart et al. (2000)]. On the other hand, several European countries have established cryo-banks for preservation of sturgeon sperm (Li et al. 2009). The creation of sperm banks is justified by the development of genetic selection programs in fish farming along with the need for protection of diversity in wild fish populations. Therefore, more detailed information about the physiological consequences of sequential or multiple sperm collection on sturgeon sperm physiology could be useful for directing conservation efforts in protecting genetic diversity of Acipenseriformes.

Sperm quality, defined as those traits of sperm that determine its capacity to fertilize eggs, is crucial for aquaculture purposes and must be monitored in fish farming to predict male reproductive success. The quality of sperm usually refers to spermatozoa motility, as a major factor determining sperm fertilizing ability (Cosson et al. 1991).

The goal of this study was to investigate the physiological consequences of multiple sperm collections on selected quantitative and qualitative parameters of sterlet (*Acipenser ruthenus*) sperm. *Acipenser ruthenus* is a common Eurasian species of sturgeon. The species has undergone significant decline, but local populations still survive in most parts of its distribution range (rivers draining to the Black, Azov and Caspian Seas; Siberia from Ob eastward to Yenisei drainages and Danubian estuaries). The sterlet constitutes an attractive model for biological studies of sturgeon because of its freshwater status, its small size and therefore low cost of maintenance and its early sexual maturation. The present work aimed to assess the ability of sterlet males to produce sperm of suitable quality over multiple collections and to evaluate the effects of multiple sperm collection on milt volume, spermatozoa concentration and protein concentration of seminal plasma as well as functional parameters of spermatozoa motility and velocity.

Material and Methods

Broodstock and sperm collection

Experiments were carried out at the Faculty of Fisheries and Protection of Waters, University of South Bohemia at Vodnany, Czech Republic, in the middle of reproductive season. Three experimental groups were used, each consisting of six different sexually mature males (6–7 years old) with an average body weight of 2 kg. Totally, the 18 individuals have been used. Prior to hormonal stimulation, fish were kept in tanks with water temperature varying between 15 and 16°C. Spermiation was stimulated by intramuscular injection of carp

pituitary powder dissolved in 0.9% (w/v) NaCl solution at a dose of 4 mg/kg body weight. Sperm was collected from the urogenital tract by catheter into 250-ml cell culture containers to avoid contamination by mucus, faeces or water, and stored 30 min at 4°C, prior to measurements.

Experimental design

The experimental groups were A: initial sperm collection at 12 h after hormone injection followed by subsequent stripping at 17 and 22 h; B: initial sperm collection at 24 h after hormone injection followed by subsequent collection at 29 and 34 h; and C: initial sperm collection at 36 h after hormone injection followed by subsequent collections at 41 and 46 h after hormone treatment. The time intervals for sperm collections have been selected based on published literature [e.g. Podushka 2003 (sterlet); Linhart et al. 2000 (paddlefish)].

The selected qualitative parameters (e.g. spermatozoa concentration, milt volume, protein concentration in seminal plasma) together with functional parameters of sterlet sperm, including spermatozoa motility and velocity, were investigated.

Evaluation of spermatozoa motility

Percentage of motile spermatozoa and spermatozoa velocity (μm/s) were determined after triggering motility under dark-field microscopy (Olympus BX 50, Tokyo, Japan) (×20 objective magnification). For triggering motility in all groups, sperm was diluted in hatchery water at 1 : 50. Parameters of hatchery water are as follows: osmolality 18–20 mOsmol/kg as well as pH 7.2–7.5. To avoid sperm sticking to the microscope slide, 0.1% (w/v) BSA (Sigma-Aldrich, St. Louis, MO, USA) was added to the swimming medium. Motility was observed and recorded immediately after dilution until 2 min post-activation using a CCD video camera (SONY, SSC-DC50AP, Tokyo, Japan) mounted on a dark-field microscope (Olympus BX 50) with the focal plane positioned close to the glass slide surface and illuminated with a stroboscopic lamp (Chadwick-Helmut, 9630, Ontario, CA, USA) set to a flash frequency of 50 Hz. Video records were obtained using a S-VHS (SONY, SVO-9500 MDP) video recorder at 25 frames/s. The positions of the spermatozoa heads were measured in five consecutive video frames at 15, 30, 45, 60 and 90 s post-activation and subsequently analysed using MICRO-IMAGE software (version 4.0.1. for Windows with a special macro created by Olympus, Prague, Czech Republic). The percentage of motile spermatozoa and their VCL were estimated as described by Rodina et al. (2004). Briefly, spermatozoa velocity was calculated in μm/s based on the length of the spermatozoa traces (Rodina et al. 2004). At least 20 spermatozoa were observed in this way.

Measurement of osmolality and protein concentration of seminal plasma

Sperm samples were centrifuged at 300 × ***g*** for 30 min; the supernatant was collected and centrifuged for 10 min at 16 000 × ***g*** and 4°C. The collected seminal plasma was stored on ice until analysis. Osmolality was measured using a Vapour Pressure Osmometer (Wescor, Logan, UT, USA) and expressed in mOsmol/kg. The protein concentration in seminal plasma was determined by the bicinchoninic acid assay (BCA), using the photometer Infinite M200 (Tecan, Mannedorf, Switzerland) (Li et al. 2010).

Measurement of milt volume and spermatozoa concentration

Milt volume was measured in ml. Prior to determination of spermatozoa concentration, sperm was diluted 100 times with an immobilizing solution composed of 10 mM Tris and 100 mM sucrose (pH 8.5) and measured in a Burker haemocytometer at ×400. The spermatozoa concentration was calculated according to methods described by Caille et al. (2006). Spermatozoa concentration was expressed as 10^9 spermatozoa/ml of sperm.

Data analysis

Percentage of motile spermatozoa and spermatozoa velocity were determined for each male (n = 6 for each group) at several time points following activation. Measurements were taken in triplicate. Prior to statistical comparisons of the tested parameters, the residuals were checked for normality (Shapiro–Wilk's test), and data were log-transformed when necessary. Statistical comparison was made by analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference test. The values of sperm velocity and percentage of motile cell were expressed as confidence intervals. The values of other parameters (sperm volume, spermatozoa concentration, osmolality and protein concentration of seminal plasma) were expressed as means ± SD (standard deviation). All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 (Stat Soft, Inc., Tulsa, OK, USA) software for Windows.

Results

Spermatozoa motility and velocity

Direct observation of undiluted spermatozoa by dark-field microscopy showed that most spermatozoa in the seminal plasma of sterlet were immotile, with the flagella straight and slightly quivering. In all experimental groups, motility of sterlet spermatozoa was activated immediately after transfer into the swimming medium and displayed the following characteristics: (i) at 15 s post-activation, 90–100% of spermatozoa were motile with mean velocity calculated at 181.12 ± 19.10 μm/s; (ii) at 90 s after activation, the velocity decreased to 130 ± 26 μm/s; and (iii) motility of some spermatozoa was maintained for up to 4 min. The spermatozoa velocity increased significantly ($p < 0.05$) with the stripping frequency. In general, the maximum percentage of motile spermatozoa was observed after the third stripping in all experimental groups (Figs 1, 2 and 3).

Protein concentration and osmolality of seminal plasma

Non-significant differences (ANOVA; $p > 0.05$) between and within experimental groups were found in protein

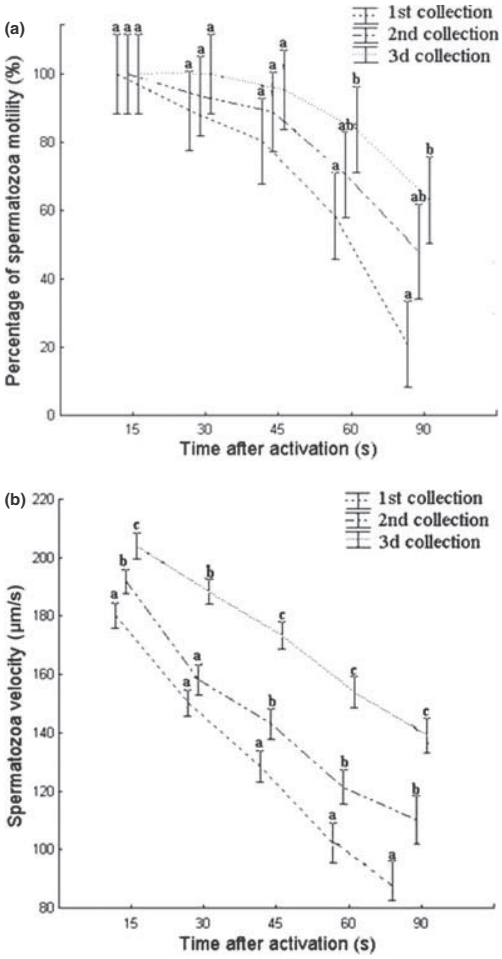

Fig. 1. Effect of multiple collections on *Acipenser ruthenus* spermatozoa motility (a) and velocity (b) in experimental group A. (12 h post-injection = first collection, 17 h post-injection = second collection, 22 h post-injection = third collection). Values with the same superscripts are not significantly different ($p > 0.05$, ANOVA)

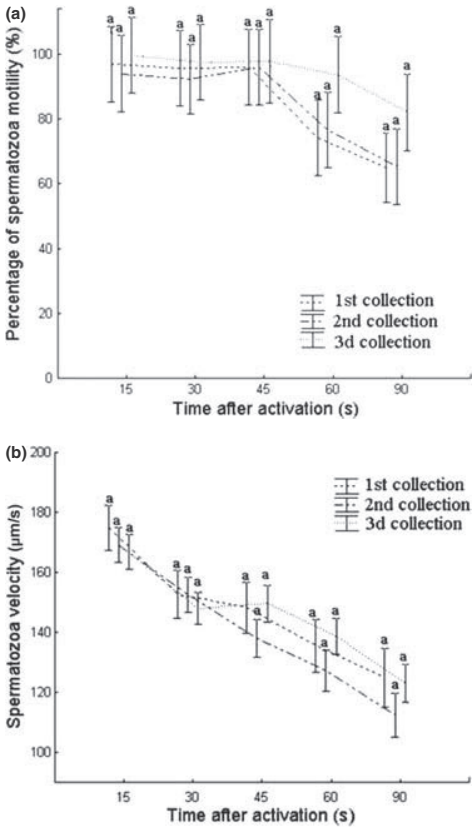

Fig. 2. Effect of multiple collections on *Acipenser ruthenus* spermatozoa motility (a) and velocity (b) in experimental group B. (24 h post-injection = first collection, 29 h post-injection = second collection, 34 h post-injection = third collection). Values with the same superscripts are not significantly different ($p > 0.05$, ANOVA)

concentration (Table 1). The osmolality of seminal plasma was significantly ($p < 0.05$) increased by the second collections in experimental group B. In groups A and C, significant changes in osmolality of seminal plasma with subsequent collections were not detected.

Milt volume and spermatozoa concentration

A high variability in the values of volume and spermatozoa concentration was observed (Table 1). Statistical analysis revealed that multiple collections significantly ($p < 0.05$) altered sperm volume in experimental groups B (from 12.74 ± 6.9 to 4.73 ± 4.67 ml) and C (from 16.44 ± 7.07 to 7.89 ± 4.44 ml). However, no significant differences among collections in group A were observed. Significant differences in spermatozoa concentration within groups were found at all collection times (ANOVA; $p < 0.05$) (Table 1).

Discussion

Spermatozoa of sturgeon and paddlefish are essentially immotile in the seminal plasma (Cosson and Linhart 1996; Li et al. 2009) but are fully activated by swimming medium. Environmental factors, such as ions, pH and osmolality, may lead to the depolarization of the cell membrane and stimulate motility of spermatozoa. As suggested by Trippel (2003), male fertilization potential is dependent on sperm quality. The predominant factors investigated in studies of sperm biology are ultrastructure and motility, the biochemistry of the spermatozoa and seminal plasma and spermatozoa metabolism (respiration and energy expenditure in motility). We found sterlet spermatozoa to be activated immediately

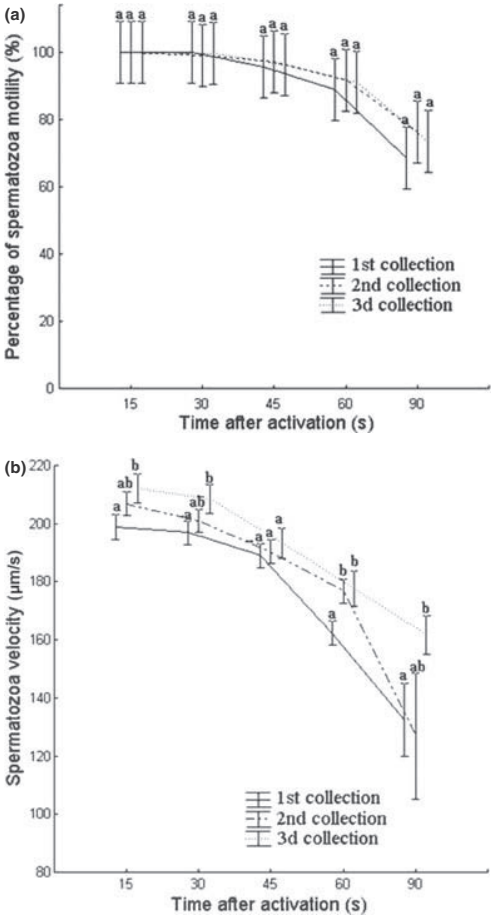

Fig. 3. Effect of multiple collections on *Acipenser ruthenus* spermatozoa motility (a) and velocity (b) in experimental group C. (36 h post-injection = first collection, 41 h post-injection = second collection, 46 h post-injection = third collection). Values with the same superscripts are not significantly different ($p > 0.05$, ANOVA)

after transfer to swimming medium irrespective of stripping frequency, and 90–100% spermatozoa became motile, a result that agrees with the previous observations (Cosson et al. 2000; Linhart et al. 2002). The maximum percentage of motile spermatozoa and highest velocity were observed after the third stripping in all experimental groups. Similar results have been reported in studies of Siberian sturgeon *Acipenser baerii* (Gallis et al. 1991), shovelnose sturgeon *Scaphirhynchus platorynchus* (Cosson et al. 2000), Persian sturgeon *Acipenser persicus* (Alavi et al. 2004) and paddlefish *Polyodon spathula* (Linhart et al. 2002).

In teleosts, a negative effect of stripping frequency has been reported on duration and intensity of sperm movement in rainbow trout *Salmo gairdneri* (Buyukhatipoglu and Holtz 1984). No effect of sequential stripping was reported on the duration of spermatozoa movement in turbot *Scophthalmus maximus* by Suquet et al. (1992). Williot et al. (2000) reported the highest percentage of motility of spermatozoa in European sturgeon (*Acipenser sturio*) that were stripped 36 h after stimulation of spermiation using injection of a commercial acetone-derived carp pituitary homogenate at 2 mg/kg body weight. Alavi et al. (2006) found a negative effect of sequential sperm collection on spermatozoa velocity and motility in Persian sturgeon.

In the current study, the volume of sperm collected was highly variable in all experimental groups, but it is apparent that sterlet was able to produce sperm throughout the experiment, even at intervals of 5 h. However, it is still not clear if sperm from each collection possessed high fertilization ability. Podushka (2003) showed that in sterlet males, beginning 12 h after hormone injection, it was possible to collect sperm every 2 h with volume of milt remaining more or less constant. In paddlefish, Linhart et al. (2000) observed that the volume of sperm produced was significantly higher from day 1 to 4.5 in LH-RHa hormone-treated fish compared to the control. Therefore, we hypothesize that in sterlet, multiple collections of sperm can be used to increase milt volume with adequate functional sperm parameters such as motility and velocity. These factors represent major criteria for artificial fish reproduction.

In the present study, multiple collections of sperm significantly altered spermatozoa concentration in all experimental groups, a result that agrees with the previous observations in Persian sturgeon (Alavi et al. 2006), paddlefish (Linhart et al. 2000) and Siberian sturgeon (Piros et al. 2002). In all experimental groups, the highest spermatozoa concentration was observed after the second stripping vs the first and third stripping. A similar result was seen in a study of Siberian sturgeon (Piros et al. 2002). The reasons for such differences in spermatozoa concentration are not clear. We suggest that the reason for decrease in sperm concentration with multiple collections could be the result of increased production of seminal plasma, which would dilute sperm and consequently lead to lower measured concentration. Other possible explanations may be sensitive to hormone treatment used as well as sensitive to environmental conditions and biological characteristics of the broodfish, as was previously noted studies of paddlefish (Linhart et al. 2000) and Siberian sturgeon (Piros et al. 2002). It is also worth to be mentioned that in the present study multiple sperm collection did significantly decreased spermatozoa concentration that is classified as an important prerequisite of successful fertilization (Kaspar et al. 2007). Therefore, from practical point of view, it is reasonable to apply this methodology during hatchery operations. At least, in terms of endangered species such as sturgeons, restricted amount of sexually mature individuals is always a limited factor during artificial reproduction.

The osmolality and composition of seminal plasma usually prevent sperm motility in fish sperm ducts (Billard 1986). The seminal plasma not only immobilizes the spermatozoa but probably also protects them

Table 1. Descriptive statistics of male sterlet, *Acipenser ruthenus* and summary of tested parameters

Experimental group	Parameters	Sperm collection time (h)		
		First	Second	Third
A		12	17	22
	Osmolality (mOsmol/kg)	57.40 ± 9.9^{aA}	66.50 ± 24.51^{aA}	57.57 ± 13.55^{aA}
	Sperm volume (ml)	5.90 ± 1.14^{aB}	5.51 ± 1.80^{aB}	4.38 ± 2.62^{aA}
	Protein concentration (mg/ml)	0.55 ± 0.27^{aA}	0.60 ± 0.10^{bA}	0.52 ± 0.14^{aA}
	Spermatozoa concentration (10^9 spz/ml)	1.08 ± 0.63^{abA}	1.47 ± 0.47^{aA}	0.84 ± 0.49^{bA}
B		24	29	34
	Osmolality (mOsmol/kg)	45.66 ± 4.45^{bB}	62.50 ± 6.68^{aA}	57.83 ± 6.73^{aA}
	Sperm volume (ml)	12.74 ± 6.9^{aA}	5.82 ± 1.91^{bB}	4.73 ± 4.67^{bA}
	Protein concentration (mg/ml)	0.50 ± 0.18^{aA}	0.56 ± 0.44^{aA}	0.57 ± 0.11^{aA}
	Spermatozoa concentration (10^9 spz/ml)	0.61 ± 0.40^{bB}	1.35 ± 0.22^{aA}	0.51 ± 0.31^{bA}
C		36	41	46
	Osmolality (mOsmol/kg)	58.66 ± 11.12^{aA}	61.50 ± 10.31^{aA}	55.00 ± 10.31^{aA}
	Sperm volume (ml)	16.44 ± 7.07^{aA}	13.83 ± 7.26^{aA}	7.89 ± 4.44^{bA}
	Protein concentration (mg/ml)	0.30 ± 0.18^{aA}	0.34 ± 0.13^{aA}	0.30 ± 0.04^{aA}
	Spermatozoa concentration (10^9 spz/ml)	1.11 ± 0.56^{aA}	1.20 ± 0.40^{aA}	0.51 ± 0.30^{bA}

In all experimental groups, the data were expressed as means ± SD. Capital letters indicate differences between groups, and small letters indicate differences between collections times. Values with the same superscripts are not significantly different ($p > 0.05$, ANOVA).

(Cosson et al. 1997). In the present study, the osmolality of seminal plasma was similar to that in other sturgeon species, such as stellate sturgeon *Acipenser stellatus* (Li et al. 2011), Russian sturgeon *Acipenser gueldenstaedtii* (Li et al. 2011), beluga *H. huso* (Li et al. 2010) and paddlefish *P. spathula* (Cosson et al. 2000; Linhart et al. 2002). Our results revealed that multiple sperm collections had no significant effect on osmolality of seminal plasma in experimental groups A (12, 17 and 22 h post-injection) and C (36, 41 and 46 h post-injection). A significantly lower value of osmolality of seminal plasma was detected in group B after the first collection (24 h post-injection). In contrast to our results, observations of paddlefish (Linhart et al. 2003) and Persian sturgeon (Alavi et al. 2006) found a decreasing trend in osmolality of seminal plasma as a consequence of multiple stripping. This leads to the conclusion that the decreasing trend may be a response to changes in secretory activity in the fish spermatic duct during the spermiation period, because the production of seminal plasma in fish (inorganic as well as organic compounds) is a secretion process of the spermatic duct epithelium (Li et al. 2009).

The impact of seminal plasma proteins on sperm function is complex and not fully understood. Accumulating evidence shows that seminal plasma plays a pivotal role in the regulation of sperm motility and in the protection against proteolytic attack during storage of spermatozoa in the reproductive system (Li et al. 2010). In general, the seminal plasma of chondrostean and teleost fish is characterized by a low concentration of proteins compared with mammalian sperm, not exceeding 2 g/l (Li et al. 2009). In the present study, the total protein concentration (0.50 ± 0.18 mg/ml) was lower than that reported for salmonids such as rainbow trout (1.74 ± 0.79 mg/ml) (Loir et al. 1990), but in agreement with concentrations reported for sturgeon (Piros et al. 2002; Li et al. 2009, 2010). No significant effect of sequential stripping on total protein in seminal plasma was detected.

The results of this study provide new data on sterlet sperm quality with respect to multiple collections, which should be beneficial for the development of aquaculture of this species. The data confirmed that multiple collections of sperm in sterlet did not negatively affect functional parameters of sterlet spermatozoa.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author Contributions

Anna Shaliutina, Otomar Linhart and Martin Hulak drafted the paper; Boris Dzyuba designed the study; and Sergey Boryshpolets and Ping Li analysed the data.

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CHAPTER 3

COMPARISON OF PROTEIN FRACTIONS IN SEMINAL PLASMA FROM MULTIPLE SPERM COLLECTIONS IN STERLET (*ACIPENSER RUTHENUS*)

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Comparison of Protein Fractions in Seminal Plasma from Multiple Sperm Collections in Sterlet (*Acipenser ruthenus*)

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Contents

Seminal plasma of sterlet *Acipenser ruthenus* was evaluated using comparative proteomics to characterize its protein fractions and to determine any influence of multiple sperm collections on these proteins. An experimental group of fish was used, in which sperm was collected three times at 5 h intervals. Protein fractions of seminal plasma were determined by SDS-gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis high-resolution gels (2D). At all stripping times, five protein bands with molecular weights of 93, 53, 48, 33 and 28 kDa were identified using SDS-PAGE. No significant differences ($p > 0.05$) in relative mass of protein bands among collections were observed. At the third collection, 20 protein spots were detected from the two-dimensional gels, compared to 17 found at the first and second collections. Ten protein spots, from the third stripping, were analysed. Screening of these spots by mass spectrometric analysis showed positive results for spot 10. Direct comparison across public databases revealed sequence similarity with two hypothetical proteins, MCAG_00854 and IscW_ISCW011489. Differences in the seminal plasma protein fractions were found at the third stripping compared to the first two. It is hypothesized that these extra proteins after the third collection could be involved in some step of intracellular mechanism which is responsible for regulating of spermatozoa motility. However, protein identification revealed no significant distinction for any protein spot and protein sequences available in public databases. These results highlighted the need for a complete genome sequences for sturgeons.

Introduction

Sturgeon (Acipenseriformes: Acipenseridae) and paddlefish (Acipenseriformes: Polyodontidae) are a unique and commercially valuable (e.g. caviar production) group of ancient fishes with late sexual maturity and unusual longevity (Bemis et al. 1997; Birstein 1993). They are highly vulnerable and have been exploited to near extinction. The sterlet *Acipenser ruthenus* of the family Acipenseridae is of relatively small size and rapid sexual maturation (Bemis et al. 1997). This species was commercially exploited in the Volga and Danube rivers into the mid-twentieth century until anthropogenic activities led to a decline in sterlet populations (Birstein et al. 1997; Billard 2000). The commercial relevance of *A. ruthenus* is related to international trade in its meat and in live juveniles for ornamental purposes. According to the Red list 2000 of IUCN, the sterlet is classified as vulnerable species (IUCN 2000).

Because of the shortage of sturgeon male broodstock, the availability of good quality sperm in sufficient amounts at the required time, along with the management of semen, ultimately determines the success of artificial reproduction on sturgeon farms. The stripping of males more than once during the reproductive season is a common practice at sturgeon hatcheries, and more information is needed about its consequences for sturgeon sperm physiology (Linhart et al. 2000; Alavi et al. 2006; Podushka 2003). Previous studies investigating the effect of multiple sperm collection showed that there were no negative effects on the per cent motility and velocity in sequential stripping (Shaliutina et al. 2011). In fact, spermatozoa velocity increased significantly ($p < 0.05$) with the stripping frequency. Moreover, the maximum percentage of motile spermatozoa was observed after the third stripping.

During the past decade, the results of several studies have suggested that the protein composition of seminal plasma is important to protection of spermatozoa during storage in the reproductive system, as well as to sperm viability (Cosson et al. 1997; Borges et al. 2005). Several authors have concluded that male fertilization potential is highly dependent, not only on sperm ultrastructure and spermatozoa motility, but also on organic components including protein composition of seminal plasma (Li et al. 2009, 2010a; Ciereszko 2008; Loir et al. 1990). There is a lack of information about protein composition of seminal plasma during multiple stripping; therefore, we applied comparative proteomics to investigate the possible effects of multiple sperm collection on protein fractions of seminal plasma in sterlet. The major aim of this study was to describe protein composition of sterlet seminal plasma and to evaluate the possible effects of multiple sperm collection on these proteins.

Material and methods

Fish and rearing conditions

Six sexually mature males (1.5–2.0 kg, 6–7 years old) were kept during the natural spawning season in 4 m^3 outdoor plastic tanks located at the hatchery of the Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Constant pond water flow was at the rate of 20 l/min and temperature was 11°C. Three weeks before stripping, the males were transferred to 4 m^3 hatchery tanks with a water flow rate of 0.2 l/s, water temperature of 15°C and 6–7 mg O_2/l, photoperiod 15 h light/9 h dark. Because of these standardized laboratory conditions, it is assumed that all fish had been subjected to common environmental effects. Moreover, fish were kept 3–4 days in such conditions without feeding prior to hormone treatment.

Sperm collection

Sterlet males (n = 6) were stimulated hormonally in April with intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at a dose of 4 mg/kg body weight. Sperm was collected from the urogenital tract by catheter (4 mm diameter) into 250 ml cell culture containers. Special care was taken to avoid contamination with urine, mucus, faeces or water. Sperm samples were stored on ice prior to analysis. Sperm was collected 24-h post-hormone injection followed by subsequent collections at 29 and 34 h.

Preparation of protein samples

Sperm samples were initially centrifuged at $300 \times g$ for 30 min followed by 10 min at $16\,000 \times g$ at 4°C according to the methods described by Li et al. (2010b). The supernatant was collected and stored at −80°C until analysis. The bicinchoninic acid assay (BCA), using the photometer Infinite M200 (Tecan, Switzerland), was applied to determine protein concentrations in the samples.

SDS-gel electrophoresis (SDS-PAGE)

As a first step, protein separation was performed by 12% polyacrylamide-bisacrylamide gel electrophoresis. Samples were re-suspended in buffer containing 65 mM Tris 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), and 5% (v/v) beta-mercaptoethanol and denatured for 3 min at 95°C prior to loading to gels. The gels were stained by Coomassie Brilliant Blue R-250. Subsequently, the gels were scanned and analysed using the GelQuant computer program, v 2.7.0 (Bio-Imaging Systems, Israel). The value of relative mass proteins (RMP) in each band was calculated as follows:

$$RMP_i = (MP_i/(MP_1 + MP_2 + \ldots MP_i)) \times 100\%$$

where RMP_i = relative protein mass; MP = protein mass; 1,2…i = band number.

Two-dimensional gel electrophoresis (2DE)

For further investigation, the seminal plasma proteins were analysed by 2DE. Isoelectric focusing (IEF) was performed on ReadyStrip IPG strips (pH 3–10, 7 cm) with PROTEAN IEF (Bio-Rad, Hercules, CA, USA). A total of 50 μg of protein was used for preliminary runs to a total volume of 125 μl of rehydration buffer (8 M Urea, 2 M Thiourea, 4% Chaps, 50 mM dithiothreitol, 0.4% IPG buffer). After IEF, the IPG strips were equilibrated according to methods described by Li et al. (2010b). Each IPG strip was laid onto a 12% SDS-PAGE gel for second dimension electrophoresis. Protein spots were visualized either by Silver staining or by Coomassie Blue R-250.

Mass spectrometric analysis

Mass spectra were measured on a matrix-assisted laser desorption/ionization reflectron time-of-flight MALDI-TOF/TOF mass spectrometer ultraFLEX III (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standards PepMix II (Bruker-Daltonics). A 5 mg/ml solution of α-cyano-4-hydroxy-cinnamic acid in 50% MeCN/0.3% acetic acid was used as a MALDI matrix. A 0.5 μl droplet of the sample was loaded onto the target, allowed to dry at ambient temperature and overlaid with a 0.4 μl of matrix solution. The positive MALDI-TOF spectra were collected in reflectron mode. MS/MS LIFT spectra of selected ions were acquired to identify protein or to confirm protein identification. The *de novo* manual sequencing for protein identification was applied.

Data analysis

Relative mass of each protein band was determined. Statistical comparison was made by the analysis of variance (ANOVA) followed by Tukey's HSD test. The values were expressed as mean ± SD. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 (Stat Soft, Inc., Tulsa, OK, USA) software for Windows.

Results and Discussion

In this study, results of SDS-PAGE were used as a first step to determine whether seminal plasma protein fractions were affected by multiple sperm collections. The seminal plasma protein fractionation on the SDS-polyacrylamide gel resulted in 5 protein bands irrespective of collection frequency. Molecular weight of detected protein bands ranged from 28 to 93 kDa (Table 1). No significant differences ($p > 0.05$) among collections in the values of relative mass of detected protein bands were observed (Table 1). During the separation of protein fractions using SDS-PAGE, proteins with the same molecular weight may overlap. To further investigate the effects of multiple sperm collection on seminal plasma proteomics, seminal plasma proteins (MCSP) were subjected to 2DE, allowing the investigation of protein fractions of seminal fluid based, not only on their molecular weight, but also on their isoelectric points (pI). The distribution of sterlet seminal plasma proteins is shown in Fig. 1a–c. After the first (24-h post-injection) and second (29-h post-injection) collection, 17 protein spots were detected, with molecular weights ranging from 26 to 84 kDa and isoelectric points (pI = 3–10). Following the third sperm collection, three additional protein spots were detected (1–3) that were not observed in the first and second stripping (Fig. 1c). The protein spots 1–3 were cut from the gel and subjected to three methods of identification (MALDI-TOF/MS matrix-associated laser desorption/ionization time-of-flight/mass spectrometry; MS/MS LIFT spectra and de novo manual sequencing). The obtained results were compared with available protein sequences deposited in public databases; but no positive sequence coverage was found. It is believed that these proteins could be involved in some step of intracellular mechanism which is responsible for the regulation of spermatozoa motility (Shaliutina et al. 2011).

Table 1. Protein patterns of *Acipenser ruthenus* seminal plasma by SDS-PAGE during multiple collections. No significant differences in relative mass of protein bands associated with different stripping were found ($p > 0.05$; ANOVA)

Sperm collection time (hours)	Protein (kDa)	Molecular weight (kDa) as determined from 6 samples	Relative mass of proteins bands (ng)
First (24)	93	93.0 ± 2.68	28.49 ± 7.4
	53	53.4 ± 0.54	48.24 ± 6.2
	48	48.4 ± 0.89	6.45 ± 3.5
	33	33.2 ± 0.44	7.12 ± 4.7
	28	28.6 ± 0.54	3.75 ± 0.4
Second (29)	93	93.5 ± 2.16	29.29 ± 2.5
	53	53.0 ± 0.63	48.03 ± 1.9
	48	48.3 ± 0.81	6.01 ± 2.7
	33	33.2 ± 0.75	6.90 ± 3.5
	28	28.5 ± 0.54	3.09 ± 0.8
Third (34)	93	93.4 ± 2.96	29.0 ± 2.6
	53	53.2 ± 0.83	49.19 ± 4.6
	48	48.4 ± 1.14	6.00 ± 3.3
	33	33.0 ± 0.70	6.70 ± 3.0
	28	28.4 ± 0.54	3.33 ± 0.7

An additional 7 protein spots (4–10) were selected for subsequent analysis. The MS-Pattern Search revealed no significant identification for any protein spot. A positive result was obtained for spot 10; direct comparison of amino acid sequences revealed sequence similarity to two hypothetical proteins (MCAG_00854 and IscW_ISCW011489) and one unnamed protein product. The lack of information on the sturgeon genome illustrated the difficulties that can be encountered in biochemical studies and highlights the need for a complete genome analysis of the main representatives in this genus.

As sturgeon farming expands for restocking and for commercial aquaculture (Billard 2000), there is an increasing need to improve the breeding process by more effective and better standardized gamete management and handling. Because of dramatic declines in stocks of endangered sterlet, the sexually mature males are stripped more than once during the spawning season for artificial insemination in hatcheries. As mentioned, the quality of sturgeon sperm after multiple collections, as determined by spermatozoa motility and velocity, has been investigated (Alavi et al. 2006; Linhart et al. 2000).

In our previous study, we showed no significant effect of multiple sperm collection on spermatozoa motility in sterlet (Shaliutina et al. 2011). Contrary to our finding, Alavi et al. (2006) found a negative effect of sequential sperm collection on spermatozoa velocity and motility in Persian sturgeon (*Acipenser persicus*). Moreover, in paddlefish (*Polyodon spathula*), Linhart et al. (2000) reported that the sperm production was significantly higher in LH-RHa hormone-treated males and reached the highest sperm production after 4.5 days. In contrast to the above-mentioned studies, Piros et al. (2002) observed that the spermatozoa concentration in Siberian sturgeon (*Acipenser baeri*) increased after the second stripping *versus* the first stripping. However, the observed increase was not significant. The reason for this difference is unclear and might be related to the differences in hormonal stimulation methods used in these studies or environmental conditions. In this light quite recently published paper by Hajirezaee et al. (2011) showed higher serum levels of testosterone (T), 11-ketotestosterone (11-KT), progesterone (P), 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20βS) and cortisol (C) after treatment with sturgeon pituitary extract, in comparison with non-spermiating males. In addition, these results showed probable involvement of these steroids in final steps of spermatozoa maturation. Therefore, we hypothesized that the hormonal treatment used for induction of spermiation together with sequential stripping can positively or negatively modified spermatozoa motility via changes in the enzymatic activities of the seminal plasma which is established subsequent to the sperm concentration.

During the past decade, the results of several studies have suggested that the protein composition of seminal plasma is crucial to the protection of spermatozoa during storage in the reproductive system as well as to sperm viability (Lahnsteiner et al. 2004; Lahnsteiner 2007).

The results of the present study confirmed that multiple collections of sperm in sterlet altered the protein pattern of seminal plasma after a third stripping. Furthermore, the altered proteins are probably involved in enzymatic pathways that regulate spermatozoa movement. In combination with our previously published results, we conclude that multiple sperm collection in sterlet did not significantly alter sperm quality, and therefore, this method could be a useful tool for artificial reproduction of sterlet, especially when access to appropriate numbers of sexually mature males is limited. The reported results should be interpreted with caution because of a lack of information on the possible

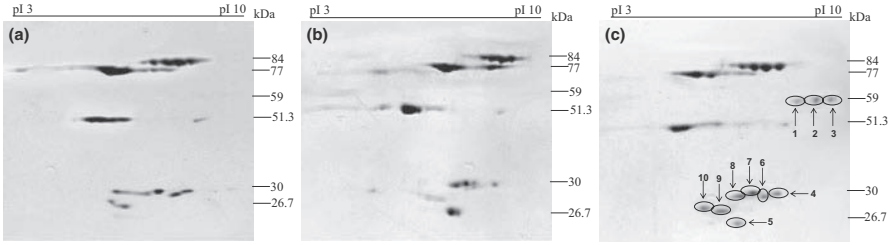

Fig. 1. Effect of multiple sperm collections on *Acipenser ruthenus* seminal plasma protein patterns with two-dimensional polyacrylamide electrophoretic gel (a: 24-h post-injection; b: 29-h post-injection; c: 34 h post-injection). The arrows indicate protein spots that were selected for identification based on their differences in expression among three stripping. Each specimen was analysed in triplicate

function of proteins that are altered by multiple sperm collections. And further studies are needed to understand the physiological relationships.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Drafted of the paper is done by the authors A Shaliutina, M Hulak, and O Linhart. Designing of this study is done by the authors A Shaliutina and B Dzyuba. Data used in this study are analysed by A Shaliutina, P Li, and M Sulc.

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CHAPTER 4

SPERMATOZOA MOTILITY AND VARIATION IN THE SEMINAL PLASMA PROTEOME OF EURASIAN PERCH (*PERCA FLUVIATILIS*) DURING THE REPRODUCTIVE SEASON

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RESEARCH ARTICLE

Molecular Reproduction & Development 79:879–887 (2012)

Spermatozoa Motility and Variation in the Seminal Plasma Proteome of Eurasian Perch (*Perca fluviatilis*) During the Reproductive Season

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SUMMARY

This study evaluated physiological and functional sperm parameters and the seminal plasma proteome of Eurasian perch (*Perca fluviatilis*) over the course of their reproductive season. Spermatozoa velocity (169.56 ± 6.53 to $158.5 \pm 7.4\,\mu m\,sec^{-1}$), percent motility ($95.89 \pm 4.28\%$ to $89.55 \pm 4.5\%$), and osmolality of seminal plasma (290 ± 5 to 297 ± 12 mOsmol kg^{-1}) remained stable throughout the reproductive season. Milt volume and protein concentration of seminal plasma gradually increased and reached the highest values late in the reproductive period. Spermatozoa concentration peaked in the mid-reproductive season ($66.90 \pm 13 \times 10^9$ spermatozoa ml^{-1}) and decreased towards the end ($54 \pm 10 \times 10^9$ spermatozoa ml^{-1}). A proteomic analysis of seminal plasma using two-dimensional polyacrylamide gel electrophoresis revealed 10 protein spots significantly altered over the course of the reproductive season. Subsequent protein characterization suggested that time in the reproductive season predominantly affected proteins involved in membrane trafficking, organization, cell motility, and oxido-reductase activity. This study provides new data on physiological properties of sperm and protein patterns of seminal plasma over the course of the reproductive season that should be considered in the development of methods for artificial reproduction of perch.

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INTRODUCTION

The Eurasian perch (*Perca fluviatilis*) has a wide distribution in northern Europe and Asia, as limited by water temperature (Toner and Rougeot, 2008). It is tolerant of a wide variety of environments, but prefers shallow, calm, and relatively nutrient-rich freshwater (Toner and Rougeot, 2008). During the past decade, perch have gained increasing attention in the freshwater aquaculture sector in Europe (Kestemon and Melard, 2000). Successful fish production is principally dependent on artificial insemination methods and on the quality of male and female gametes.

Abbreviations: 2DE, two-dimensional polyacrylamide gel electrophoresis.

Nevertheless, more information on gamete physiology and regulation at a molecular level is required in perch aquaculture. Recently it has been proposed that, in addition to the traditional spermatozoa quality variables of motility, velocity, and the osmolality of seminal plasma (Kaspar et al., 2007, 2008), the protein composition of the seminal plasma also plays an important role in fertilization (Li et al., 2009).

Among those fish species studied, perch has one of the highest seminal plasma protein concentrations (Lahnsteiner et al., 1995). Several protein groups have been identified including lipoproteins (Loir et al., 1990), metalloproteases, serine proteases (Kowalski et al., 2003), transferrin (Wojtczak et al., 2005; Li et al., 2010a), and anti-proteases (Ciereszko et al., 2000). A suggested function for these protein groups is the protection of spermatozoa against proteolytic enzyme activity during the reproductive season (Ciereszko et al., 2000; Lahnsteiner et al., 2004). Several studies have shown interspecies differences in seminal plasma protein composition (Li et al., 2011) as well as a significant effect of sperm collection method on protein composition of seminal plasma (Shaliutina et al., 2012ab). The role of seminal plasma protein groups, however, is not well understood.

The aim of the present study was to investigate variation over the course of the reproductive period in Eurasian perch spermatozoa concentration, osmolality, and protein concentration of seminal plasma, as well as functional variables such as spermatozoa motility and velocity. Proteomic methods were used to study protein patterns of seminal plasma as well as to determine possible functions.

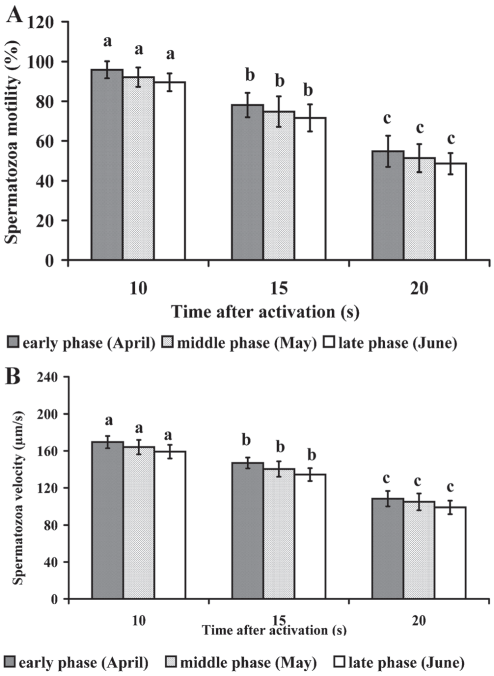

Figure 1. Changes in *Perca fluviatilis* spermatozoa motility (**A**) and velocity (**B**) during the spawning season (n = 10). Values with the same superscripts are not significantly different ($P > 0.05$, ANOVA).

RESULTS

Spermatozoa Motility and Velocity

Observation of undiluted sperm by dark field microscopy showed that most spermatozoa in the seminal plasma were immotile, with flagella straight and slightly quivering. Throughout the reproductive season, a majority of sperm samples showed 95–100% motile spermatozoa within 10 sec of dilution in activation medium. At all sampling times, the percent of motile spermatozoa and spermatozoa velocity decreased rapidly as a function of time post-activation. No significant differences (ANOVA; $P > 0.05$) in spermatozoa velocity or percent of motile cells were detected throughout the reproductive season (Fig. 1A and B).

Milt Volume and Spermatozoa Concentration

The volume of collected milt gradually increased (Fig. 2A) during the reproductive season, and significant differences among collections (ANOVA; $P < 0.05$) were observed. The lowest volume of milt (2.07 ± 1.09 ml) was found in the early phase of the reproduction season (April) and the highest (5.43 ± 2.64 ml) late in the reproductive season (June). Spermatozoa concentration (Fig. 2B) showed a bell-shaped curve with the maximal value in mid-phase season ($66.90 \pm 13 \times 10^9$ spermatozoa ml^{-1}).

Protein Concentration and Osmolality of Seminal Plasma

The mean seminal plasma osmolality was 290–297 mOsmol kg^{-1}, with small variation (ANOVA; $P > 0.05$) within each phase (Fig. 3A). Significant differences (ANOVA; $P < 0.05$) in protein concentration were detected among collection times (Fig. 3B). The maximum protein concentration in the seminal plasma (6.2 ± 2.39 mg ml^{-1}) was recorded in June, whereas the minimum was detected at the beginning of the reproductive season in April (4.60 ± 1.79 mg ml^{-1}).

Protein Separation by two-dimensional Gel Electrophoresis and Gel Imaging

To characterize changes over the course of the reproductive season in seminal plasma proteome, seminal plasma proteins were subjected to two-dimensional electrophoresis (2DE). No significant variability in amount or localization of protein spots detected was revealed among the seminal plasma in different males (10 males) within the

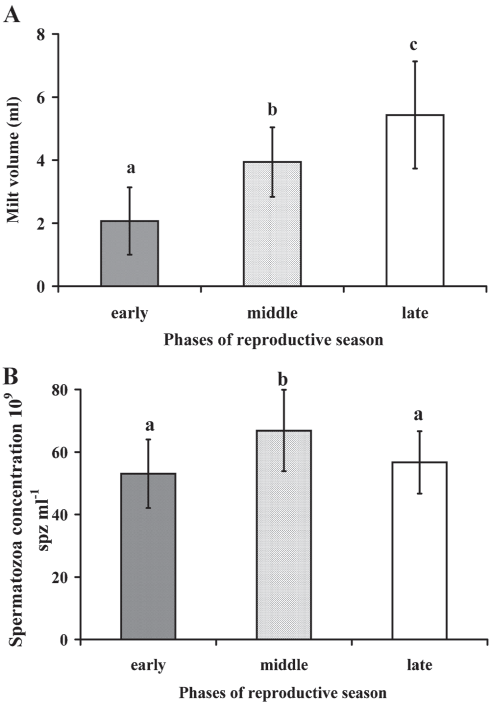

Figure 2. Changes in *Perca fluviatilis* milt volume (**A**) and spermatozoa concentration (**B**) during the spawning season (n = 10). Data were expressed as means ± standard deviations. Values with the same superscripts are not significantly different ($P > 0.05$, ANOVA).

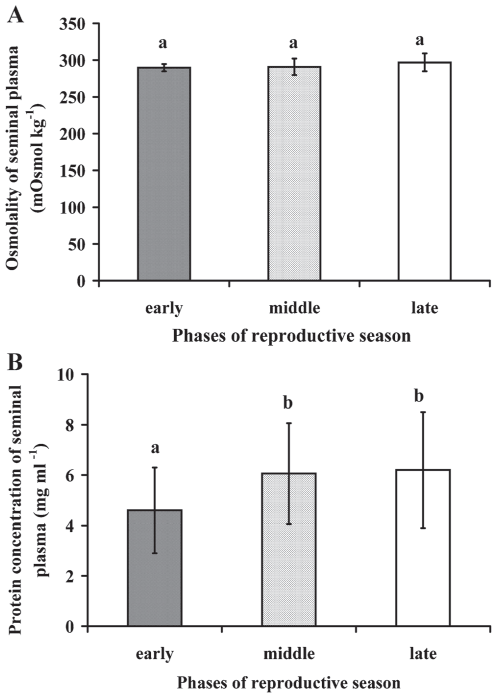

Figure 3. Changes in *Perca fluviatilis* seminal plasma osmolality (**A**) and protein concentration (**B**) during the spawning season (n = 10). Data were expressed as means ± standard deviations. Values with the same superscripts are not significantly different ($P > 0.05$, ANOVA).

same phase of reproductive season. On the other hand, 2DE gels produced in early (April) and in the mid/late (May–June) phases of the reproduction season resulted in 96 and 106 protein spots, respectively, with molecular masses of 25–250 kDa and isoelectric points (pI) of 4.7–5.9 (Fig. 4). In the mid- to late-phase of the reproductive season, six protein spots (nos. 3, 4, 6, 7, 9, and 10) were found that were not seen at the initial sampling. The intensity of these spots increased towards the end of the spawning period. The intensity of protein spots 2, 5, and 8 was higher at the end of the reproductive season than at the early and mid-phase. Protein spot 1 was detected only at the end of the reproductive season.

Identification of Protein Spots by Mass Spectrometry

Seminal plasma protein spots 1–10 were cut from the gel and subjected to matrix-associated laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF/MS). First, all protein spots were identified by Mass Fingerprinting. No significant hits of protein identification using Peptide Mass Fingerprinting was observed with any obtained dataset of m/z signals. The proteins spots were further examined by the MS/MS Ion search MASCOT tool or by manual interpretation with de novo sequence tag homology from selected m/z signals (Table 2). The results were compared to available protein sequences deposited in public databases. No significant hits of protein identification using MASCOT MS/MS Ion Search Approach was observed for spots 1, 2–6 or 7. Although sample 8 exhibited low homology with the peptide FNRPFMVIITER [serpin 3–5-like (*Oreochromis niloticus*; gi|348516246), the manual annotation of the MS/MS spectra excluded this result as a false positive identification. Two signals of spots 9 and 10 revealed significant identification as hemopexin-like, warm temperature acclimation protein 65-2 and partial-and-warm temperature acclimation-related-65 (Table 1). Because many m/z signals revealed abundant spectra with high signal/noise ratio but no protein identification, manual, de

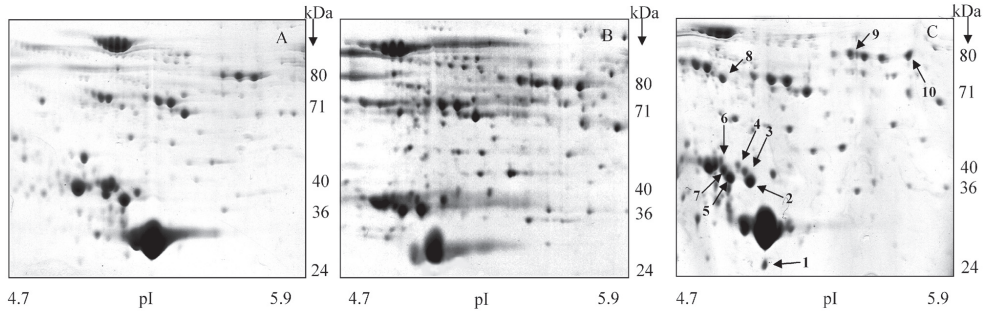

Figure 4. Effect of reproductive season on *Perca fluviatilis* seminal plasma protein patterns with 2DE. (**A** = early phase of reproductive season, **B** = middle phase of reproductive season, and **C** = late phase of reproductive season). Molecular weight marker is on the right.

novo sequence determination was performed with a sequential tag search (Table 2).

DISCUSSION

Spermatozoa velocity, motility, and osmolality of seminal plasma were stable during the entire reproductive season. Milt volume and protein concentration gradually increased and reached the highest levels late in the reproductive season. Spermatozoa concentration peaked in mid-phase and decreased towards the end of spawning season. Our results showed significant differences in seminal plasma proteome over the course of the reproductive season.

Spermatozoa motility is a major prerequisite for successful fertilization (Li et al., 2009). In the present study, no

TABLE 1. Protein Identification by De Novo Sequencing and Sequential Tag Homology Search Approach

Spot no.	Protein name [organisms]	Accession no.	MW (kDa)/pI	MS/MS (m/z)	Sequence
1	Pleckstrin-like protein domain-containing family f member 3 [*Ictalurus furcatus*]	ADO28110	24.1/5.2	1494.72	NSEVQFTMLNRR
1	Rnmtl1-b protein, partial [*Danio rerio*]	AAI29485	24.1/5.2	1622.811	NSEVQFTMLNRRK
1	Unnamed protein product [*Tetraodon nigroviridis*]	CAG11926	24.1/5.2	1690.979	MPLQPASPPDLNAALR
1	Coronin-7 [*Danio rerio*]		24.1/5.2	2732.333	QNDGFAAVWWEFDWK
2–6	MATE efflux family protein *Prevotella amnii*	ZP_07627789	36–41/4.94–5.15	2425.193	E[IL]FTAWV[IL]VW
2–6	Predicted: geminin-like [*Oreochromis niloticus*]	XP_003444213	36–41/4.94–5.15	2843.371	DAQDENYDDDILFGGNVK
2–6	Transposase [*Bifidobacteruim longum*]	322690212	36–41/4.94–5.15	1537.789	DAMPDPR
7	Dual specificity protein phosphatase 18 [*Salmo salar*]	NP_001135353	39.5/4.94	1365.767	EVEYIHIVHVK
7	Carbonic anhydrase like precursor [*Danio rerio*]	NP_001038604	39.5/4.94	1882.033	STLSGGGLPTTYK
7	Unnamed protein product [*Tetraodon nigroviridis*]	CAG00932	39.5/4.94	1271.711	EDITEMTNYR
7	Soluble lytic murein transglycosylase precursor [*Orientia tsutsugamushi*]	189183499	39.5/4.94	1980.027	QWNEAVLT
8	Protein Z-dependent protease inhibitor-like [*Oreochromis niloticus*]	XP_003453155	68/4.95	2145.153	WVDVDETGLSAAASTALGEPR
8	Binding-protein-dependent transporters inner membrane component *Paenibacillus elgii*	ZP_09078075	68/4.95	1522.853	PPFVMGLVNSR
8	myb-related protein A-like [*Oreochromis niloticus*]	XP_003443664	68/4.95	1872.867	DRAVLSEMMGTDMFNR

The MS/MS sequential tags were searched against a NCBInr 2011.10.10 protein database subset of the all taxonomy group using MS/PatternProteinProspector™ software with no enzyme and two maximal miss-matched amino-acid residues.

TABLE 2. Protein Identification by Mascot MS/MS Ion Search Approach Mascot Score

Spot no.	Protein name [organisms]	Accession no.	MW (kDa)/pI	MS/MS (m/z)	Sequence
9–10	Hemopexin-like [*Oreochromis niloticus*]	XP_003440941	83/5.44–5.62	1126.625	R.DGIHAFPITR.L
9–10	Warm temperature acclimation protein 65-2, partial [*Perca flavescens*]	ADX97154	83/5.44–5.62	1317.692	EYAFAGPIYMR
9–10	Warm-temperature-acclimation related-65 [*Oryzias latipes*]	gi|66267674	83/5.44–5.62	2352.161	K.EIQEDFPGVPTHLDAAVECPKG

(Probability based score is $-10 \times \text{Log}(P)$, where P is the probability that the observed match is a random event. Scores greater than recorded significant hit were significant ($P < 0.05$). The MS/MS peak lists were searched against a NCBInr 20100910 protein database subset of the Chordata taxonomy group using MASCOT™ software with the following settings: enzyme chemistry—trypsin, missed cleavages 2, carbamidomethyl modification of cysteine, variable single oxidation of methionine, peptide mass tolerance ±50 ppm, and fragment mass tolerance ±0.7 Da.

significant differences in the percent of spermatozoa showing motility throughout the spawning season were observed. Typically, at 10 sec post-activation, 95–100% of sperm cells became motile irrespective of the time of sperm collection in the spawning period. Spermatozoa motility ceased after 30 sec post-activation. A similar trend has been observed for spermatozoa velocity. Studies of several fish species have reported either a decreasing trend in spermatozoa motility or stability as the reproductive season progressed (Rideout et al., 2004; Suquet et al., 2005; Babiak et al., 2006). Therefore, it is likely that variation in spermatozoa motility is species-specific, but can also depend on rearing and environmental conditions. While high grading in spermatozoa motility parameters constitutes a major prerequisite of successful fertilization, proof that a high fertilizing capacity of spermatozoa relates to high motility indices is still needed in perch. Further, low egg fertility, as observed at early and late stages of the reproductive season, is less reliable to estimate sperm fertilizing ability. Consequently, the elucidation of sperm fertilizing ability in relation to the various phases of the reproductive season is still needed for a better understanding of the precise role of the seminal plasma proteins composition.

The mechanism regulating sperm hydration during spermiation plays a major role in determining the milt volume in fish (Mylonas et al., 1997). In the current study, the minimum milt volume was recorded at the beginning of the spawning season and gradually increased toward the end of the season. Our results are in accord with other studies that declared gradual increase of milt volume from the early to the end phase of the reproductive season (Piironen, 1985). Several studies have reported the highest milt volume at the peak of the reproductive season with a subsequent decline towards the end of the period (Shangguan and Crim, 1999; Verma et al., 2009). It has also been proposed that variation in milt volume may be related to sperm hydration (''thinning''), which is a characteristic of the final stages of sperm maturation in fish (Ciereszko, 2008).

Seasonal trends in spermatozoa concentration vary among fish species (Lubzens et al., 1997; Hulak et al., 2008a). In the present study, spermatozoa concentration peaked in the mid-spawning phase and decreased at the end of the season. The concentration of spermatozoa has been traditionally used for the assessment of sperm quality (Li et al., 2009). We suggest that the use of perch sperm from mid-spawning period may ensure optimal fertilization success in artificial reproduction. Further research is required to confirm this hypothesis.

The osmolality and composition of seminal plasma usually prevent sperm motility in fish sperm ducts (Billard, 1986). In the present study, the osmolality of seminal plasma was not found to vary throughout the spawning period. Similar results have been reported in *Barbus barbus* (Alavi et al., 2008). In contrast, several other studies reported significant variation in osmolality of seminal plasma during the reproductive period in various fish species (Kruger et al., 1984; Verma et al., 2009). Variation in seminal plasma osmolality can be a response to changes in secretory activity in the fish sperm duct during the spawning period (Li et al., 2009), but in contrast, it can also be used as a predictive indicator of sperm contamination by urine (Linhart et al., 2003; Hulak et al., 2008b).

Seminal plasma of fish mainly contains mineral compounds and low concentrations of organic substances (Li et al., 2009). Accumulating evidence shows that seminal plasma plays a pivotal role in protection against proteolytic attack on spermatozoa during storage in the reproductive system (Li et al., 2010c). In the present study, the total protein concentration of seminal plasma increased significantly from the early to the end of the reproductive period. Several authors have suggested that the increasing trend in protein concentration of seminal plasma could be a result of disturbance of the blood/gonad barrier leading to the transfer of these proteins from blood to the reproductive system (Lessard et al., 2000; Cao et al., 2003; Krol et al., 2006). Our results showed a significant effect of time in the reproductive season on the seminal plasma proteome. Investigation revealed proteins that, based on function, could be categorized into three main groups:

The first group is composed of proteins crucial to stability of the spermatozoa membrane as well as for a protective effect against oxidative stress and cytotoxic compounds. Spot 1 corresponds to a protein containing

the Pleckstrin-like protein domain (family f member 3) which belongs to the PH-like super family of proteins; its main molecular function would be metal ions binding and phospholipid binding (Chen et al., 2010); as a result this could influence the stability of spermatozoa membrane structure. Geminin-like protein and transposase, detected in spots 2–6, belong to the MATE efflux family of proteins, a large class of membrane proteins present in the cells of most organisms (Nancy, 2001). These proteins bind to a variety of potentially cytotoxic compounds and remove them from the cell, providing protection against cytotoxic effects or oxidative stress (Zhelenova et al., 2000).

The second group consists of proteins, involved in sperm motility regulation. Dual specificity protein phosphatase from spot 7 belong to family of phosphatases involved in the regulation of spermatozoa motility and protein tyrosine phosphorylation during activation of the spermatozoa of pig, horse, and dog (González-Fernández et al., 2009). It has also been shown that lytic murein transglycosylase precursor (spot 7) and carbonic anhydrase-like precursor (spot 7) are key enzymes responsible for conversion of CO_2 to HCO_3^- in a pH/HCO_3^--dependent manner mediated by carbonic anhydrase, a mechanism that plays a key role in the motility control of porcine and flatfish spermatozoa (Tajima et al., 1987; Inaba et al., 2003). Coronin (spot 1) represents a conserved family of actin cytoskeleton regulators that promote cell motility and modulate actin-dependent processes (Rauchenberger et al., 1997). Z-dependent protease inhibitor, from spot 8 (also known as a plasma serpin), is known as important participant in spermatozoa cytoskeleton assembly (Alireza et al., 2005) and spermatozoa motility acquisition (Zhao et al., 2011). The alterations in these five proteins during the reproductive season could be associated with stable spermatozoa motility during the spawning period. Their precise role in regulation of spermatozoa motility should be investigated.

The final (third) group of proteins identified in the present study contain unnamed protein products as well as several proteins, such as warm-temperature-acclimation-associated protein (Wap65) and myb-related protein, which are highly conserved across fish genera or homologous to mammalian proteins but have unknown or hypothetical functions.

This study provides new information on the quality and functional parameters of Eurasian perch sperm and the seminal plasma proteome over the course of the spawning season. The data confirmed that spermatozoa velocity, motility, and osmolality of seminal plasma were stable during phases of the perch reproduction period, while peak sperm volume and protein concentration was reached late in the season. Protein patterns in seminal plasma varied throughout the season. Approximately half of the altered proteins are involved in enzyme pathways that can regulate spermatozoa motility. The information on the physical and chemical characteristics of Eurasian perch sperm reported in this study should help to improve management and optimize the development of protocols for artificial reproduction of this species.

MATERIALS AND METHODS

Rearing Conditions and Semen Collection

The reproduction and culture of Eurasian perch was carried out at the Faculty of Fisheries and Protection of Waters, University of South Bohemia at Vodnany in the Czech Republic. Ten sexually mature males (3 years old, 183.56 ± 25 g mean body weight, 20.15 ± 1.54 cm mean total length) were used for the study. Fish were marked by fluorescent elastomeric injection and held in a hatchery tank supplied with flowing pond water at 7–10°C in April–May and 11–15°C in May–June under natural photoperiod. Prior to stripping, fish were anesthetized in carnation oil (0.03 ml L^{-1}). Sperm samples were collected in 5-ml syringes by abdominal massage. No hormone treatment was used for the induction of spermiation. Special care was taken to avoid contamination with mucus, feces or water. Sperm samples were stored on ice (0–4°C) in closed assay tubes until processing.

Sperm Samples Collection

Sperm samples from 10 experimental males were collected in the early phase of the reproductive season (April 26, 2010), in the mid-phase of the reproductive season (May 17, 2010), and late phase of the reproductive season (June 7, 2010).

The time interval between stripings was 3 weeks. The selected physiological variables of sperm: spermatozoa motility and velocity; spermatozoa concentration; sperm volume; protein concentration; and osmolality in seminal plasma together with protein composition of seminal plasma were measured.

Spermatozoa Motility Assessment

Spermatozoa velocity (μm sec^{-1}, measured only on motile spermatozoa) and the percent of motile sperm cells were determined after triggering spermatozoa motility under dark-field microscopy (Olympus BX 50, Tokyo, Japan) (20× objective magnification). For triggering, 1 μl of sperm was diluted with 49 μl of distilled water on a glass slide prepositioned on the microscope stage. To prevent spermatozoa from sticking to the microscope slide, 0.1% bovine serum albumin (Sigma–Aldrich, St. Louis, MO) was added to the swimming medium. Spermatozoa motility was recorded with a CCD video camera (SONY SSCDC50AP, Tokyo, Japan) mounted on a microscope illuminated with ExposureScope®. The successive positions of the heads of each spermatozoon were detected and followed in five successive frames using a video recorder (SONY SVHS, SVO-9500 MDP, Tokyo, Japan), and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). The trajectory of the spermatozoa head movement was recorded and traced from five overlapping successive frames. Spermatozoa motility and velocity were calculated as described by Rodina et al. (2007). Measurement of spermatozoa motility for each sample was conducted in triplicate.

Evaluation of Milt Volume and Spermatozoa Concentration

The milt volume was expressed in milliliters (ml). Spermatozoa concentration of each sperm sample was estimated microscopically using a Burker cell hemocytometer (Meopta, Prague, Czech Republic) at $\times 200$ magnification with an Olympus BX 50 phase contrast microscope (Olympus, Japan). Prior to determination of spermatozoa concentration, sperm was diluted 10,000 times with a physiological saline solution. A droplet (10 μl) of diluted sperm was placed on a hemocytometer (depth 0.1 mm) with a coverslip. The spermatozoa concentration was calculated according to methods described by Caille et al. (2006). Spermatozoa concentration was expressed as 10^9 spermatozoa ml^{-1}.

Measurement of Osmolality and Protein Concentration of Seminal Plasma

Osmolality of seminal plasma was measured using a vapor pressure osmometer (Wescor, Logan, UT) and expressed in mOsmol/kg. The bicinchoninic acid assay (BCA), using the photometer Infinite M200 (Tecan, Mannedorf, Switzerland) for reading, was applied to determine the protein concentrations in the samples.

Preparation of Protein Samples

Sperm samples were initially centrifuged at 300*g* for 30 min followed by 10 min at 16,000*g* at 4°C according to methods described by Li et al. (2010b). The supernatant was carefully collected and divided into two portions: a first one was stored on ice for measurement of osmolality and protein concentration in seminal plasma, and a second stored at −80°C for protein separation analysis.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2DE) and Data Analysis

The seminal plasma proteins were analyzed by 2DE. Isoelectric focusing (IEF) was performed on ReadyStrip IPG strips (pI range 4.7–5.9, 7 cm) with PROTEAN IEF (Bio-Rad). A total of 50 μg of protein was used for preliminary runs in a total volume of 125 μl of rehydration buffer (8 M urea, 2 M thiourea, 4% Chaps, 50 mM dithiothreitol, 0.4% IPG buffer). Electrical current conditions for the separation was set as follows: passive rehydration for 10 hr; isolelectric focusing, 250 V for 1 hr, 500 V for 1 hr, 1000 V for 1 hr, and 5,000 V for 3 hr (gradient). After isoelectric focusing, the IPG strips were equilibrated in the first step in a solution containing 6 M urea, 29.3% glycerol, 2% SDS, 75 mM Tris–HCl pH 8.8, and 2% (w/v) dithiothreitol for 15 min, and in the second step with a solution containing 2.5% (w/v) iodacetamide replacing dithiothreitol for another 15 min. Each IPG strip was laid onto a 12% SDS–polyacrylamide gel for second dimension electrophoresis. Protein spots were visualized by Coomassie Brilliant Blue R-250 (Applichem, Darmstadt, Germany) staining.

The stained gels were scanned and analyzed by Nonlinear 2D software (USA). Average gels for each phase of the reproductive season (early, middle, and late) were derived from three replicates. Protein spots were detected and matched automatically, followed by manual adjustment. The necessary criteria for determination of differential expression of proteins at the time points during the reproductive period were as follows: (i) spots present at one sampling that were not observed in another and (ii) the differences were similar in the three replicate analyses ($P < 0.05$). In addition, spots were analyzed with adjusted spot filtration settings, and spots with normalized volumes smaller than 0.05 were excluded.

In-Gel Digestion and Mass Spectrometry

Spots of interest were excised from gels and cut into small cubes (approx. 1 mm^3), and de-stained using 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile. After complete de-staining, the gel was washed with water, dehydrated in acetonitrile, and rehydrated in water. The gel was partially dried using a SpeedVac concentrator and subsequently reconstituted with cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 10% acetonitrile, and sequencing grade trypsin (50 ng μl^{-1}; Promega, Madison, WI). Digestion was carried out overnight at 37°C. The resulting peptides were extracted with 40% MeCN/0.4% acetic acid. Following extraction, the peptides were subjected directly to mass spectrometry analysis.

Mass spectra were measured on a matrix-assisted laser desorption/ionization reflectron time-of-flight MALDI-TOF/TOF mass spectrometer ultraFLEX III (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra were calibrated externally using the mono-isotopic $[M+H]+$ ion of peptide standards PepMix II (Bruker-Daltonics). A 5 mg ml^{-1} solution of α-cyano-4-hydroxy-cinnamic acid in 50% MeCN/0.3% acetic acid was used as a MALDI matrix. A 0.5-μl sample was loaded onto the target, and the droplet was allowed to dry at ambient temperature and over-laid with 0.4 μl of matrix solution. The positive MALDI-TOF spectra were collected in reflectron mode. MS/MS LIFT spectra of selected ions were obtained to identify protein or to confirm identification. MALDI-MS and MS/MS spectra were interpreted with the MASCOT program (http://www.matrixscience.com/) in conjunction with the molecular weight (MW) and isoelectric point (pI) of proteins in the gels.

For those proteins that were not identified by MALDI-MS, the de novo manual sequencing for protein identification was applied. De novo manual sequence determination was performed with sequential tag search (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mspattern).

Statistical Analysis

Prior to statistical comparisons of the tested parameters, all data were tested for normality and homogeneity of variance (Kolmogorov test and Bartlett test, respectively) and log-transformed if necessary. Values including

spermatozoa concentration, sperm volume, protein concentration, and osmolality of seminal plasma, were expressed as means ± standard deviation (n = 10) and analyzed by factorial analysis of variance (ANOVA). Percent motile spermatozoa and spermatozoa velocity were determined for each male at several time points following activation. Measurements were conducted in triplicate. Statistical comparison was made by ANOVA followed by Tukey's HSD test for each analyzed parameter. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

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CHAPTER 5

EFFECT OF SHORT-TERM STORAGE ON QUALITY PARAMETERS, DNA INTEGRITY, AND OXIDATIVE STRESS IN RUSSIAN (*ACIPENSER GUELDENSTAEDTII*) AND SIBERIAN (*ACIPENSER BAERII*) STURGEON SPERM

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Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm

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ABSTRACT

The sperm of Russian sturgeon (*A. gueldenstaedtii*) and Siberian sturgeon (*A. baerii*) was used to evaluate the effects of short-term (liquid) storage on functional parameters (spermatozoa motility and velocity), DNA integrity and oxidative stress indices. Spermatozoa showed >50% motility during 6 days of storage with an average velocity of 133.12 ± 15.4 to 87.9 ± 11.23 $\mu m\ s^{-1}$ in both species. No motile spermatozoa were recorded after nine days of storage. Analysis of Russian sturgeon sperm showed no significant differences in DNA damage expressed as percent tail DNA and Olive Tail Moment for first three days of storage. In Siberian sturgeon significant differences in DNA damage were detected after two days of storage. The level of oxidative stress indices (TBARS, CP) and antioxidant activity (SOD) increased significantly with storage time in both species. Results of this study can be utilized for successful reproduction management and cryopreservation protocols of these endangered species.

1. Introduction

Sturgeon is among the world's most valuable wildlife resources. These northern hemisphere fishes can be found in large river systems, lakes, coastal waters, and inner seas throughout Eurasia and North America (Birstein and DeSalle, 1998). Most of the world's sturgeon populations have experienced significant decline, mainly due to overfishing, habitat destruction, and pollution (Pikitch et al., 2005). At the same time, sturgeon has become a popular species for aquaculture production. The major hindrance to the culture of sturgeon juveniles is the challenge in simultaneously obtaining viable gametes of both sexes.

To exceed this limitation, various sperm storage methods are frequently used in fish farms. Short-term or liquid storage is the most common technique. Non-diluted sperm storage (Park and Chapman, 2005), sperm storage after dilution with simple ionic medium (Glogowski et al., 2002) or storage after dilution with multi-component media containing a big range of additives such as antibiotics (Brown and Mims, 1995) or antioxidants (Stoss, 1983) are applied as different methodological approaches. In general, sperm is maintained at 4 °C either with aeration or in the presence of CO_2 for several days before fertilization ability decreases (Billard et al., 2004). In aquaculture, refrigerated storage is a simple and inexpensive procedure often needed to deal with logistics of large-scale hatchery operations. Cryopreservation is a powerful tool that allows sperm to be stored indefinitely. This method has been recognized as the most appropriate way for gene banking aimed to conserve specific genetic diversity (Cabrita et al., 2010).

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Any sperm storage procedure must assure the intact preservation of sperm DNA integrity. Several studies have claimed that DNA damage can occur during cold as well as frozen storage, both in mammals and fish (Fraser et al., 2007; Li et al., 2008a), and these DNA alterations may have consequences for the development of offspring (Kopeika et al., 2004). Identification and characterization of DNA damage events could allow storage procedures to be improved by modulating protocols with the objective of diminishing DNA fragmentation.

DNA fragmentation during sperm storage has been ascribed to oxidative stress (Li et al., 2010b). This is a detrimental process affecting spermatozoa structure and function, from plasma membrane composition to the mitochondria and cytoskeleton. It is known that DNA integrity can be impaired in different ways by oxidation. Strand breaks can be generated and nitrogen bases oxidized (Box et al., 2001). Moreover, DNA damage in spermatozoa could be caused by many factors, from sperm aging (Catriona et al., 2011) to the effects of the freezing process (Li et al., 2010c) or UV irradiation (Dietrich et al., 2005). Spermatozoa are sensitive to such stresses, because they possess limited endogenous antioxidant protection while presenting abundant substrates for free radical attack in the form of unsaturated fatty acids and DNA (Koppers et al., 2010). When the production of reactive oxygen species (ROS) by the sperm mitochondria is excessive, the gamete's limited endogenous antioxidant defenses are rapidly overwhelmed and oxidative damage induces lipid peroxidation in the spermatozoa, with a resultant loss of fertilizing potential and vitality (Aitken et al., 1998). Oxidative damage has an important impact on sperm physiology and the study of its nature and effects is of great importance in the field of gamete biology. It will be useful to precisely identify whether oxidative stress occurring during sperm storage affects DNA integrity and whether other mechanisms are involved.

In our study storage of undiluted sperm was used to reveal the possible sources of sperm quality degradation during *in vitro* storage. We evaluated the effect of short-term storage on the physiology and DNA integrity of Russian and Siberian sturgeon sperm with respect to spermatozoa motility and velocity and oxidative stress.

2. Material and methods

2.1. Broodstock handling and collection of gametes

The breeding and culture of Russian sturgeon and Siberian sturgeon were carried out at Fischzucht Rhonforelle GmbH & Co.KG, Gersfeld, Germany. Six males of *A. gueldenstaedtii* (6–8 kg, 7 years old) and six males of *Acipenser baerii* (5–6 kg, 6 years old) were used. Prior to hormone stimulation, fish were kept in tanks with water temperature varying between 14 and 15 °C. Spermiation in both species was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at 5 mg/kg of body weight, 48 h before sperm collection. Semen was collected from urogenital papilla by aspiration through a plastic catheter (5–7 mm diameter) connected to a 20 mL syringe. Special care was taken to avoid contamination with mucus, feces, or water. Sperm samples were stored on ice (0–4 °C) until processing.

2.2. Experimental protocol

To investigate the effect of short-term (liquid) storage of sturgeon spermatozoa on DNA integrity, motility characteristics, and oxidative stress indices, the experimental protocol for both Russian sturgeon (*A. gueldenstaedtii*) and Siberian sturgeon (*A. baerii*) was designed to follow standard protocols that are applied for short-term storage of sturgeon spermatozoa under hatchery conditions as follows:

Sperm samples from each species ($n = 6$ per each species) were divided into 250 mL cell containers and kept under aerobic conditions at 4 °C. From each sample, aliquots were removed at 24 (1 day), 48 (2 days), 72 (3 days), 144 (6 days), and 216 (9 days) h after collection, for assessment of functional parameters of sperm, including spermatozoa motility and velocity and DNA integrity, as well as oxidative stress indices and antioxidant activity.

2.3. Sperm motility and velocity recording

Sperm activity was video recorded using dark-field microscopy (Olympus BX 50; stroboscopic lamp Strobex 9630, Chadvick-Helmut, USA) to evaluate motility and velocity. Using a CCD video camera (Sony, SSC-DC50AP), the microscopic field was transferred to a video monitor and recorded with a S-VHS system (Sony, SVO-9500 MDP). The strobe frequency was set to automatic register with video frames (50 Hz) for sperm velocity measurement. Motility and velocity were examined at 20x objective magnification immediately after mixing 1 μL sperm with 49 μL distilled water +0.1% BSA on a glass slide pre-positioned on the microscope stage. The final dilution was 1:10 000. The BSA was added to prevent sperm heads from sticking to the glass slide (Rodina et al., 2007). Within 10 s of mixing, sperm swimming activity was recorded for 2 min. The focal plane was positioned near the glass slide surface.

Successive positions of sperm heads were analyzed from video frames using Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Velocity and percent motility were calculated from sperm head positions on five successive frames with three colors (frame 1 red, frames 2–4 green, and frame 5 blue). Twenty to 40 sperm cells were counted for each frame. Sperm that moved were visible in three colors, while non-moving sperm were white. The percent of motile sperm and sperm velocity was calculated as described (Boryshpolets et al., 2009).

2.4. Oxidative stress and antioxidant indices analyses

Sperm samples were centrifuged at $5000 \times g$ at 4 °C for 10 min. The supernatant was carefully collected and discarded. The spermatozoa pellet was diluted with 50 mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5 mM EDTA, to obtain a spermatozoa concentration of 5×10^8 cells mL^{-1}, then homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic,

Berlin, Germany). The homogenate was divided into two portions: one in which thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP) was measured and a second that was centrifuged at 12 000 × *g* for 30 min at 4 °C to obtain the post mitochondrial supernatant for the antioxidant enzyme activity assay.

The TBARS method described by Lushchak et al. (2005) was used to evaluate sperm lipid peroxidation (LPO). Its concentration was calculated by absorption at 535 nm and a molar extinction coefficient of 156 mM cm^{-1}. The content of TBARS was expressed as nanomoles per 10^8 cells. Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the method described by Lenz et al. (1989). The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM cm^{-1}, expressed as nmol per 10^8 cells.

Total superoxide dismutase (SOD) activity was determined by the method of Marklund and Marklund (1974) and was assessed spectrophotometrically at 420 nm.

2.5. Assessment of DNA damage

The single-cell gel electrophoresis (Comet) assay was performed on the protocol described by Li et al. (2008a). Unless otherwise stated, molecular grade, DNase-free reagents (Sigma Aldrich, USA) were used throughout. Microscope slides (OxiSelectST; Cell Biolabs, INC. USA) were used for Comet assay, and each slide was prepared in the following manner: 50 μL of sperm (6×10^6 cells mL^{-1}) were diluted in 5 mL of PBS (phosphate buffer solution). Diluted samples (200 μL) were mixed with 700 μL of 0.8% NuSieve GTG low melting temperature agarose (OxiSelectST; Cell Biolabs, INC. USA). Finally, 50 μL of this mixture was added to the slide and allowed to solidify for 1 h.

After 1 h the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 10% dimethylsulfoxide, and 1% Triton X-100, pH 10). The slides were treated with proteinase K (20 mM Tris–HCl, 1 mM CaCl2, and 50% glycerol, pH 7.4) and incubated overnight at 32 °C (Li et al., 2008a). After the proteinase K solution was drained, the slides were immersed in a horizontal gel tank filled with alkaline buffer (300 mM sodium hydroxide, 1 mM EDTA, pH 12.3) for 20 min to allow the DNA to unwind. The buffer level was adjusted to approximately 0.25 cm above the slides, and electrophoresis was carried out for 20 min at 35 V and 170 mA. The slides were drained well, dehydrated by dipping into absolute ethanol for 5 min, and air-dried for storage. For comet visualization 50 μL of Vista Green DNA Staining Solution (OxiSelectST; Cell Biloabs, INC. USA) was loaded onto the slides that were subsequently covered with a coverslip and analyzed with an Olympus BX50 fluorescence microscope at 20× magnifications. A total of 100 cells were scored for each sample, and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the tail) and tail DNA content (% tail DNA) were measured. Olive Tail Moment (tail length × % tail DNA content) was calculated using the formula below:

$$M_{\text{Tail Olive}} = (|CG_{\text{Tail}} - CG_{\text{Head}}|) \times \%DNA_{\text{Tail}},$$

where $M_{\text{Tail Olive}}$ is the Olive Tail Moment, CG_{Tail} the center of gravity of the tail, CG_{Head} the center of gravity of the head, and % DNA_{Tail} is the percent of migrated DNA in the tail compared to the head.

2.6. Statistical analysis

Normality and the homogeneity of variance of all data were first tested by the Kolmogorov test and the Bartlett test, respectively. Percent motile spermatozoa and spermatozoa velocity were determined for each male ($n = 6$ per each species) at several time points following activation. Measurements were conducted in triplicate. Statistical comparison for percent motile spermatozoa and spermatozoa velocity values was made by analysis of variance (factorial ANOVA) followed by Tukey's HSD test. Data obtained from the Comet assay were log-transformed if necessary. Values include TBARS, CP, SOD level, and percent of DNA damage were expressed as means ± SD. Differences in these values among times of storage for the same species were analyzed by one-way ANOVA, followed by Tukey's HSD test. Differences between species at the same time of storage were analyzed using *t*-test. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

3. Results

3.1. Spermatozoa motility and velocity

After sperm activation a majority of sperm samples from males of both species showed 100% motility of spermatozoa within 10 s. During the first three days of sperm storage the percentage of spermatozoa motility displayed the following values: (i) at 10–30 s post-activation, 95–100% of spermatozoa were motile in both analyzed species (Fig. 1A and B) and no significant differences ($P > 0.05$) between fresh and stored samples were observed; (ii) at 40 s post-activation, significant differences ($P < 0.05$) between fresh and stored samples were observed in sperm samples from Russian sturgeon but not in samples from Siberian sturgeon (Fig. 1A and 1B); (iii) during the progress of the motility period, the percentage of actively swimming cells in both analyzed species significantly ($P < 0.05$) decreased with time and it reached 60% at 90 s after activation in both analyzed species (Fig. 1A and B). The period of motility was maintained for 5 min in both species.

A significant decline in percent of motile spermatozoa ($P < 0.05$) was observed after six days of storage for all analyzed samples of both species and percentage of motile spermatozoa demonstrated the following characteristics: (a) at 10 s post-activation 80% of spermatozoa were motile in Russian sturgeon and 78% in Siberian sturgeon, respectively; (b) during the progress of the motility period the percentage of motile spermatozoa decline to 28% at 90 s after activation in Russian sturgeon and to 26% in Siberian

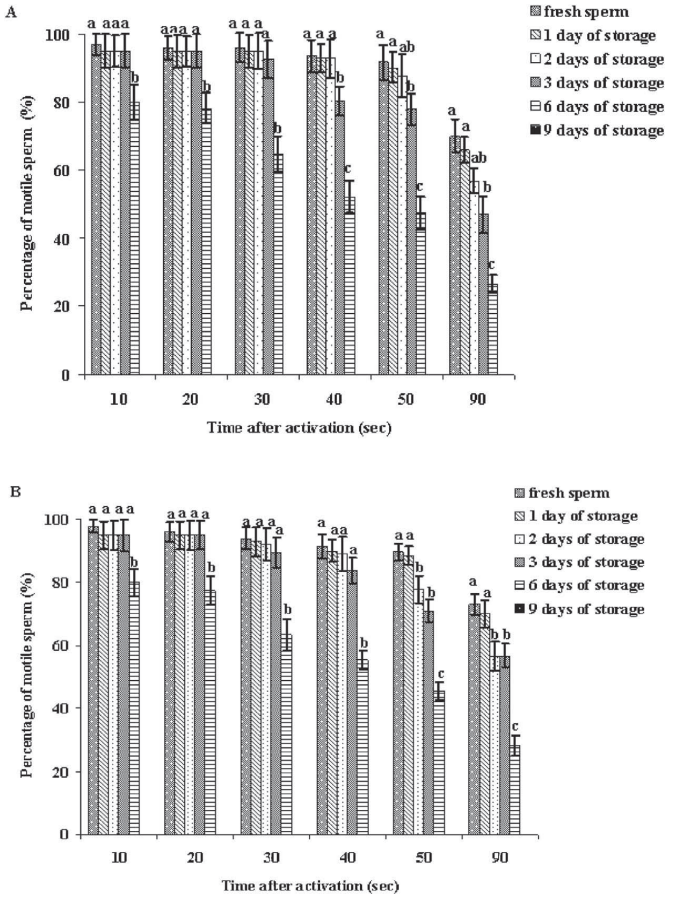

Fig. 1. Effect of short-term storage on *A. gueldenstaedtii* (A) and *Acipenser baerii* (B) spermatozoa motility. Data are presented as means ± SD, $n = 6$ per each species and time of storage. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

sturgeon. In addition, a period of motility lasting at 2 min in both analyzed species. After nine days of storage, no motile spermatozoa were observed in either species.

Estimation of spermatozoa curvilinear velocity (VCL) showed a normal distribution and analysis of variance (ANOVA) showed that the significant differences ($P < 0.05$) in spermatozoa velocity between fresh and stored samples were apparent after two days of sperm storage and decline significantly ($P < 0.05$) with time of storage in both species. After the two days of storage, the spermatozoa velocity demonstrated the following characteristics: (a) at 10 s post-activation it reached 173 ± 16 μm/s in *A. gueldenstaedtii* and 165 ± 14 μm/s in *A. baerii* (Fig. 2A and B); (b) after 90 s post activation, the velocity decreased to 105 ± 14 μm/s in Russian sturgeon and 100 ± 13 μm/s in Siberian sturgeon. After six day of storage, the velocity decreased to 133 μm/s at 10 s post activation and to 90 μm/s at 90 s post activation for all analyzed samples of both species.

3.2. Oxidative stress and antioxidant responses

The level of TBARS increased significantly in spermatozoa of Russian sturgeon after six days of sperm storage and after three days of storage in spermatozoa of Siberian sturgeon. No significant differences in level of TBARS between species were observed (Fig. 3A). In addition, the level of CP increased significantly in spermatozoa of Russian sturgeon after two days of storage and in Siberian sturgeon after three days of storage. Moreover, level of CP in spermatozoa was significantly higher in Russian sturgeon comparing to Siberian sturgeon during six days of storage (Fig. 3B). In Russian sturgeon sperm, the measured levels of TBARS and CP varied from 0.33 ± 0.06 to 0.75 ± 0.15 and from 40.1 ± 4.25 to 74 ± 7.3 nmol/10^8 sperm cells, respectively. The antioxidant activity expressed by total SOD activity significantly increased within storage time and no statistical differences in activity of SOD between

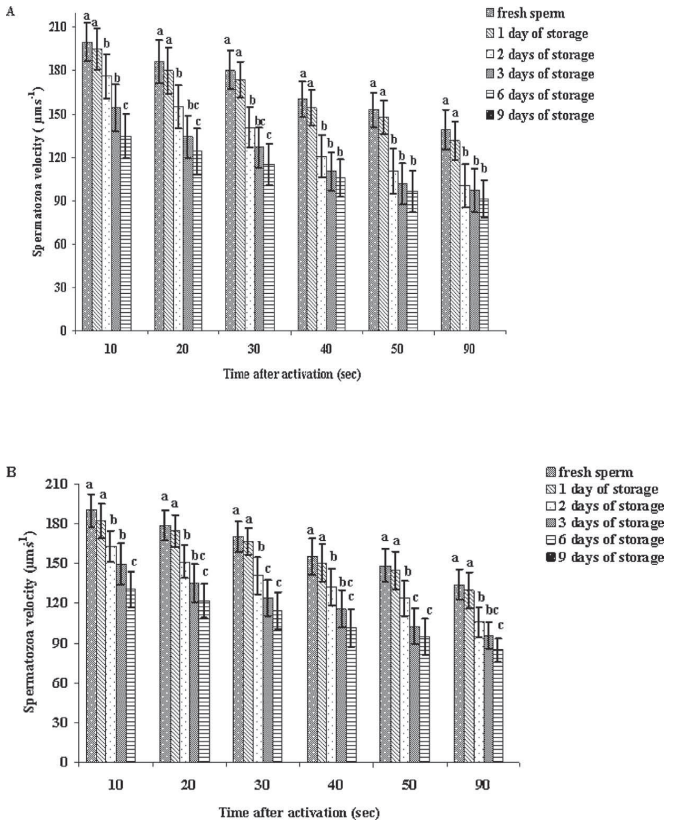

Fig. 2. Effect of short-term storage on *A. gueldenstaedtii* (A) and *Acipenser baerii* (B) spermatozoa velocity. Data are presented as means ± SD, $n = 6$ per each species and time of storage. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Russian and Siberian sturgeon spermatozoa were detected (Fig. 3C).

3.3. Effect of storage on DNA fragmentation

DNA evaluation by Comet assay reported a basal percentage of tail DNA of 5% ± 1.5 in *A. gueldenstaedtii* sperm and 8.5% ± 2.5 in *A. baerii* sperm (Fig. 4A). A significantly higher percentage of tail DNA was found in samples exposed to short-term storage than in fresh ones (22% ± 4.5 in *A. gueldenstaedtii* sperm and 15% ± 5.3 in *A. baerii* sperm). In Russian sturgeon, no significant differences in DNA damage expressed as % tail DNA and Olive Tail Moment during the first 3 days of storage were observed. Subsequently the level of DNA damage in spermatozoa of Russian sturgeon significantly increased. In Siberian sturgeon, the level of DNA damage expressed as % tail DNA and Olive Tail Moment significantly increased after tree days of storage. Significant between-species differences in % tail DNA and Olive Tail Moment were detected after 2 and 3 days, respectively (Fig. 4A and B).

4. Discussion

As a first step toward understanding how short-term storage might alter sperm physiology, DNA integrity, and oxidative stress indices of Russian and Siberian sturgeon sperm, spermatozoa motility and velocity parameters were evaluated.

Our results showed that the spermatozoa of both species were fully capable of being activated immediately after transfer to swimming medium, and 95–100% of spermatozoa became motile. During the first 3 days of storage the percent of spermatozoa motility remained stable in both analyzed species. More specifically, no significant differences between fresh and stored samples during the first 30 s post activation were observed. Moreover, after this period, spermatozoa motility declined. In addition a significant decline in spermatozoa velocity started after two days of storage in both analyzed species. Similar results have been reported in sterlet (*A. ruthenus*) by Dettlaff et al. (1993). Dettlaff et al. (1993) reported that the fertilizing capacity of stored sperm was 5 days during refrigerated storage. In comparison with our study

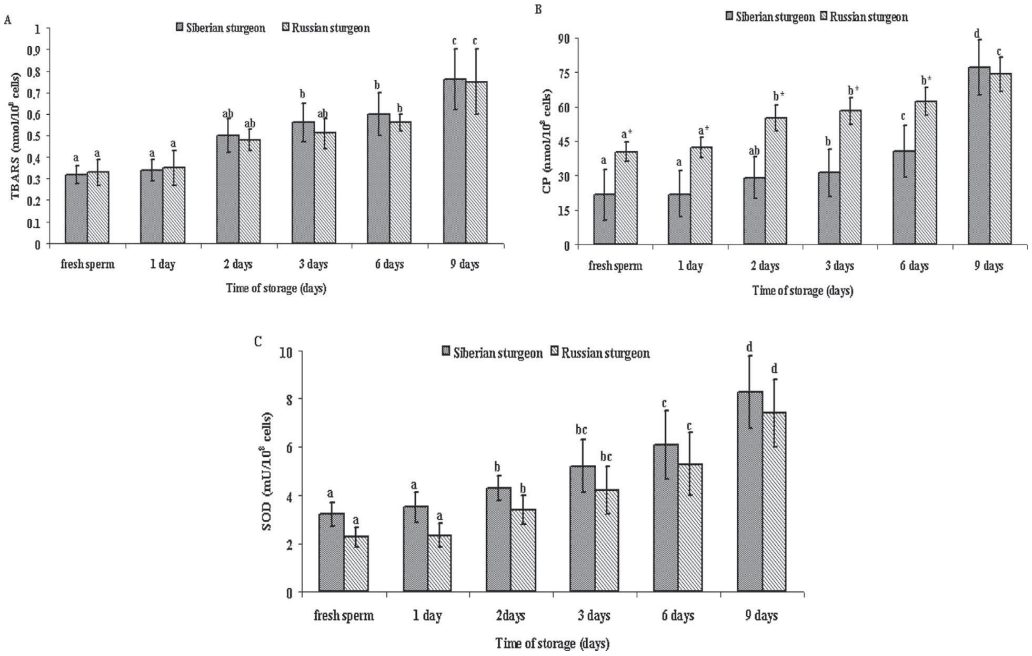

Fig. 3. Effect of short-term storage on oxidative stress indices expressed by TBARS (A); CP (B) and SOD (C) in *A. gueldenstaedtii* and *Acipenser baerii* sperm. Data are presented as means ± SD, $n = 6$ per each species and time of storage. Different letters indicate significant differences among times of storage for the same species (ANOVA, $P < 0.05$); * indicate significant differences between species at the same time of storage (*t*-test, $P < 0.05$).

the sperm of both species were able to become motile during 6 days of storage under aerobic conditions at 4 °C (refrigerated storage). In contrast, DiLauro et al. (1994) reported that in Atlantic sturgeon (*A. oxyrinchus*) 40% of spermatozoa were motile after 17 days storage in plastic bags with daily replenishment of oxygen. Linhart et al. (1995) observed 97% of spermatozoa motility in paddlefish (*Polyodon spathula*) after 16 h storage at 24 °C in a simple saline solution. After nine days of storage, no motility was detected in either of the species in the present study. Oxidative stress has been presumed as the main reason of decline in these functional characteristics (Aitken and Baker, 2006). Disequilibrium between ROS and the spermatozoa antioxidant system can cause metabolic or functional disorders, reducing sperm motility and increased lipid peroxidation (LPO) and carbonylation of proteins (Li et al., 2009).

The lipid composition of the spermatozoa membrane makes it susceptible to oxidative damage, because of their relatively high polyunsaturated fatty acid (PUFA) content (Trenzado et al., 2006). Lipid peroxidation can trigger the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, resistance to osmotic shock, and fertilization potential (Shiva et al., 2011). In addition, protein oxidization could have deleterious effects on sperm function, with plasma membrane structural proteins being affected as well as proteins with enzymatic activity (Domínguez-Rebolledo et al., 2010). Lipid peroxidation is particularly important for aquatic animals, since they normally contain greater amounts of highly unsaturated fatty acids (HUFA) than do other species. Lipid peroxidation has been reported to be a major contributor to the loss of cell function under oxidative stress (Storey, 1996) and has usually been indicated by TBARS in fish (Oakes and Van der Kraak, 2003). Our results showed that the level of TBARS increased significantly after six days of sperm storage in Russian sturgeon and after three days in Siberian sturgeon. In contrast to our results, Li et al. (2010b) found an increasing level of LPO (expressed as TBARS levels) associated with the thawing process after cryopreservation of common carp (*Cyprinus carpio*) spermatozoa, indicating that the ROS generation was primarily related to the freeze/thaw process rather than the effect of equilibration. The CP is a result of protein oxidation. The formation of CP is nonreversible, causing conformational changes, decreased catalytic activity in enzymes, and ultimately resulting, owing to increased susceptibility to protease action, in breakdown of proteins by proteases (Zhang et al., 2008). In the present study we observed that the level of CP increased significantly in spermatozoa of Russian sturgeon after two days of storage and in Siberian sturgeon after three days of storage. Subsequently, the level of CP significantly increased with storage time. Based on our results, we hypothesize that in Russian sturgeon and Siberian sturgeon the cause of the loss of spermatozoa motility and velocity during short-term storage was oxidative stress that significantly interrupted

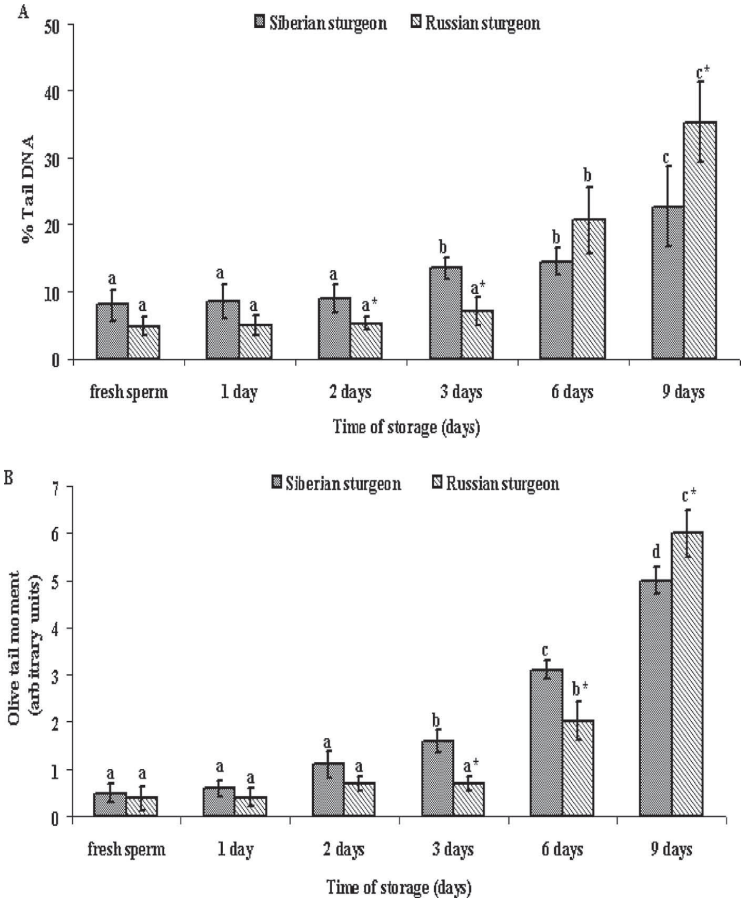

Fig. 4. Percent of tail DNA (A) and Olive Tail Moment (B) in *A. gueldenstaedtii* and *Acipenser baerii* sperm, during short-term storage ($n = 6$ per each species). Columns represent mean values from 600 cells, 100 per male. Data are presented as means ± SD. Different letters indicate significant differences among times of storage for the same species (ANOVA, $P < 0.05$); * indicate significant differences between species at the same time of storage (t-test, $P < 0.05$).

cellular metabolism (*e.g.* oxidative phosphorylation) of spermatozoa, leading to subsequent decline of motility parameters. Additionally, in our study we observed significant differences in the level of CP between species, but future investigation is required for the elucidation of the reasons.

Fish sperm possess an antioxidant system consist of enzymatic (glutathione peroxidase and reductase, catalase, superoxide dismutase) and non-enzymatic (α-tocopherol, ascorbic acid, β-carotene, selenium, zinc) components that are capable to counteract the damaging effects of ROS and protect the cell structure (Li et al., 2009). However, a limited amount of information is available about the precise mechanism of action of antioxidant systems in fish sperm (Lahnsteiner et al., 2010). In the present study the antioxidant activity expressed by total SOD activity was significantly increased after two days storage in sperm of both species. However, Li et al. (2010b) showed that SOD activity in carp spermatozoa was neither activated nor inhibited by the freeze/thaw process, indicating that the antioxidant defense system in spermatozoa is species specific. Based on our results we argue that the level of antioxidant enzymatic activity is insufficient to prevent cellular damage and DNA fragmentation caused by oxidative stress resulting from short storage during more than three days in Russian sturgeon and two days in Siberian sturgeon, respectively. Several studies, have demonstrated that it is possible to reduce damaging effects of ROS by the addition of various antioxidant compounds to the freezing media prior to cryopreservation of fish sperm (Lahnsteiner et al., 2011). However, the effect of each antioxidant is species-specific, improving different parameters of sperm quality depending on the type of antioxidant and concentration used (Cabrita et al., 2011). Therefore we argue

that as a future step in investigation of various effects of short term or cold storage on sturgeon spermatozoa should be focused on the application of antioxidants that will be capable of preventing deleterious effects of storage on qualitative sperm parameters during the short term storage or cryopreservation.

In the present study, the Comet assay method was used to evaluate the effect of short-term sperm storage on DNA integrity. Our results showed that during short-term storage no significant differences in DNA damage expressed as percent tail DNA and Olive Moment during the first three days of storage of Russian sturgeon sperm were observed. However results recorded after six days of storage showed a significant increase in level of DNA damage. In sperm of Siberian sturgeon the significant increases in DNA damage were observed after three days of storage. Pérez-Cerezales et al. (2009) reported that classical cold storage procedures (~2 h at 4 °C with aeration) significantly increased DNA damage in rainbow trout (*Oncorhynchus mykiss*) sperm. Labbe et al. (2001), using the Comet assay, demonstrated that the DNA stability in rainbow trout sperm was slightly affected by cryopreservation. Cabrita et al. (2005), using the same method, found a significant increase in fragmented DNA in cryopreserved rainbow trout sperm, while in gilthead sea bream (*Sparus aurata*) varying effects on DNA integrity were related to differing dilutions following cryopreservation. The differences observed in the abovementioned studies could be also related to the chromatin structure of the analyzed species or the obtaining of the sperm at different times during the reproductive season. Differences between species detected in our study in the level of DNA damage during short-term storage could not be explained directly from our results because a little information in this field is available. Additional experiments are required to investigate possible existence of species-specific cellular mechanisms responsible for stability of spermatozoa chromatin during *in vitro* storage.

In conclusion, the results of the present study provide new data on Russian and Siberian sturgeon sperm quality with respect to short-term storage and indicate that the decline in sperm quality can provide novel information about integrity of the DNA molecule is likely caused by oxidative stress and accumulation of LPO and CP in sperm cells resulting from cold storage of sperm. The data presented here suggest that application of antioxidants during liquid storage or cryopreservation of fish spermatozoa could prevent cellular injuries caused by oxidative stress.

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CHAPTER 6

SPERMATOZOA MOTILITY, CRYORESISTANCE, AND FERTILIZING ABILITY IN STERLET *ACIPENSER RUTHENUS* DURING SEQUENTIAL STRIPPING

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Spermatozoa motility, cryoresistance, and fertilizing ability in sterlet *Acipenser ruthenus* during sequential stripping

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ABSTRACT

We describe spermatozoa characteristics from sequential collections in sterlet, *Acipenser ruthenus*, following a single dose of carp pituitary extract (CPE). Sperm production and spermatozoa fertilizing ability, percent motility, and curvilinear velocity (VCL) were investigated in fresh and frozen/thawed sperm. Sperm was collected by two procedures: (A) stripping 3 times per 24 h at 3 h intervals on 3 consecutive days beginning 12 h after CPE treatment, and (B) stripping 3 times over 6 h beginning 36 h after CPE treatment. Spermatozoa motility and VCL were evaluated by video microscopy, and sperm production was measured as volume and concentration. Sperm samples were frozen by a conventional freezing procedure in a cryoprotective medium containing 10% methanol. Fertilization was conducted using a ratio of 10^5 spermatozoa/egg. Both sequential stripping procedures yielded larger volumes of viable spermatozoa than did a single collection. Sperm parameters such as density and volume varied widely depending on collection time. The highest numbers of spermatozoa per individual were collected 15–42 h post-CPE treatment in (A) and at 42 h post-CPE treatment in (B) ($85 \pm 4\%$ and $64 \pm 5\%$ of total spermatozoa count, respectively). Median percent motility in spermatozoa before cryopreservation was 26–100% and 5–67% post-thaw. Fertilization rates obtained with frozen/thawed spermatozoa were 13–76% (median value). A significant increase in spermatozoa motility parameters and fertilizing ability at the second collection on each day was observed. Sequential stripping and spermatozoa cryopreservation in combination could improve the efficiency of sturgeon aquaculture.

1. Introduction

Sturgeon farming is considered a promising area of aquaculture with a growing world market for black caviar and sturgeon meat. Modern intensive sturgeon farming in Europe was predicted by Williot et al. (2001). Now worldwide production of cultured sturgeon has increased from 2500 metric tons in 1999 to 25,600 metric tons in 2008 (FAO, 2008), and aquaculture caviar production increased from 1.69 metric tons in 2003 to 27.32 metric tons in 2007 (Wuertz et al., 2009). Twenty-five of 27 sturgeon species are listed as extinct, critically endangered, endangered, or vulnerable (Billard and Lecointre, 2001). Thus sturgeon farming involves rare natural species and has potential for considerable development.

Although sturgeon farming has a history of more than one hundred years, the basis for intensive artificial reproduction and methods of *in vitro* gamete manipulation were developed only in the second half of the 20th century (Dettlaff et al., 1993). The importance of sperm storage to efficient husbandry was emphasized by Billard (2001). Several methods of hypothermic storage and cryopreservation have been developed for sturgeon sperm (Billard et al., 2004), allowing the use of sperm some time after collection. The effectiveness of male exploitation depends on collecting the maximum amount of spermatozoa with high fertilization capability that can be used immediately for direct egg fertilization or be cryopreserved for delayed fertilization. In sturgeon the possibility of repeat sperm collections after a single hormone treatment (sequential collections) has been extensively studied (Podushka, 2003). Sperm volume obtained through multiple collections at intervals of 2–3 h has been reported to be approximately fivefold that of a single collection, but no information about its quality or characteristics has been reported. Time intervals of 12 h (Alavi et al., 2006) and 24 h (Linhart et al., 2000) between collections have been applied in other studies, with precise measurement of sperm production indices and spermatozoa motility parameters. A cryoresistance assessment of spermatozoa obtained from 14 sequential strippings at 6 h intervals showed low motility in thawed spermatozoa, including in those exhibiting high motility prior to freezing (Kopeika and Kopeika,

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2008). However, no data on the fertilizing ability of spermatozoa obtained through sequential stripping have been published.

Testes of sturgeon possess no externally opening sperm ducts. Spermatozoa pass through urinary collecting ducts (Wrobel and Jouma, 2004), where they are believed to mix with urine. Research is needed to clarify the influence of this mixing process on spermatozoa motility, fertilizing ability, and cryoresistance.

The aim of the present study was to describe spermatozoa characteristics of sturgeon sperm samples obtained through multiple collections. Two procedures, differing in time post-hormone treatment of the initial stripping, were used. This time span was considered a possible determinant of spermatozoa quality influenced by the duration of residence in the urinary duct.

The sterlet, *Acipenser ruthenus*, was selected as a model species because of ease of handling due to its small size. The spermatozoa cryopreservation procedure was based on original studies (Glogowski et al., 2002; Urba´nyi et al., 2000) that reported high post-thaw fertilizing ability of sturgeon spermatozoa.

2. Materials and methods

2.1. Fish and rearing conditions

Sterlet broodstock (3–5 years old, weight 0.67–1.18 kg) were kept during the natural spawning season in outdoor 4 m^3 plastic tanks with a constant flow rate of 20 l min^{-1} pond water and temperature of 8–12 °C, located at the hatchery of the Research Institute of Fish Culture and Hydrobiology. Prior to hormone treatment, fish were moved to a closed water recirculation system with water temperature elevated to 15 °C within 24 h and held 3–4 days without feeding.

2.2. Procedures for sperm and urine collection

Ten male sterlet were injected intramuscularly with carp pituitary extract (CPE) at 4 mg kg^{-1} before being stripped. Females were injected with the same extract at 36 (0.5 mg kg^{-1}) and 24 h (4.5 mg kg^{-1}) before stripping (Dettlaff et al., 1993).

Sperm was collected at the urogenital papilla by aspiration using a plastic catheter (4 mm diameter) connected to a 10 ml syringe. Two procedures were used:

(A) sperm was collected from 5 of the males (first group of fish) 3 times per day at 3 h intervals on 3 consecutive days beginning 12 h after CPE treatment. As males were injected at 21.00 h, daily collection took place at 09.00, 12.00, and 15.00 h (12, 15, 18 h post-CPE treatment on day 1; 36, 39, 42 h post-CPE treatment on day 2; and 60, 63, 66 h post-CPE treatment on day 3).
(B) sperm was collected from the second group of 5 males (second group of fish) 3 times on a single day beginning 36 h after CPE treatment (36, 39, 42 h).

Sperm from (B) collected at 36 h was considered the control group, as this is the time routinely used for stripping in commercial practice (Gela et al., 2008). During each collection, the available sperm was thoroughly removed from the sperm–urinary tract.

Urine was collected by catheterization of the uro-genital papilla 5 days after hormone injection, when no spermatozoa were found by microscopic observation of the undiluted sample.

2.3. Evaluation of sperm velocity and percent motility

Sperm was diluted at a ratio of 1:50 with water from tanks in which the fish were kept, and spermatozoa motility was immediately measured until it ceased, using a CCD video camera (Sony, SSCDC50AP) mounted on a dark-field microscope (Olympus BX50, 200) and illuminated with a stroboscopic lamp (Chadwick- Helmut, 9630, USA) set to a flash frequency of 50 Hz. Video recordings were obtained (Sony SVHS, SVO-9500 MDP) at 25 frames s^{-1} and analyzed to estimate spermatozoa curvilinear velocity (VCL, μm s^{-1}) and percent motile using Olympus micro-image software (Olympus Micro Image 4.0.1. for Windows, Hamburg Germany). To compute VCL and percent motile spermatozoa at 10 s post-activation, head tracks were generated from five successive video frames, and VCL was calculated as length of spermatozoa head track divided by the time elapsed between the first and fifth video frame. Ten to 50 spermatozoa were evaluated in each frame. Spermatozoa with velocity lower than 3 μm s^{-1} were considered immotile and excluded from further analysis (Rodina et al., 2008).

Spermatozoa velocity and percent motility were also determined in urine as described above, with urine replacing water. In these trials, spermatozoa samples obtained from three males were assayed for motility in each of the three urine samples.

2.4. Evaluation of spermatozoa production indices, seminal fluid, and urine osmolality

Spermatozoa concentration of each sperm sample was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on an Olympus BX 50 phase contrast microscope (Olympus, Japan).

Spermatozoa number (SN) was computed as spermatozoa concentration multiplied by the volume of each sperm sample. Collected SN per kg body weight was calculated as SN divided by body weight.

Total spermatozoa production (TSP) was computed as the sum of SN for each male. Percent spermatozoa collected per stripping (PS) was calculated by:

$$PS = SN * TSP^{-1} * 100.$$

Seminal fluids (SF) were obtained after centrifugation at 10 000 *g* for 10 min.

Osmolalities of SF and urine were evaluated using a Vapor Pressure Osmometer 5520 (Wescor, USA) and expressed in mOsm kg^{-1}.

2.5. Cryopreservation of sperm

Sperm from each stripping of each male was individually frozen. Before freezing the samples were diluted 1:1 in an extender consisting of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0 (Glogowski et al., 2002) containing 10% methanol. The diluted sperm was placed into 0.5 ml straws (CRYO-VET, France), transferred to a styrofoam box containing liquid nitrogen, placed 3 cm above liquid nitrogen level on a styrofoam raft for 20 min, and then plunged into liquid nitrogen. Thawing was conducted in a water bath at 40 °C for 6 s. Thawed sperm was used immediately for motility or fertilization assays of spermatozoa.

2.6. Fertilization assay

Eggs from three females pooled in equal parts were used. To 2 g eggs (approximately 160 eggs), 8 ml of tank water was added along with sperm. Based on spermatozoa concentration in the sample, the volume of sperm was adjusted to obtain a 10^5 spermatozoa/egg ratio. After 2 min mixing, the fertilized eggs were incubated at 16 °C. As spermatozoa for experiments were collected over an extended period, it was not possible to control the fertilizing ability of fresh spermatozoa using the same batch of eggs; thus we recorded fertilizing ability of frozen–thawed sperm only. The use of eggs from three females in our experimental design had the potential to reduce variations in fertilization rates related to egg quality. The quality of eggs was evaluated by insemination with fresh spermatozoa from other males applying the same spermatozoa/egg ratio. Live embryos were counted at the eyed stage after 4 days incubation (stage 4), and dead eggs were removed. Yolk

sac larvae were counted at day 8 of incubation. Fertilization and hatching rates were expressed as the percent of live embryos and yolk sac larvae at days 4 and 8 of incubation, respectively.

2.7. Data presentation and statistical analysis

Five males were used for each stripping procedure. Measurements of SF osmolality, VCL, percent motility, fertilization, and hatching rates were conducted in triplicate for each male, while spermatozoa concentration and sperm volume were estimated from a single measurement.

The values of parameters were checked for distribution characteristics and homogeneity of dispersion using the Shapiro–Wilk's and Levene's tests, respectively. Spermatozoa VCL before and after cryopreservation and SN showed normal distribution with similar dispersion values. For these values the mean values with a 95% CI were presented in figures and mean ± standard deviation are described in text. To compare spermatozoa velocity before and after cryopreservation, parametric ANOVA was applied, and Tukey's honest significant difference (HSD) test was used to identify differences among subgroups. When values (motility, sperm volume, spermatozoa concentration, and sperm production indices) were not normally distributed, they are presented as median and interquartile range in figures and as min–max ranges when described in the text. Nonparametric statistics using the Kruskal–Wallis test followed by the Mann–Whitney U-test were conducted for comparison among groups. Correlations among spermatozoa parameters were evaluated using Spearman rank analysis.

Statistical significance was considered at $P<0.05$. All analyses and plotting were conducted using Statistica V9.1 computer program (Statsoft Inc, USA).

3. Results

3.1. Sperm production indices with sequential stripping

Parameters such as spermatozoa concentration and volume of sperm sample varied widely among collections. Spermatozoa concentration ranged from 0.1 to 48.4 10^8 spz*ml^{-1} and volume of sperm sample from 1.8 to 24.0 ml. These variations precluded statistical analysis using parametric methods (Levene's test, $P<0.05$, Shapiro–Wilk's test $P<0.05$). With nonparametric Kruskal–Wallis ANOVA, significant differences were found in volume of sperm sample, spermatozoa concentration, percent spermatozoa collected per stripping, and seminal fluid osmolality.

Volume of sperm samples collected on the first day (12, 15, and 18 h post CPE treatment) for (A) was significantly higher than the volume of sperm samples collected on the second day (36, 39, and 42 h post CPE treatment). Volume of sperm samples collected on the third day (66 h post CPE treatment) was significantly lower than the volume of sperm samples collected on the second day (Fig. 1A, $P<0.05$, Mann–Whitney U-test, $n=5$). In (B), a significant decrease in the volume of sperm samples collected was observed at the third stripping (Fig.1B, $P<0.05$, Mann–Whitney U-test, $n=5$). Significant increases in spermatozoa concentration were found after the first stripping with both procedures (Fig. 2A and B). A similar increase over day 1 values was seen on the second day of stripping (36 and 39 h post-CPE treatment, Fig. 2A), while no significant changes compared to day 2 were observed on day 3 (60, 63, and 66 h post-CPE treatment, Fig. 2A).

Analysis of spermatozoa production showed a significant increase in PS at the second stripping for both procedures (Fig. 3). In (A), PS was not significantly altered with stripping at 15–42 h post-CPE treatment; however a decrease was observed 60–66 h post-CPE treatment (Fig. 3A). In (B), PS was significantly greater at 39 h post-CPE treatment compared with 36 and 42 h (Fig. 3B).

Collected spermatozoa number (SN) for (A) and (B) were $3.73*10^{11}$ and $1.49*10^{11}$, respectively, and were 12.4 and 5.0 times higher than for spermatozoa collected 36 h post-CPE treatment in (B) ($0.30*10^{11}$ spermatozoa), the control group. The highest SN was found 15–42 h post-CPE treatment in (A) and 42 h post-CPE treatment in (B) (85 ± 4% and 64 ± 5% of total spermatozoa production, respectively).

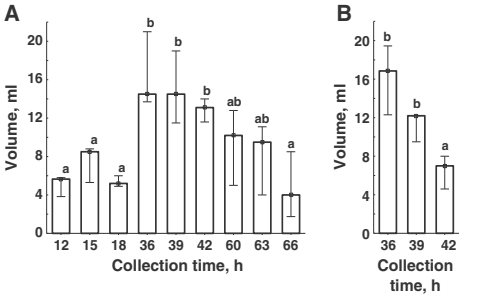

Fig. 1. Volume of sperm samples collected during sequential stripping. A: Procedure (A) sperm was collected 3 times per day at 3 h intervals on 3 consecutive days starting 12 h post-CPE treatment (12, 15, 18, 36, 39, 42, 60, 63, and 66 h). B: Procedure (B) sperm was collected 3 times in 1 day starting 36 h post-CPE treatment (36, 39, 42 h). Median value with 25% and 75% percentiles is presented, values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

3.2. Spermatozoa motility

Spermatozoa motility was observed in both tank water and urine, while no motility was observed in seminal fluid. Significant decreases in percent motile spermatozoa were observed after freeze-thawing for all sperm samples (Fig. 4A–B). Motility in spermatozoa samples before cryopreservation ranged from 26 to 100%, while post-thaw motility was from 5 to 67%. The lowest percent motility was observed at 12 and 60 h post-CPE treatment in both fresh and frozen–thawed samples. Frozen–thawed samples collected at 12 h post-CPE treatment shown significantly lower motility ($P<0.05$, Mann–Whitney U-test, $n=5$) from all other samples except those collected at 60 h post-CPE treatment.

Estimation of spermatozoa curvilinear velocity (VCL) showed normal distribution, and parametrical methods of ANOVA were used for comparison of mean values obtained for the groups. In sperm samples before freezing, VCL was significantly increased from 12 h post-CPE treatment (99 ± 19 μm s^{-1}) to 18 h post-CPE treatment (178 ± 37 μm s^{-1}), while, in samples collected after 36 h post-CPE treatment, significant reduction in VCL was observed only at 60 h post-CPE treatment (163 ± 29 μm s^{-1}) in (A) (Fig. 5A). There was no significant difference in VCL in (B). Freezing and thawing led to significant decrease of VCL in

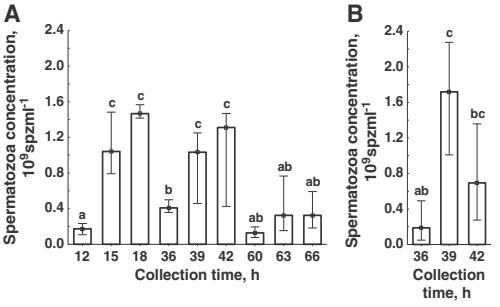

Fig. 2. Spermatozoa concentration in samples collected during sequential stripping. A: Procedure (A). B: Procedure (B). Median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

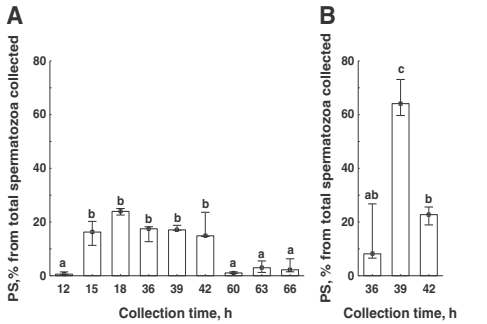

Fig. 3. Percent of spermatozoa collected per stripping calculated for samples obtained during sequential stripping. A: Procedure (A). B: Procedure (B). Median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

spermatozoa samples collected by (A) at 12, 18 and 60 h post-CPE treatment. The same effect was observed in (B) (Fig. 5B).

Spermatozoa motility was observed in sperm collected 36 h post-CPE treatment diluted in urine. Percent motile spermatozoa ranged from 80 to 100% whether diluted with pond water or urine. VCL of spermatozoa activated by urine was 183 ± 31 μm s^{-1} and did not differ significantly from those activated by water (190 ± 22 μm s^{-1}).

3.3. Seminal fluids and urine osmolality

Gradual increase of seminal fluid osmolality was observed on the first day of stripping with subsequent decrease on days 2 and 3 in (A) (Fig. 6A, $P<0.05$, Mann–Whitney U-test, $n=5$). No significant change in seminal fluid osmolality was observed in sperm from (B) (Fig. 6B).

Osmolality of urine was 25 ± 7 mOsm kg^{-1} and was significantly lower than osmolality of seminal fluid obtained at any time of stripping (Mann–Whitney U-test, $n=5$).

3.4. Fertilization assays with sperm samples after freeze-thawing

After insemination by fresh spermatozoa, fertilization rate ranged from 90 to100% ($n=3$) showing a high viability of eggs used in the experiments. Fertilization and hatching rates using frozen–thawed spermatozoa are shown in Figs. 7 and 8, respectively. The parameters showed similar trends. While, in all cases, hatching rates were lower than corresponding values of fertilization rates, no significant differences

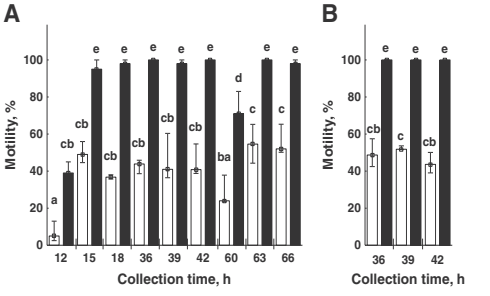

Fig. 4. Spermatozoa motility percent in samples collected during sequential stripping. A: Procedure (A). B: Procedure (B). Median values with 25% and 75% percentiles are presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test). □ — post-thaw motility, ■ — motility before freezing.

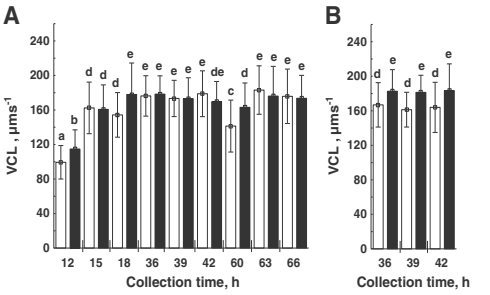

Fig. 5. Curvilinear velocity before and after freeze-thawing in sperm samples collected during sequential stripping. A: Procedure (A). B: Procedure (B). Mean with S.D. are presented; values with different letters are significantly different ($P<0.05$, Tukey's test). □ — post-thaw velocity, ■ — velocity before freezing.

were found between median values of these two parameters in sperm samples collected at the same time post-hormone treatment. Significant increases in both parameters were observed in the samples from the second collection on day 1 and day 3 in (A) (Figs. 7A and 8A) and from the second collection in (B) (Figs. 7B and 8B) compared with the first daily collection.

Results of analysis of the correlation among all spermatozoa parameters studied are presented in Table 1 with only significant ($P<0.05$) values shown. Fertilization and hatching rates correlated significantly with spermatozoa concentration, volume, count per stripping, post-thaw motility, and post-thaw VCL, although correlation coefficients were low (0.292–0.538). Spermatozoa count per stripping correlated with sperm volume, seminal fluid osmolality, and spermatozoa concentration with higher correlation coefficients (0.617–0.874). Spermatozoa concentration correlated with sperm osmolality with coefficient 0.722.

4. Discussion

It is clearly established that appropriate hormone treatment stimulates spermiation in sturgeon for a relatively long period (Alavi et al., 2006; Kopeika and Kopeika, 2008; Linhart et al., 2000; Podushka, 2003). In the present study, we demonstrated that, by using sequential stripping, it is possible to collect an increased amount of spermatozoa possessing both high fertilization ability and cryo-resistance.

As our experimental fish were kept in appropriate spawning conditions, hormone treatment with pituitary extract probably resulted in accelerated sperm production by release of fully developed spermatozoa from spermatocysts into the sperm ducts (spermiation) followed by

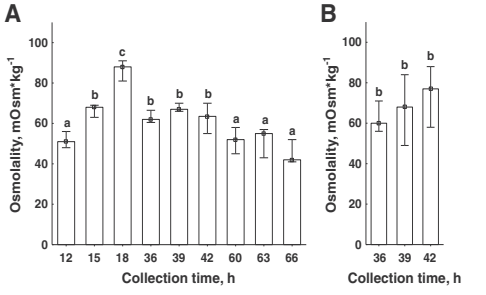

Fig. 6. Seminal fluid osmolality in sperm samples collected during sequential stripping. A: Procedure (A). B: Procedure (B). Median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

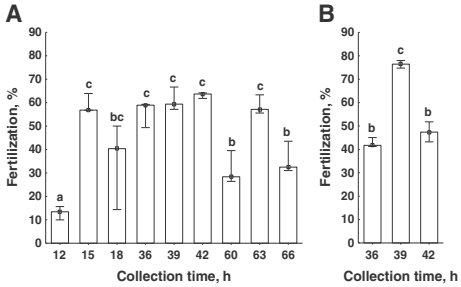

Fig. 7. Fertilization rate of eggs, fertilized by frozen–thawed sperm samples collected during sequential stripping. A: Sperm samples collected in procedure (A), B: sperm samples collected in procedure (B) (median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test)).

production of seminal fluid (Mylonas et al., 2010; Schulz et al., 2010). As the precise sequence of sperm formation events has not been described in sturgeon, we assume that the wide variation in spermatozoa concentration during sequential stripping could be related to temporal asynchrony of spermatozoa release from spermatocysts relative to seminal fluid production. This mode of milt production could be the source of variation in the amount of spermatozoa obtained per stripping observed in our study. Similar results have been shown in paddlefish (Linhart et al., 2000).

In the present study more detailed information about milt production was obtained from measurements of spermatozoa volume and spermatozoa concentration. Increase in milt volume appeared 36 h after hormone treatment, while increased spermatozoa concentration was observed as early as 15 h post-CPE treatment, and a significant increase in seminal fluid osmolality was observed 15 and 18 h post-CPE treatment. Thus, we suggest that spermiation, as a response to hormone treatment, could be initiated before the increase of seminal fluid production associated with the phenomenon known as milt hydration. While it is certain that milt hydration is a hormone regulated process, it is not clear how the involved hormones exert their effects (Schulz and Miura, 2002). Increased spermatozoa concentration in sperm stripped 15 and 39 h after CPE treatment in (A) and 39 h post-CPE treatment in (B) (second strippings per day) suggests that sperm collection itself could stimulate the process of spermiation. While this suggestion is in accordance with results described previously

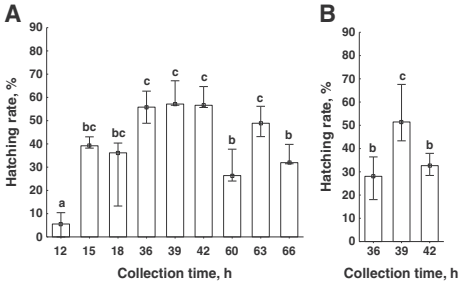

Fig. 8. Hatching rate of embryos obtained with frozen–thawed sperm samples collected during sequential stripping. A: Sperm samples collected in procedure (A), B: sperm samples collected in procedure (B). Median value with 25% and 75% percentiles is presented; values with different letters are significantly different ($P<0.05$, Mann–Whitney U-test).

(Podushka, 2003), more detailed study is required to understand the physiological basis of this phenomenon.

The specific structure of the sturgeon sperm-urinary excretory system, with a urogenital junction created by testes seminal ducts and opisthonephros (Hoar, 1969; Wrobel and Jouma, 2004), makes unambiguous determination of the source of milt volume increase almost impossible. Most likely, collected sturgeon milt consisted of a mixture of seminal fluid and urine. As we found no description of urine composition during spermiation in sturgeon, some related information should be mentioned. Both urine and seminal fluid of various sturgeon species are hypotonic compared to blood plasma (Krayushkina and Semenova, 2006; Potts and Rudy, 1972), and osmolality of urine measured in our study was significantly lower than osmolality of seminal fluid. Also, both fluids contain K^+ and Ca^{2+} ions, which have been shown to reciprocally control spermatozoa motility in sturgeon (Gallis et al., 1991; Linhart et al., 2002). Urine possesses similar concentrations of K^+ and Ca^{2+} ions (Krayushkina and Semenova, 2006) while, in seminal fluid, the concentration of K^+ ions ranges from two (Linhart et al., 2003) to 30 times (Toth et al., 1997) that of Ca^{2+}. Motility of sturgeon spermatozoa can be initiated in a wide range of environmental osmolalities [0–120 mOsm l^{-1}], but osmolalities of 25 and 50 mOsm kg^{-1} were found to produce only 60 and 10% motility, respectively, in sterlet (Lahnsteiner et al., 2004). The most likely reason is that both low osmolality and low K^+/Ca^{2+} ratio in urine determine its ability to activate spermatozoa motility, as observed in our study, as well as by Park and Chapman (2005). To investigate whether urine dilution of sperm occurs and has any effect on spermatozoa parameters, urine-specific compounds should be quantified, since variations in SF osmolality may not necessarily be due to urine contamination. To detect the underlying processes, additional studies are necessary.

As spermatozoa motility in fish can be activated several times by a gradual osmolality decrease (Boryshpolets et al., 2009; Dzyuba et al., 2011), it is likely that the first activation step may occur immediately upon spermatozoa release from testes into the urogenital tract. We suggest that low osmolality of seminal fluid observed in our study and previously described (Alavi et al., 2006; Linhart et al., 2003) is related to dilution of sperm with urine, and changes in osmolality of seminal fluid, in turn, could result from the extent of this dilution. High regression coefficients between spermatozoa concentration, spermatozoa number per stripping, and osmolality support this supposition, while more investigations are needed to confirm it.

While the process of sperm production in sturgeon is unclear, our study demonstrated that multiple collections provide the possibility of increasing the amount of spermatozoa with high motility parameters, cryoresistance, and fertilizing ability. Although sperm samples obtained at different stages of multiple collection differed in the parameters studied, only samples collected in the first stripping at 12 h after hormone treatment were of significantly lower quality compared to the control with respect to motility, VCL, and fertilizing ability. These results were similar to previously described observations of low percent motility in spermatozoa of Siberian sturgeon collected 12 h post-hormone treatment (Kopeika and Kopeika, 2008). In contrast to our observation, no decreased motility was observed in the early stages of hormonal stimulation of spermiation in paddlefish (Linhart et al., 2000). The increase in percent motile spermatozoa following post-CPE treatment is not fully understood, but spermatozoa maturation as a process of "development from non-functional gametes to mature spermatozoa fully capable of vigorous motility and fertilization" (Schulz and Miura, 2002) could be involved. Spermatozoa maturation as a sequence of events is described in a few teleosts (rainbow trout, chum salmon, and Japanese eel) but not in sturgeon. From the studies of these species, it is obvious that spermatozoa maturation is under the control of the endocrine system. This process includes the increase of seminal plasma pH in the sperm duct, which results in elevation of intra-spermatozoa cAMP levels (Schulz et al., 2010). We have not found in literature any description of physiological processes in sturgeon underlying spermatozoa maturation.

Table 1
Regression analysis of indexes of sperm samples collected in both procedures of stripping*.

	Sperm osmolality	Spermatozoa amount per stripping	Post-thaw motility	Post-thaw VCL	Fertilization rate	Hatching rate	Spermatozoa concentration	Volume of sperm
Sperm osmolality		0.652			0.292		0.722	
Spermatozoa amount per stripping	0.652		0.388	0.324	0.538	0.452	0.874	0.617
Post-thaw motility		0.388		0.385	0.347	0.292	0.312	0.333
Post-thaw VCL		0.324	0.385			0.330		0.318
Fertilization rate	0.292	0.538	0.347			0.838	0.497	0.356
Hatching rate		0.452	0.292	0.330	0.838		0.391	0.415
Motility percentage before freezing		0.278		0.362	0.391	0.366		0.412

* Spearman rank order coefficients, for which $P<0.05$, and $n=60$, are presented.

Our results describing changes of spermatozoa motility parameters during sequential stripping could be considered a promising model for future, more detailed, studies of the spermatozoa maturation process in sturgeon. Significant positive correlations between spermatozoa count per stripping and post-thaw motility, post-thaw VCL, fertilization, and hatching rates suggest that mature spermatozoa are produced during the increase in sperm production associated with sequential stripping.

Spermatozoa motility and VCL values of fresh and frozen–thawed samples, osmolality of seminal fluid, and rates of fertilization and hatching for embryos obtained with frozen–thawed sperm collected at different times of sequential stripping were in the same range as previously described in sturgeon (Alavi et al., 2006; Billard et al., 2004; Dzyuba et al., 2010; Lahnsteiner et al., 2004; Piros et al., 2002). However, in our study we found significant changes in spermatozoa fertilizing ability associated with sequential stripping. Increased fertilizing ability of spermatozoa collected at the second stripping of day 1 of both procedures and at the second stripping on day 3 of (A) was observed. The low correlation coefficients among spermatozoa motility, VCL, and fertilization and hatching rates indicate that spermatozoa motility parameters are not unambiguous determinants of fertilizing ability. This was shown in (B), when spermatozoa collected 39 h after hormone treatment demonstrated motility and VCL similar to that collected 36 h and 42 h after hormone treatment, but resulted in a significantly higher fertilization rate. Spermatozoa of sturgeon differ from those of freshwater teleost fish by the presence of an acrosome, and correct timing of the acrosome reaction is considered to be a crucial for fertilization success (Ginzburg, 1972; Psenicka, et al., 2008). Observed differences in fertilizing ability of spermatozoa may be associated with the acrosome maturation stage, and more investigation is needed to determine the functional state of the acrosome in spermatozoa collected at different stages of sequential stripping procedures.

5. Conclusion

Long-term sperm production after a single hormonal treatment in sturgeons is the basis of effective sequential stripping of males. Sequential stripping could be an appropriate model for future study of the spermatozoa maturation processes in sturgeon. While sperm samples collected at different stages of sequential stripping varied in motility parameters and fertilizing ability, these variations are not limiting for the most effective exploitation of the male fish. The initial stripping may stimulate production of spermatozoa possessing the highest motility and fertilizing ability. For artificial sturgeon breeding the identification of periods of the most intensive spermatozoa production is important. When sperm sampling begins within a short (12 h) period after hormonal stimulation, sperm from the first collection consists of spermatozoa of inadequate quality, but this collection stimulates a quick production of high quality spermatozoa.

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CHAPTER 7

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Sperm quality could be defined as the capacity of the sperm to successfully fertilize eggs which will further develop into a normal embryo. Nevertheless, for both aquaculture and research practical reasons, alternative and accurate methodologies are needed to evaluate fish sperm quality. Specific methodologies for sperm analysis must be accessible to allow the researcher to study the effects of *in vivo* sperm storage (in the reproductive duct) or *in vitro* storage (cryopreservation, short-term storage) and to understand putative causes for the decrease in sperm fertilizing ability in species showing potential reproductive dysfunction.

EFFECT OF *IN VIVO* STORAGE ON SPERM QUALITY PARAMETERS

In the present study a multiple sequential sperm stripping and sperm collection during the reproductive period were used as methodological approaches to study *in vivo* sperm storage.

Sperm Production

Sperm production indexes, including sperm volume, spermatozoa concentration and osmolality of seminal plasma are highly variable among fish species (Lubzens et al., 1997). Moreover these parameters depend on time of sperm storage in the reproductive tract (Koldraset al., 1996). Our current studies demonstrated that duration of *in vivo* storage significantly altered sperm volume. More specifically, the volume of sperm from multiple sperm collection was highly variable in all experimental groups; however it appears that, in case of sterlet, most specimens were able to produce sperm throughout the experimental period investigated, even for intervals lasting 5 h (Shaliutina et al., 2012b). Regarding the reproductive season, the minimum milt volume was recorded at the beginning of the spawning period and gradually increased toward the end of the season (Shaliutina et al., 2012a). We observed also that variation in milt volume may be related to sperm hydration ("thinning"), which is a characteristic of the final stages of sperm maturation in fish (Ciereszko, 2008).

As for sperm volume, spermatozoa concentration was also changing during periods corresponding to the above-mentioned methodological approaches. Our results show that during multiple collections of sperm, the highest spermatozoa concentration was observed after the second stripping as compared to the first and third stripping. A similar result was seen in a study on Siberian sturgeon (Piros et al., 2002). However, the exact reasons for such differences in spermatozoa concentration are not clear. Nevertheless, we suggest that the decrease in sperm concentration resulting from multiple collections could be the result of increased production of seminal plasma, which would dilute sperm and consequently lead to lower values of the measured concentration. Other possible explanations may be related to the sensitivity to hormone treatment used as well as sensitivity to environmental conditions and biological characteristics of the broodfish, as was previously noted in studies on paddlefish (Linhart et al., 2000). Seasonal trends in spermatozoa concentration vary among fish species (Hulak et al., 2008a). Our results showed that spermatozoa concentration peaked in the mid-spawning phase and decreased at the end of the season.

The osmolality of seminal plasma usually prevents sperm motility in fish sperm ducts (Billard, 1986). In the present study, the osmolality of seminal plasma was not found to vary neither throughout the spawning period nor at subsequent collection. In contrast, several other studies reported significant variation in osmolality of seminal plasma in various fish species as a consequence of multiple stripping and reproductive periods (Kruger et al., 1984; Verma et al., 2009). This suggests that the variation in seminal plasma osmolality can be a response to changes in secretory activity in the fish sperm duct during the spermiation period (Li et al., 2009), while these features can also be used as a predictive indicator of sperm contamination by urine (Linhart et al., 2003; Hulak et al., 2008b).

Sperm motility and velocity

The quality of sperm usually refers to percentage of motile spermatozoa and velocity of swimming ones, both being recognized as major factors determining sperm fertilizing ability (Cosson et al., 1991). The time period of sperm storage in the reproductive duct is identified as being initial factor that may be responsible for variation in sperm motility parameters.

In our current study (Shaliutina et al., 2012b), we found that sterlet spermatozoa can be activated immediately after transfer to a proper swimming medium irrespective of stripping frequency, in which conditions, 90–100% spermatozoa became motile, a result that agrees with previous observations (Cosson et al., 2000; Linhart et al., 2002). Moreover, the maximum percentage of motile spermatozoa and highest velocity were observed after the third stripping. In contrast to our results a negative effect of stripping frequency has been reported regarding the duration and intensity of sperm movement in some teleosts species (Buyukhatipoglu and Holtz, 1984). However, our data confirmed that multiple collections of sperm in sterlet did not negatively affect functional parameters of spermatozoa.

Concerning reproductive period, no significant differences in the velocity and percentage of motile spermatozoa throughout the spawning season were observed. On the other hand, studies dealing with several fish species have reported either a decreasing trend in spermatozoa motility or stability as the reproductive season progressed (Suquet et al., 2005; Babiak et al., 2006). Therefore, this whole set of data leads to hypothesize that variation in spermatozoa motility parameters is mostly species-specific, but also depends on the duration of sperm storage in the reproductive tract.

EFFECT OF *IN VIVO* STORAGE ON PROTEINS COMPOSITION OF SEMINAL PLASMA

During the past decade, the results of several studies have suggested that the proteins composition of seminal plasma is crucial to the protection of spermatozoa during storage in the reproductive system as well as to sperm viability (Lahnsteiner et al., 2004). The data of our current study (Shaliutina et al., 2013a) showed that sterlet seminal plasma protein profiles were affected by sequential sperm stripping. Moreover, 20 protein spots were detected in the two-dimensional electrophoresis gels at the third stripping, while 17 were observed at the first and second collections. We hypothesized that the extra proteins above-observed after the third collection could

be involved in some step of an intracellular mechanism, which would be responsible for regulating spermatozoa motility. However, identification of these protein spots did not reveal any significant similarity with any of the protein sequences available from public databases. Hence, these results highlighted the need for complete sturgeon's genome sequencing in order to obtain correct understanding of the precise roles of seminal plasma proteins.

As for the sequential sperm stripping, time in the reproductive season also changed the protein profiles of seminal plasma (Shaliutina et al., 2012a). Ten altered protein spots in perch seminal plasma over the course of reproductive period were identified. Moreover, based on the putative function of these identified proteins, they were categorized into three main groups. The first group is composed of proteins crucial for the stability of the spermatozoa membrane as well as for a protective effect against oxidative stress and cytotoxic compounds. Among them, the Pleckstrin-like protein domain (family f member 3) was detected, which belongs to the PH-like super family of proteins. Its main molecular function would be of metal ions binding and phospholipid binding (Chen et al., 2010). We hypothesized that the observed Pleckstrin-like protein domain-containing family f member 3 could influence the stability of spermatozoa membrane structure during the spermiation period. In addition, geminin-like protein and transposase were revealed. Both proteins belong to the MATE efflux family of proteins, a large class of membrane proteins present in the cells of most organisms (Nancy, 2001). These proteins bind to a variety of potentially cytotoxic compounds and remove them from the cell, providing protection against cytotoxic effects or oxidative stress (Zhelenova et al., 2000).

The second group consists of proteins involved in sperm motility regulation. Dual specificity protein phosphatase belong to the large family of phosphatases involved in the regulation of spermatozoa motility and protein tyrosine phosphorylation during activation of the spermatozoa of pig, horse, dog (Gonzalez-Fernandez et al., 2009). It has also been shown that lytic murein transglycosylase precursor and carbonic anhydrase-like precursor are key enzymes responsible for conversion of CO_2 to HCO^-_3 in a pH/HCO^-_3-dependent manner mediated by carbonic anhydrase, a mechanism that plays a key role in the motility control of porcine (Tajima et al., 1987) and flatfish spermatozoa (Inaba et al., 2003). Coronin represents a conserved family of actin cytoskeleton regulators that promote cell motility and modulate actin-dependent processes (Rauchenberger et al., 1997). Z-dependent protease inhibitor, also known as a plasma serpin, is as important participant in spermatozoa cytoskeleton assembly (Alireza et al., 2005) and spermatozoa motility acquisition (Zhao et al., 2011). Therefore, in our study the alterations in these five proteins during the reproductive season could be associated with stable spermatozoa motility during the spawning period. However, their precise role in the regulation of spermatozoa motility should be more deeply investigated.

The third group of proteins identified in the present study contains both unknown proteins and several already described proteins, such as warm-temperature-acclimation-associated protein (Wap65) and myb-related protein, which are highly conserved across fish genera or homologous to mammalian proteins but have only hypothetical functions.

Taken together, the results of the present study confirmed that *in vivo* storage of sperm considerably changed the protein profiles of seminal plasma. Furthermore, some of the proteins that we identified are probably involved in enzymatic pathways that control spermatozoa movement.

INFLUENCE OF DIFFERENT *IN VITRO* STORAGE METHODS ON SPERM QUALITY

Short-term *in vitro* storage

In this work (Shaliutina et al., 2013b), alterations in sperm physiology, DNA integrity, and oxidative stress indices of Russian and Siberian sturgeon sperm during short-term *in vitro* storage were studied. The results show that during the first 3 days of storage the percentage of motile spermatozoa remained stable in both analyzed species. Moreover, after this 3 days period, sperm motility declines. In addition a significant decrease in spermatozoa velocity appeared already after two days of storage in both analyzed species. Similar results have been published in case of sterlet (*A. ruthenus*) by Dettlaff et al. (1993), who reported that the fertilizing capacity of stored sperm was lasting about 5 days during short-term storage. A comparison with our study shows that the sperm samples of both species were able to activate their motility for 6 days period of storage. However, after nine days, no motility was detected in either of the species. We hypothesized that oxidative stress is presumably the main reason of decline in these functional characteristics as already proposed by Aitken et al. (2006). Disequilibrium between ROS and the spermatozoa antioxidant system can cause metabolic or functional disorders, reducing sperm motility and increased lipid peroxidation (LPO) and carbonylation of proteins (Li et al., 2010).

In mammals the effect of reactive oxygen species (ROS) on spermatozoa has been well characterizied: it may cause lipid peroxidation of spermatozoa membrane; damage in the mid-piece, axonemal structure and DNA as well as malfunctions of capacitation and acrosomal reaction; loss of motility and fertility (Gagnon et al., 1991). Similar to mammalian spermatozoa, fish spermatozoa contain high level of polyunsaturated fatty acid (PUFA), which is particularly susceptible to oxidative damage (Trenzado et al., 2006). Nevertheless, the exact role of ROS induction in fish spermatozoa remains unclear. Our results have shown that the level of thiobarbituric acid reactive substances (TBARS) in spermatozoa increased significantly after six days of sperm storage in Russian sturgeon and after three days in Siberian sturgeon. Moreover, the level of carbonyl derivatives of proteins (CP) increased in spermatozoa of Russian sturgeon after two days of storage and in Siberian sturgeon after three days of storage. Subsequently, the level of CP significantly increased with storage time.

The antioxidant system in mammalian sperm has been well studied (Alvarez and Storey, 1989). However, a limited amount of information is available about the precise mechanism of action of antioxidant systems in fish sperm (Lahnsteiner et al., 2010). In the present study the antioxidant activity which was expressed as the total SOD activity, significantly increased after two days storage in sperm of both species. In contrast to our study, Li et al. (2010) showed that in carp spermatozoa, SOD activity was neither activated nor inhibited by the freeze/thaw process, which indicates that the antioxidant defense system in spermatozoa is species specific. Therefore, based on our results, we argue that the level of antioxidant enzymatic activity is insufficient to prevent cellular damage and DNA fragmentation caused by oxidative stress resulting from short-term storage during more than three days in sturgeon species.

DNA damage can occur during cold as well as frozen storage, both in mammals and fish spermatozoa (Fraser et al., 2007), and these DNA alterations may have

consequences for the development of offspring (Kopeika et al., 2004). The present study demonstrated that during short-term storage no differences in DNA damage during the first three days of storage in Russian sturgeon sperm were observed. However results recorded after six days showed an increase in level of DNA damage. In sperm of Siberian sturgeon, the increases in DNA damage were observed after three days of storage. These results are in agreement with previous studies in which classical cold storage procedures significantly increased DNA damage in rainbow trout sperm (Pérez-Cerezales et al., 2009). The differences observed in the above-mentioned studies could be also related to the chromatin structure of the analyzed species or the obtaining of the sperm at different time periods during the reproductive season. Hence, this study indicates that the decline in sperm quality can be associated with integrity of the DNA molecules, which is likely caused by oxidative stress and accumulation of LPO and CP in sperm cells resulting from short-term storage of sperm.

Application of cryopreservation as long-term method of *in vitro* storage

It is clearly established that appropriate hormone treatment stimulates spermiation in sturgeon for a relatively long period (Kopeika and Kopeika, 2008; Linhart et al., 2000; Podushka, 2003). This long period of spermiation allows collecting sperm subjected to different time of *in vivo* storage by sequential stripping. Our study (Dzyuba et al., 2012) elucidates that, sperm samples, collected by such sequential stripping followed by long term *in vitro* storage (cryopreservation), possess both high fertilization ability and cryo-resistance. However, in our study we found significant changes in spermatozoa fertilizing ability associated with different *in vivo* storage time. Increased fertilizing ability of spermatozoa collected at the second stripping was observed, allowing to speculate, that decrease in sperm *in vivo* storage is favorable for sperm cryoresistance. We conclude that even though sperm samples collected at different stages of sequential stripping varied in motility parameters and fertilizing ability, these variations are not a limiting factor for the most effective exploitation of the sterlet males in fisheries practice. In addition the initial stripping would stimulate production of spermatozoa possessing the highest motility and fertilizing ability.

CONCLUSIONS

In this thesis, which includes five publications, the descriptions of sperm motility parameters and biochemical characteristics of teleostean and chondrostean fish species during *in vivo* and *in vitro* storage have been addressed. The main findings of these papers has contributed significantly to a better understanding of the reproductive biology of the teleostean and chondrostean, which should be beneficial to the development of aquaculture of these species.

The following conclusions were obtained from the work presented in this thesis:

1. Sperm production characteristics such as milt volume and spermatozoa concentration were highly variable during *in vivo* sperm storage in the models of teleostean and chondrostean species that we have used. Moreover, all motility

parameters remain stable within the different time periods of sperm storage in the reproductive tract.

2. Protein patterns in seminal plasma vary during *in vivo* sperm storage in the models of chondrostean and teleostean fish species we used. Approximately half of the altered proteins are probably involved in enzyme pathways that can regulate spermatozoa motility.
3. During the period of storage at 4 °C, the declines in motility parameters of sturgeon sperm as well as in DNA integrity were probably due to corresponding observed increases in the oxidative stress and accumulation of LPO and CP in sperm cells resulting from short-term *in vitro* storage.
4. Sturgeon sperm samples subjected to different time of *in vivo* storage possess both high fertilization ability and cryo-resistance. These findings are the base for the most effective exploitation of sturgeon males in fisheries practice.

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ENGLISH SUMMARY

Fish sperm motility parameters and total proteins profiles in seminal plasma during *in vivo* and *in vitro* storage

Anna Shaliutina

The effect of gamete storage on sperm quality has received considerable attention in recent years. Previous studies have shown that spermatozoa stored *in vivo* or *in vitro* for a long time can lost their motility and fertilization capacity. Moreover, it have been concluded that male fertilization potential is highly dependent, not only on spermatozoa motility parameters, but also on organic components including protein composition of seminal plasma. On the other hand, spermatozoa motility and protein profiles of seminal plasma are highly dependent on conditions of storage. Therefore, additional data about effects of *in vivo* and *in vitro* storage on quality sperm parameters and protein composition of seminal plasma are essential for development of fish artificial reproduction methods.

In the current work the influence of *in vitro* and *in vivo* storage on parameters of sperm motility, DNA integrity, antioxidant defensive system and seminal plasma protein composition were studied.

Two methodological approaches for study of *in vivo* sperm storage were applied.

As the first approach (chapter 2–3), the multiple sequential sperm stripping after single hormonal stimulation in sterlet (*Acipenser ruthenus*) was chosen. The objectives of these experiments were to investigate the effect of multiple sperm collections on selected sperm quantitative and qualitative parameters. Results showed a significant increase in spermatozoa motility and velocity when increasing number of sperm stripping. The maximum percentage of motile spermatozoa and highest velocity were observed at the third collection. No significant effect of multiple collections on protein concentration and osmolality of seminal plasma were found. Data obtained from our proteomic studies showed that seminal plasma protein profiles were affected by such sequential stripping. Moreover, 20 protein spots were detected from the two-dimensional electrophoresis gels at the third stripping, compared to 17 found at the first and second collections. However, protein identification revealed no significant distinction for any protein spot and protein sequences available in public databases. These results highlighted the need for complete sturgeons genome sequences required for correct understanding of the precise roles of seminal plasma proteins.

As the second approach for *in vivo* storage studies (chapter 4), sperm stripping during the reproductive period of perch (*Perca fluviatilis)* was applied. Results demonstrated that spermatozoa velocity, percent motility, and osmolality of seminal plasma remained stable throughout the reproductive season. Milt volume and protein concentration of seminal plasma gradually increased and reached the highest values late in the reproductive period. Spermatozoa concentration peaked in the mid-reproductive season and decreased towards the end. A proteomic analysis of seminal plasma revealed 10 protein spots significantly altered over the course of the reproductive season. Subsequent protein characterization suggested that time in the reproductive season predominantly affected proteins involved in membrane trafficking and organization, cell motility, and oxido-reductase activity.

To investigate the consequences of *in vitro* storage several methods of sperm preservation were used in the present study.

Short-term (hypothermic, 4 °C) storage was applied as the first method (chapter 5) to evaluate the effect of *in vitro* storage on sperm motility parameters, DNA damage and oxidative stress indices in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon spermatozoa. Consequently, spermatozoa showed more than 50% motility over 6 days of storage. No motile spermatozoa were recorded after nine days of storage. Analysis of Russian sturgeon sperm showed no significant differences in DNA damage for the first three days of storage, whereas, in Siberian sturgeon significant differences in DNA damage were detected after two days. The level of the oxidative stress indicators, namely thiobarbituric acid reactive substances, carbonyl derivatives of proteins and total superoxide dismutase activity increased significantly with time of *in vitro* storage in both species, which demonstrates occurrence of oxidative stress during *in vitro* sperm storage. We suggest that application of antioxidants during *in vitro* storage of sturgeon spermatozoa would prevent cellular injuries caused by oxidative stress. The application of cryopreservation technology to sperm was the second method of *in vitro* storage studied in the present work (chapter 6). The cryoresistance assessment of sterlet (*Acipenser ruthenus*) spermatozoa obtained from sequential strippings showed that spermatozoa motility, velocity and fertilization rates were decreased when the freeze/thawing procedure is applied. However sequential stripping combined with spermatozoa cryopreservation would be a way to improve the efficiency of sturgeon male exploitation aquaculture.

As a conclusion, the results of this study provide new data on sperm quality and quantity parameters of chondrostean and teleostean fish species with respect to *in vivo* and *in vitro* storage capacities, which should be beneficial for the development of aquaculture of these species. The data confirmed that protein patterns in seminal plasma varied during *in vivo* storage, depending on time of sperm collection. Furthermore, the altered proteins are probably involved in enzymatic pathways that regulate spermatozoa movement. In practice, the results presented in this thesis should help to improve management and optimize the development of protocols for artificial reproduction.

CZECH SUMMARY

Parametry motility spermií ryb a celkové proteinové profily semenné plazmy během *in vivo* a *in vitro* uchování

Anna Shaliutina

Vlivu uchování gamet na kvalitu spermatu byla věnována značná pozornost v průběhu několika předchozích let. Předchozí studie ukázaly, že spermie uchovávané po delší dobu *in vivo* či *in vitro* mohou ztrácet pohyblivost a fertilizační schopnost. Mimoto bylo dokázáno, že fertilizační potenciál samců je vysoce závislý nejen na parametrech pohyblivosti spermií, ale také na složení proteinů semenné plazmy. Pro vývoj a zdokonalení metod umělé reprodukce ryb je nezbytné získat další poznatky o vlivu *in vivo* a *in vitro* uchovávání na parametry kvality spermatu a na proteinové složení semenné plazmy.

Tato práce byla zaměřena na studium vlivu *in vivo* a *in vitro* uchování na pohyblivost spermií, integritu DNA, oxidativní stres a v neposlední řadě také na složení semenné plazmy.

Pro studium vlivů uchování spermatu *in vivo* bylo použito dvou metodologických postupů. V prvním případě bylo zvoleno mnohonásobné sekvenční odebírání spermatu od jesetera malého (*Acipeser ruthenus*) po jediné hormonální stimulaci. Cílem tohoto postupu bylo ověřit vliv mnohonásobného odebírání spermatu na vybrané kvantitativní a kvalitativní parametry. Bylo zjištěno, že se zvyšující se frekvencí odběru spermatu došlo i k signifikantnímu nárůstu procenta pohyblivosti a rychlosti pohybu spermií studovaného druhu. Naproti tomu nebyl zaznamenán průkazný vliv mnohonásobných odběrů na koncentraci a osmolatilu semenné plazmy jesetera malého. Následnými proteomickými rozbory byl prokázán vliv mnohonásobných odběrů na proteinové složení semenné plazmy. Mimoto, bylo nalezeno 20 proteinových spotů pomocí dvoudimensionální gelové elektroforézy u vzorku ze třetího odběru spermatu, zatímco u prvního a druhého odběru bylo nalezeno pouze 17 proteinových spotů. Nicméně, nebyly nalezeny žádné rozdíly v sekvencích získaných z těchto proteinových spotů. Tyto zjištěné skutečnosti ještě více poukazují na nutnost sekvenování genomu jeseterovitých ryb pro lepší porozumění role proteinů semenné plazmy.

Vícenásobné odebírání spermatu okouna říčního (*Perca fluviatilis*) v průběhu celé reprodukční sezóny bylo aplikováno jako druhý přístup pro studium vlivu uchování spermatu *in vivo* na jeho kvalitu. Zjištěné výsledky ukázaly, že rychlost pohybu spermií, procento pohyblovosti spermií a osmolalita semenné plazmy zůstávají neměnné v průběhu celého reprodukčního období. Oproti tomu, množství spermatu a proteinová koncentrace semenné plazmy se postupně zvyšovaly a dosáhly nejvyšších hodnot na konci reprodukčního období. Hodnota koncentrace spermií dosáhla svého vrcholu v polovině reprodukčního období a poté se snižovala až do jeho konce. Souběžná analýza proteinového složení ukázala, že čas odběru spermatu v průběhu reprodukčního období ovlivnil především membránové transportní proteiny a jejich organizaci, dále pak pohyblivost spermií a oxidativně redukční aktivitu.

Pro vyhodnocení dopadu uchování spermatu *in vitro* bylo v této studii využito několika postupů a způsobů konzervace.

V prvním případě bylo provedeno krátkodobé *in vitro* uchování spermatu jesetera ruského (*Acipenser gueldenstaedtii*) a jesetera sibiřského (*Acipenser baerii*) při

hypotermických podmínkách (4 °C). Vyhodnocovány byly vlivy tohoto uchování na parametry motility spermií, úrovně poškození DNA a na indikátory oxidativního stresu. Po šesti dnech *in vitro* uchování byla zjištěna 50% pohyblivost, zatímco po uplynutí devíti dnů byla již pohyblivost spermií studovaných druhů nulová. U jesetera ruského nebylo zaznamenáno žádné poškození DNA v průběhu prvních tří dnů uchování, kdežto u jesetera sibiřského bylo zaznamenáno prokazatelné poškození DNA již v po dvou dnech *in vitro* uchování. Úroveň indikátorů oxidativního stresu u spermatu obou druhů, konkrétně reaktivních substancí kyseliny thiobarbiturové, proteinových karbonylových derivátů a úroveň aktivity celkových superoxidativních dismutáz, prokazatelně narůstala v průběhu celého *in vitro* uchování. Předpokládáme, že aplikace antioxidantů v průběhu uchování spermatu jeseterovitých ryb *in vitro* by zabránila buněčnému poškození v důsledku oxidativního stresu. Kryoprezervace byla druhou metodou uchování spermatu v podmínkách *in vitro*, která byla studována v této práci. Hodnocení kryorezistence spermií jesetera malého získaných se sekvenčního odběru ukázalo, že v důsledku zmrazování a rozmrazování spermatu dochází k poklesu motility spermií, jejich rychlosti a fertilizačnímu potenciálu. Ačkoliv sekvenční odběry spermatu v kombinaci s jeho zmrazováním mohou být jednou z cest ke zvýšení efektivnosti stále se rozšiřujícího chovu jesetera v akvakulturních podmínkách.

Výsledky této studie poskytují ucelený pohled na kvalitativní a kvantitativní parametry spermatu chrupavčitých a kostnatých druhů ryb s ohledem na jeho *in vivo* a *in vitro* uchovávání. Tyto výsledky by měly být přínosem pro zdokonalení akvakulturního chovu výše zmíněných druhů. Získaná data potvrdila, že proteinové složení semenné plazmy se v průběhu uchování spermatu *in vivo* mění v závislosti na době jeho odběru. Dále je pravděpodobné, že na regulaci pohybu spermií se podílejí proteinové změny enzymatických drah. Výsledky prezentované v této studii by měly napomoci optimalizovat postupy a protokoly umělé reprodukce ryb.

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LIST OF PUBLICATIONS

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Shaliutina, A., Hulak, M., Li, P., Sulc, M., Dzuyba, B., Linhart, O., 2013. Comparison of protein fractions in seminal plasma from multiple sperm collections in sterlet (*Acipenser ruthenus*). Reproduction in Domestic Animals 48, 156–159.

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Shaliutina, A., Gazo, I., Cosson, J., Linhart, O., 2013. Comparison of oxidant and antioxidant status of seminal plasma and spermatozoa of several fish species. In: Aquaculture Europe 2013, Trondheim, Norway, 9–12 August 2013

Dzyuba, B., Cosson, J., Boryshpolets, S., **Shaliutina, A.,** Bytyutskyy, D., Flajšhans, M., Dzyuba, V., Rodina, M., Linhart, O., 2012. Sperm fertilizing ability and sensitivity to hypotonic stress after cryopreservation in sterlet (*Acipenser ruthenus*). In: Domestication in Finfish Aquaculture, Olsztyn-Mragowo, Poland, 23–25 October 2012.

Gazo, I., Linhartova, P., **Shaliutina, A.,** Hulak, M., 2012. The effect of environmentally related concentrations of vinclozolin on DNA integrity and oxidative stress indices in sterlet (*Acipenser ruthenus*) sperm. In: AQUA 2012-Global Aquaculture Securing Our Future, Prague, Czech Republic, 1–5 September 2012.

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Shaliutina, A., Hulak, M., Dzyuba, B., Policar, T., Linhart, O., 2012. Characterization of semen quality at the different time of spawn in perch (*Perca fluviatilis*). In: AQUA 2012-Global Aquaculture Securing Our Future, Prague, Czech Republic, 1–5 September 2012.

Shaliutina, A., Hulak, M., Gazo, I., Linhart, O., 2012. Effect of long-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) sturgeon sperm. In: Domestication in Finfish Aquaculture – Innova Fish, Olsztyn, Poland, 23–25 October 2012.

Shaliutina, A., Dzyuba, B., Hulak, M., Boryshpolets, S., Li, P., Linhart, O., 2011. Evaluation of spermiation indices and comparison of protein profiles of seminal plasma during multiple sperm collection in sterlet *Acipencer ruthenus.* In: 3rd International Workshop on the Biology of Fish Gametes, Budapest, Hungary, 7–9 September 2011.

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Pond aquaculture	2010
Applied hydrobiology	2010
Fish reproduction	2011
Ichthyology and systematics of fish	2011
Basic of scientific communication	2012
English language	2012
Czech language	2012

Scientific seminars	**Year**
Seminar days of RIFCH	2009 2010 2011 2012

International conferences	**Year**
3rd International Workshop on the Biology of Fish Gametes, Budapest, Hungary, September 7–9 (Oral presentation)	2011
2nd International conference NACEE, St. Petersburg, Russia, September 11–13 (Oral presentation)	2011
1st International caesar Conference, Bonn, Germany, October 5–7 (Poster presentation)	2011
AQUA 2012-Global Aquqculture, Prague, Czech Republic, September 1–5 (Poster presentation)	2012

Domestication in Finfish Aquaculture – Innova Fish, Olsztyn, Poland,October 23–25 (Poster presentation)	2012
Aquaculture Europe 2013, Trondheim, Norway, August 9–12 (Poster presentation)	2013
Foreign stays during Ph.D.	**Year**
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Prof. Kazuo Inaba, Shimoda Marine Research Center of the Tsukuba University, Shimoda, Japan (2 months, proteomics technology)	2012
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KNOWLEDGE OF LANGUAGES

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