Ultrastructure of spermatozoa and fertilization process in sturgeon

Ultrastruktura spermií a fertilizačního procesu jeseterů

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Chapter 1

General Introduction

1.1 Introduction

A spermatozoon has the function to transport the male haploid chromosome set into the oocyte. For this purpose it is composed of four different compartments, the acrosome, the head, the midpiece, and the flagellum (Callard and Callard, 1999; Knobil and Neill, 1999). The acrosome has lytic activities to enable the entrance of the spermatozoon into the oocyte through egg envelope, the head contains the nucleus and therefore the DNA material. In the midpiece the mitochondria are located, which deliver the energy for flagellar beating. The flagellum itself is the motor of the spermatozoon. The centriolar complex consists of the proximal and the distal centriole whereby the latter is the basal body of the flagellum. This centriolar complex anchors the flagellum at the sperm cell and is normally located in close vicinity to the nucleus.

Fish gametes show a great variety in morphology and ultrastructure, including the number and location of different organelles (Baccetti et al., 1984; Baccetti, 1986; Jones and Butler, 1988). Species-specific morphological and physiological features have been shown in spermatozoa and eggs of several fish species, which reflect differences in functional capabilities (Babin et al., 2007; Alavi et al., 2008). Morphology and fine structure are considered to be the major sources of information in comparative spermatology (Baccetti, 1986; Jamieson, 1991, 1999; Lahnsteiner and Patzner, 2007).

In *Chondrichthyes* the centriolar complex is not located at the nucleus, but at the caudal end of the midpiece. Consequnently also the flagellum originates at the caudal end of the midpiece. This very specific organization is found in no other fish group. The elasmobranch spermatozoon is filiform and typically composed of the four above mentioned compartments (acrosome, nucleus, midpiece, and flagellum) (Jamieson, 1991; Mattei, 1991). The midpiece is well developed and contains a large number of mitochondria. Glycogen particles are characteristic for the midpiece and in some species also for the flagellum.

In the *Osteichthyes* the sperm structure is much more diverse (Jamieson, 1991; Mattei, 1991). Spermatozoa of the *Actinistia*, *Dipnoi*, *Cladistia*, and *Chondrostei* have an acrosome, and the head is elongated. The nucleus is pervaded by a channel in its longitudinal axis, which contains filaments (rods). In the Actinistia, the mitochondria are not located in a separated midpiece but surround the caudal base of the nucleus. A separate midpiece is found in *Dipnoi*, *Cladista* and *Chondrostei*. The midpiece is small in species with external fertilization (*Dipnoi*, *Cladistia*, and *Chondrostei*) but well developed and of similar size as in elasmobranch fish in species with internal fertilization (*Actinistia*).

Spermatozoa of the *Neopterygii* do not have an acrosome. This is the only common structural feature of spermatozoa in this systematic group. Within the *Neopterygii* the *Teleostei* are the most diverse group of fish with about 20,000 species. The teleost spermatozoon consists of head, midpiece, and tail. The head is variable in form and smaller than in the *Chondrichthyes* ($\leq$ 10 µm). The midpiece is small, too. It contains the mitochondria and is pervaded by a cytoplasmic canal. This is an invagination of the plasma membrane of the midpiece region, which separates the cytoplasm of the midpiece almost completely from the flagellum. The centriolar complex consists of proximal and distal centriole and is located in an invagination of the nucleus, the nuclear notch. It has often complex stabilization structures anchoring the flagellum at the nucleus or in the cytoplasm of the midpiece. Commonly, the flagellum has a 9 x 2 + 2 microtubule pattern and may contain lateral side fins, which are considered as paddles to enhance the force of flagellar beating (Lahnsteiner and Patzner, 2007).

Spermatozoa of teleost fish can be divided into two groups. Species with internal fertilization (e.g. *Cottidae*, *Embiotocidae*, *Poeciliidae*) have an elongated nucleus and a

relatively big midpiece region with numerous mitochondria. The head of a spermatozoon of a species with internal fertilization measures around 10 μm, the midpiece 5 μm and the tail 30-40 μm. However, this statement cannot be generalized as some species with internal fertilization (*Goodeidae*, *Sebasticus marmoratus*) have a sperm morphology typical of species with external fertilization. Teleost species with external fertilization have a more simple sperm organization. These sperm types are termed "aqua sperm" (Jamieson, 1991) and commonly exhibit a hyper motile behavior for 60-90 sec in teleost (Zuromska and Markowska, 1984; Linhart et al., 2003) remarkably similar to that so-called "hyper motility" of mammalian spermatozoa in the vicinity of eggs before fertilization (Cosson et al., 1999).

1.2 Sturgeon spermatozoa in comparison with those of teleost

The number of spermatozoa released by the male is enormous: up to 500 m^3 in Russian sturgeon *Acipenser gueldenstaedti* or 1000 cm^3 in kaluga *Huso dauricus*). One cubic centimeter of sperm contains up to $1x10^{10}$ spermatozoa, usually from $1x10^9$ to $4x10^9$ (Dettlaff et al., 1993).

The spermatozoa are immobile in the ejaculate and activated the moment water is added. At first, they perform quick forward motion; during this period, a spermatozoon of *Huso huso* moves at a rate of 0.1 $mm.s^{-1}$. Then the forward motion of the spermatozoa gradually slows down, turns into uncoordinated oscillating movement and, finaly, ceases altogether. The spermatozoa of sturgeons retain their motility in water for a considerably longer time than those of teleosts that spawn in fresh water: for example the spermatozoa of beluga *Huso huso* (Ginsburg, 1968) swim in water up to 13 min, but those of teleost (e.g. common carp *Cyprinus carpio*, Musselius, 1951) only one min.

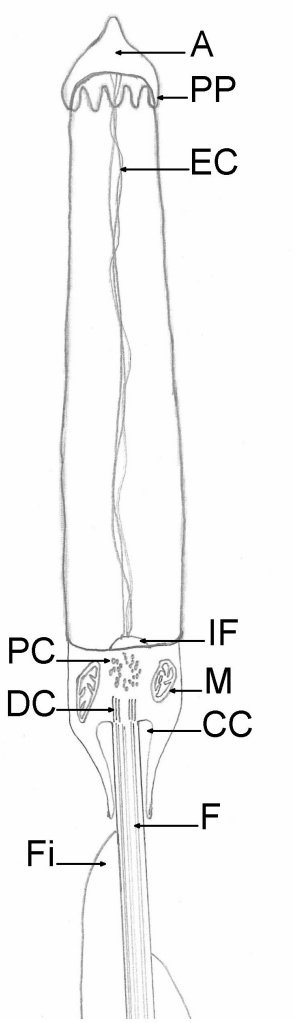
Spermatozoa of sturgeon fishes show characteristic differences compared to those of teleost fishes (Jamieson, 1991) in terms of morphology, presence of acrosome (Cherr and Clark, 1984; Psenicka et al., 2007; Wei et al., 2007), sperm behavior (Cosson et al., 2000) and biochemistry with the presence of acrosin- or trypsin-like activities (Ciereszko et al., 1994, 1996). The defining structure of the sturgeon sperm cell constitutes composed of three major parts; (a) an elongated head with acrosome, (b) a cylindrical midpiece and (c) a flagellum (See Dettlaff et al., 1993 for general information). Scheme of tench spermatozoa as representative of teleost and those of sturgeon is shown on picture 1a,b. The overview on variation of spermatozoa of diferent sturgeon species is given in the Chapter 2 (Figure 7) and within one species Chapter 3.

1.2.1 Acrosome

The lack of acrosome in teleost fishes is compensated for by the presence of a micropyle, through which the sperm penetrates into the egg (Lahnsteiner and Patzner, 1999). Interestingly, sturgeon spermatozoa contain the acrosome forming a long fertilization filament while the corresponding eggs have several micropyles (Debus et al., 2002; Linhart and Kudo, 1997). Schema (fig. 2) shows species with the fertilization filament and the permeability to their egg. The acrosome, possibly functioning to expedite egg penetration during fertilization, is considered to be derived from the Golgi apparatus and commonly cap-shaped (Nicander, 1970). The acrosome terminates with posterolateral projections. DiLauro et al., (2000) mentioned briefly the longer posterolateral projections may be an adaptation that provides a more secure anchorage to the egg in fast flowing water during spawning and fertilization.

The Chapter 2 is also focused on morphology of sturgeon spermatozoa acrosome, confirmation of presence and localization of acrosin protein, the Chapter 3 describes methodology for acrosome integrity screening, the Chapter 4 shows the influence of different

cryoprotectant on the acrosome and the Chapter 5 brings out its function during fertilization process.



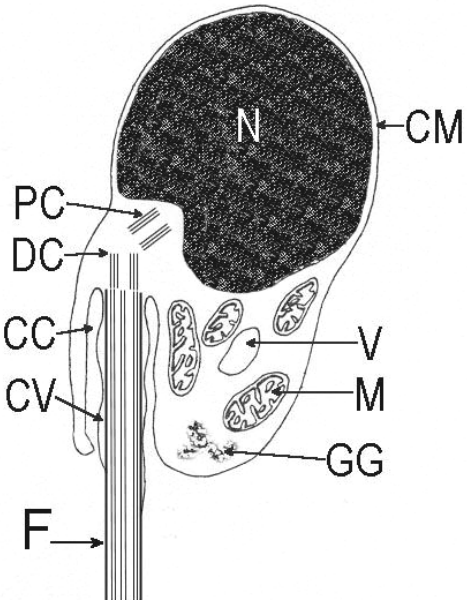


Figure 1 shows basic structure of surgeon (a, righ) and teleost (tench) spermatozoa (b, left).

A – acrosome
PP - posterolateral projections
EC – endonuclear canals
IF- implantation fossa
N – nukleus
CM – cytoplasmic membrane
PC – proximal centriole
DC – distal centriole
M - mitochondria
CC – cytoplasmatic canal
GG – glycogen granules
V - vesicle
CV – cytoplasmic vesicle
F – flagelum
Fi - fin

1.3.2 Nucleus

The basic task of spermatozoan head is to transfer genetic material localized in nucleoplasma to egg. Optimal shape and size of spermatozoan head is a prerequisite for good penetration of spermatozoon throughout the micropyle (Ginsburg, 1968). Different shapes of spermatozoan head occur in chondrostean and teleostean fishes with external fertilization (Figure 1a,b). Regular, ball-shaped spermatozoan head was found in northern pike *Esox lucius* (Mattei, 1969); big spermatozoan head in silver carp *Hypophtalmichthys molitrix* (Billard, 1970; Emaljanova and Makeeva); elongated one in *Mimagoniates barberi* (Pecio and Rafinski, 1994); crescent-shaped one in white-spotted conger eel *Conger myriaster* (Okamura and Motonobu, 1999). Generally, the spermatozoan head in fishes is relatively small (2-4 μm) in relation to the total size of spermatozoon. The exceptions were observed at European eel *Anguilla anguilla* spermatozoa (Gibbons et al., 1983; Ginsburg, 1968) and at *chondrostei* with elongated sperm head up to 10 μm in length and about 1 μm in width (Cherr and Clark, 1984, 1985; Ciereszko et al., 1994, DiLauro et al., 1998, 1999, 2000, 2001; Gibbson et al., 1983; Ginsburg, 1968; Ginsburg, 1977; Psenicka et al., 2007, 2009a; Xu & Xiong, 1988).

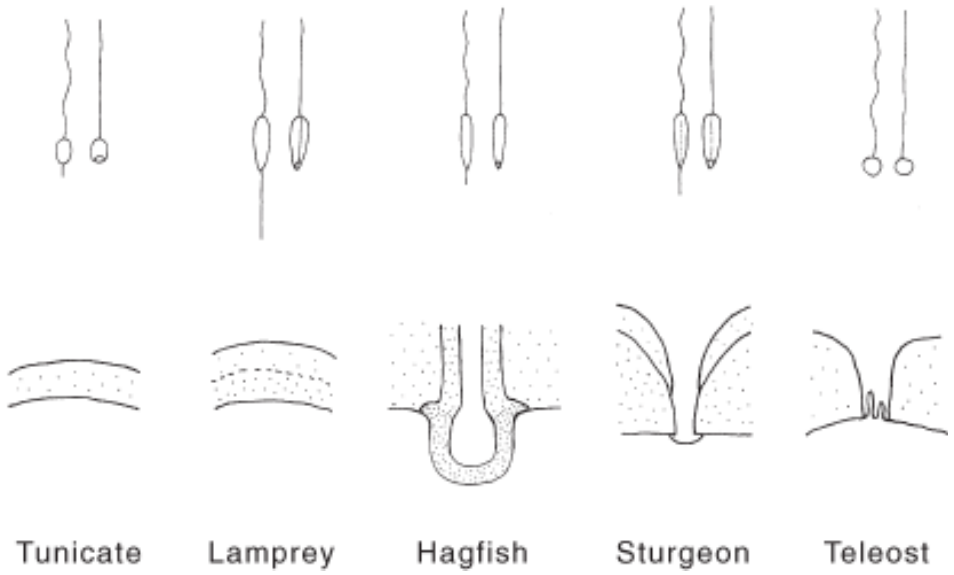


Figure 2. Schematic drawings of the fertilization site or micropylar region of eggs and the anterior structure of sperm before and after the acrosome reaction in chordates (Morisawa, 1999a). Illustrations of eggs (lower side) were drawn after Holland et al. (1988) for tunicates; Nicander et al. (1968) for the lamprey; Morisawa (1999b) for hagfish, bottom of micropylar region in a very late stage of oogenesis; Cherr and Clark (1982) and Dettlaff et al. (1993) for the sturgeon; and Kudo (1980) for teleosts. Outside of the egg, upper open space; egg coat (vitelline membrane, egg envelope or chorion), dotted. Those of sperm (upper side) were after Holland et al. (1988) for tunicates; Jaana and Yamamoto (1981) for the lamprey; Morisawa (1995) for intact sperm and Morisawa (1999a) for reacted sperm of hagfish; Cherr and Clark (1984) for sturgeon; and Mattei (1970) for teleosts. Intact sperm, with straight flagellum; reacted sperm, with waved flagellum.

In sturgeon spermatozoa differences in its shape and size are evident, for example between nucleus length of sterlet *Acipenser ruthenus* (3.30 μm) and white sturgeon *Acipenser transmontanus* (9.21 μm). Inter-specific diversity in size and shape of sturgeon sperm cell nuclei has evolutionary and genetic implications (DiLauro et al., 1999). The membrane-lined endonuclear canals (ECs) traverse the nucleus from the acrosomal end to the basal nuclear fossa region so called implantation fossa. Except for the Atlantic sturgeon *Acipenser oxyrhynchus* DiLauro et al., 1998) that has two ECs, all other sturgeon species have three (only seldom two or four) ECs in spermatozoa nucleus (Cherr and Clark, 1984; DiLauro et al., 2000, 2001; Ginsburg, 1977; Psenicka et al., 2007; Wei et al., 2007). DiLauro et al. (1999), Cherr and Clark (1984) and Jamieson (1991) believed that the materials in nuclear canals would play a perforatorial role during penetration of sperm into the egg.

1.2.2 Midpiece

The midpiece is firmly linked with head and it contains proximal and distal centriole and mitochondrial segments. Both centrioles consisted of nine peripheral triplets of microtubules. The midpiece is thus similar to that of a mammalian spermatozoon, except for the fact that fish spermatozoa contain fewer mitochondria and its flagellum is separated from the midpiece by a cytoplasmic canal (Lahnsteiner, 2003). The midpiece appeared to be asymmetric and the nucleus was never oriented in the same plane as the joint of flagellum, as observed in many cyprinid species. The angles between proximal and distal centriole are species specific within cyprinids (Baccetti et al., 1984; Psenicka et al., 2006). Distal centriole in sturgeon spermatozoa is located adjacent to the proximal centriole and the axoneme rises posteriorly to the distal centriole. In sturgeon the proximal centriole is parallel to the plane of

head and flagellum and the distal centriole is perpendicularly orientated to the plane of spermatozoon.

Next species specific feature in fish is number of mitochondria. Only a single mitochondrion was found in the spermatozoan midpiece in European perch *Perca fluviatilis*. On the other hand, more than 20 mitochondria were found in the midpiece of ide *Leuciscus idus* (Ginsburg, 1968). In cyprinids generally, the number of mitochondria varied from 2 to 10 on the particular species (Baccetti et al., 1984). Three to six and three to eight mitochondria have been reported in sturgeon (Psenicka et al., 2007). Mitochondria are the main source for ATP-generation during sperm motility (Billard et al., 1999).

1.2.3 Flagellum (tail)

Flagellar length in teleost varies from 25 μm (tench, Psenicka et al., 2006) to 94 μm in channel catfish Ictalurus punctatus. Contrariwise flagellum within sturgeons has from 33 (Chinese sturgeon *A. sinensis*, Wei et al., 2007) to 42 (sterlet, Psenicka et al., 2009a). The fibrillar part of flagellum consists of nine peripheral doublets and a central pair of singlet microtubules arranged as in most fish spermatozoa, but contrariwise Sperm flagella of fish belonging to *Anguilliformes* and *Elopiformes* present a "9+0" structure without central microtubules and the outer arms (Gibbons et al., 1985; Okamura and Motonobu, 1999; Todd, 1976).

The other peculiarities in the flagellum were reported for some species: cells with two flagella were found in plainfin midshipman *Porichtis notatus* (Stanley 1969) and channel catfish *Ictalarus punctatus* (Jaspers et al., 1976). Along the flagella, the plasma membrane often formed one or two lateral fins (Billard, 1970; Nicander, 1970), preferentially oriented along the horizontal plane defined by the central microtubules (Billard, 1970). Such a modification of the flagellum could improve the efficiency of the flagellar propulsion as discussed by Afzelius (1978). This general form was documented for fish belonging to *Poeciliidae, Janysiidae, Patolontidae* and *Embiotocidae* (Billard, 1970; Lanhsteiner, et al., 1997; Stanley, 1969; Van Deurs, 1975).

1.3 Structure of sturgeon eggs

According to the distribution of yolk and lipid inclusions and the structure of cortical granules, the mature egg does not differ from the oocytes which start to mature. The cortical layer structure has changed, the length of microvilli has been markedly reduced, and they no longer enter so called the zona radiate tubules. But the most importent character of oocyte maturation is germinal vesicle (GV) migration to animal pole (Dettlaff, et al., 1993). Figure 4 shows the position of GV of oocyte of female, which is ready for hormonal stimulation of artificial reproduction (Gela et al., 2008). The main differences between a mature egg and oocyte lies in fact that there is no GV in the former and, after the GV breaks down during maturation, the karioplasm spreads over the animal cytoplasm without mixing with it completely (Dettlaff et al., 1993).

The number of eggs deposited by the females of Acipenserid fish is very high (up to 4.1 mil in kaluga). The eggs of most animals have one or more investments that must be transferred by the spermatozoon before gamete union can occur. In addition to provide protection, in some animals these coats may have other functions such as attachement to a substrate (adhesivevennes) and sperm activation. The majority of fish eggs are covered by a tough, proteinaceouse envelope that is extracellularly derived. This envelope, or chorion, is the main protective structure of the unfertilized egg and, later, of the developing embryo. Morphological studies on teleost eggs have shown that the chorion is perforated by a small

opening, the micropyle, which allows spermatozoa passage to the enclosed egg. The spermatozoa in most teleosts can enter the egg only through single micropyle (although two or more micropyles are found on the wolffish *Anarhichus lupus* and the ocean pout *Macrozooarces americanus*, Yao et al., 1995), which is funnel-shaped and is located at the animal pole. In teleosts a species specific feature of the sperm entry site has been shown (Kudo, 1991). The diameter of the inner aperture of the micropyle enables entering only a single spermatozoon.

In sturgeons the number of micropyles varies markedly in different females of a species, and also in the eggs of one female (Podushka, 1993). Therefore it does not allow for definite species identification (Debus et al., 2002). The number of micropyle has been reported as 52 micropyles in the Russian sturgeon (Ginsburg, 1968) or 3 to 16 in the Siberian sturgeon *Acipenser baerii* (Debus et al., 2002).

The eggs of sturgeon are globular or, mostly, somewhat elongated. The eggs are brownish-gray. The color of the different region of the egg varies, reflecting the polarity of its internal structure. The part of the egg facing upward after fertilization (animal region) is usually lighter than the lower uniformly colored vegetal part. In the center of the animal area, there is a light polar spot surrounded by dark concentric rings (Figure 3).

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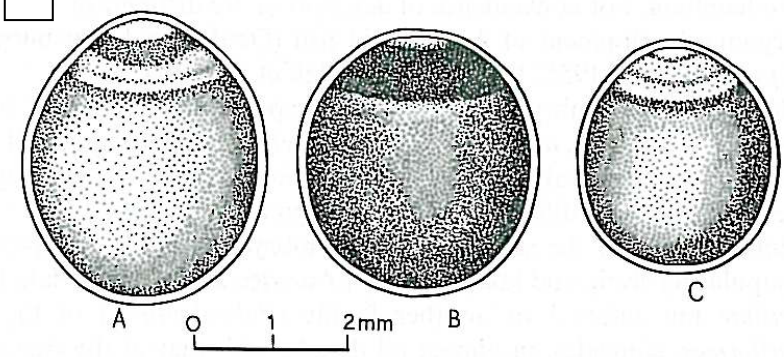

Figure 3 shows lateral view of eggs of *Huso huso* (A), *A. gueldenstaedti colchicus* (B), and *A. stellatus* (C) (Dettlaff and Ginsburg, 1954).

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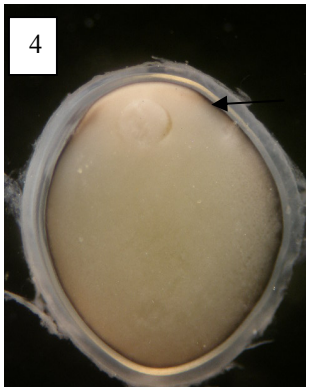

Figure 4. The optimal position of germinal vesicle (arrow) of *A. ruthenus* oocyte for spawning (Gela et al. 2008).

Older investigations on egg envelopes of acipenserids, mainly made by Russian authors (e.g. Vorobyeva et al., 1986), described three main layered structures. These envelope descriptions vary considerably between investigators. For better orientation in following text, we prefer to simplify the nomenglature. We will label the layer most proximal to the oolema "layer 1" (L1) and the layer exterior to L1, "layer 2" (L2). These two layers were described by Dettlaff et al. (2003) as zona radiata interna and externa. The layer adjacent to the exterior of L2 will be labed "layer 3" (L3). Dettlaff et al. (1993) describe this layer as jelly coat, but we use this mark for the outermost soluble layer according to (Cherr and Clark 1985). The L1 and L2 layers display a filamentous material and contain projections with spaces around them. The L3 contains canals filling with the jelly. Later the jelly is washed due to water Psenicka et al., 2009b).

Total thickness of layers was measured to be 70 μm in stellate sturgeon *Acipenser stellatus*. This parameter can be species specific (Vorobyeva et al., 1986). However, the thickness of the membranes in the animal pole region is lower than in the rest of the egg surface (Dettlaff and Ginsburg, 1954). In contrast, most teleost egg envelopes thickness ranges 2 μm to 50 μm. One to two layers of cortical granules are located under the egg layers.

The release of cortical granules components upon sperm penetration blocks polyspermy, and creates a single layer called "perivitelline space" in most of fish eggs (Cherr and Clark, 1985; Dettlaff et al., 1993; Psenicka et al., 2009b). Mature eggs of several fish species respond to sperm penetration by forming an earlier fertilization cone at the sperm entry site, which block polyspermy before initiation of the cortical reaction (Kudo, 1980, Kudo and Sato, 1985; Linhart and Kudo, 1997, Psenicka et al., 2009b).

IN THIS THESIS

The overall aim of present thesis was describe ultrastructure of sturgeon spermatozoa, function of their acrosome and process of fertilization.

The specific objectives were to:

- Describe and compare the ultrastructure of sturgeon spermatozoa with those of teleost.
- Find the best way of monitoring of acrosomal reaction and describe the process of acrosomal reaction in sturgeon
- Evaluate influence of different ways of cryopreservation on sturgeon spermatozoa viability, motility and acrosomal integrity.
- Investigate the fertilization process and function of acrosome for penetration in sturgeon.
- Describe the ultrastructure of sturgeon eggs, the changes after activation and fertilization, and prevention of polyspermy.

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Chapter 2

Morphology and ultrastructure of Siberian sturgeon, *Acipenser baerii*, spermatozoa using scanning and transmission electron microscopy

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Morphology and ultrastructure of Siberian sturgeon (*Acipenser baerii*) spermatozoa using scanning and transmission electron microscopy

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Abstract

Background information. Available data concerning the sperm morphology of teleost fishes demonstrate wide variation. In the present study, the spermatozoa of Siberian sturgeon (*Acipenser baerii* Brandt, 1869), a chondrostean fish, was investigated. In contrast with teleost fish, chondrostean spermatozoa have a head with a distinct acrosome, whereas other structures, such as a midpiece and a single flagellum, are present in spermatozoa of most species.

Results. The average length of the head including the acrosome and the midpiece was 7.01±0.83 mm. Ten posterolateral projections derived from the acrosome were present on a subacrosomal region, with mean lengths of 0.94±0.15 μm and widths of 0.93±0.11 μm. The nucleus consisted of electrodense homogeneous nuclear chromatin. Three intertwining endonuclear canals, bound by membranes, traversed the nucleus longitudinally from the acrosomal end to the basal nuclear fossa region. There were between three and six mitochondria, two types of centrioles (proximal and distal) in the midpiece and two vacuoles composed of lipid droplets. The flagellum (44.75±4.93 μm in length), originating from the centriolar apparatus, had a typical 9+2 eukaryotic flagellar organization. In addition, there was an extracellular cytoplasm canal between the cytoplasmic sheath and the flagellum.

Conclusions. A principal components analysis explained the individual morphological variation fairly well. Of the total accumulated variance, 41.45% was accounted for by parameters related to the head and midpiece of the sperm and the length of the flagellum. Comparing the present study with previous studies of morphology of sturgeon spermatozoa, there were large inter- or intra-specific differences that could be valuable taxonomically.

Key words: *acrosome, flagellum, scanning electron microscopy, Siberian sturgeon* (*Acipenser baerii), spermatozoon, transmission electron microscopy*

Abbreviations used: PCA, principal components analysis; PLP, posterolateral projection; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

1. Introduction

Spermatozoa structure in fish is of interest mainly because specific morphological differences among sperm reflect differences in functional capabilities and phylogeny (Afzelius, 1978; Jamieson, 1991; Mattei, 1991). High inter- and intra-specific diversities have been reported, which relate to whether fertilization is internal or external and whether fertilization is dependent on life history (Jamieson, 1991).

Chondrostean spermatozoa differ basically from those of teleost fish by having an acrosome (Cherr and Clark, 1984; Dettlaff et al., 1993; Ginsburg, 1968; Lahnsteiner et al., 2004; Psenicka et al., 2006), having a longer motility period (Alavi et al., 2004; Billard et al., 1999; Cosson et al., 2000; Toth et al., 1997) and possessing acrosin (Ciereszko et al., 1994; Piros et al., 2002).

The sturgeon sperm cell structure can be differentiated into three major regions: (i) an elongated head with a front part called an acrosome and its subacrosome, (ii) a cylindrical midpiece with the centriolar complex, and (iii) a flagellum with the 9+2 microtubular structure (Cherr and Clark, 1984; DiLauro et al., 1998, 1999, 2000, 2001; Ginsburg, 1968;. The nucleus occupies most of the head volume; it usually contains three endonuclear canals that lead from the implantation fossa to the acrosome. The acrosome often forms several posterolateral projections which have been observed in pallid sturgeon *Scaphirhynchus albus* Forbes and Richardson, 1905 (DiLauro et al., 2001).

The mitochondria, the implantation fossa and the centriolar complex are located in the midpiece region. The fossa and proximal centriole are apparently attached by a structure similar to the fibrous body (Afzelius, 1978).

The flagellum consists of nine peripheral doublets and a central pair of singlet microtubules that are arranged as found in most fish spermatozoa, with the exception of the sperm flagellum in the European eel (Todd, 1976; Gibbons et al., 1985) and white-spotted conger eel *Conger myriaster* (Okamura and Motonobu, 1999) which have a 9+0 microtubule structure and lack the outer arms.

The plasma membrane is folded into one or two lateral fins or ridges along most of the flagellum length of sturgeon sperm (pallid sturgeon; DiLauro et al., 2001), as found in teleosts such as rainbow trout *Oncorhynchus mykiss* Walbaum, 1792, *Poeciliidae, Janysiidae, Patolontidae* and *Embiotocidae*, (Stanley, 1969; Billard,1983; Van Deurs, 1975; Lanhsteiner et al., 1997, respectively). These fin structures appear to be preferentially orientated along the horizontal plane defined by the central microtubules (Billard, 1970), and could help increase the efficiency of wave propagation (Cosson et al., 2000).

Siberian sturgeon has been increasingly considered for commercial aquaculture in Europe (Williot et al., 2000a), but, in spite of several studies on reproductive physiology (Le Menn and Pelissero, 1991), gamete physiology and biochemistry (Billard et al., 1999; Galli et al., 1991; Glogowski et al., 2002; Williot et al., 2000b) and cryopreservation (Billard et al., 2000; Kopeika et al., 1994; Tsvetkova et al., 1996), there are currently no data regarding the ultrastructure of its sperm. However, Billard et al. (2000) described the effect of cryopreservation on flagellar structure using an image analysis system based on light optical observations.

The present study has investigated and described the ultrastructure of Siberian sturgeon (*Acipenser baerii*) spermatozoa using SEM (scanning electron microscopy) and TEM (transmission electron microscopy).

2. Results

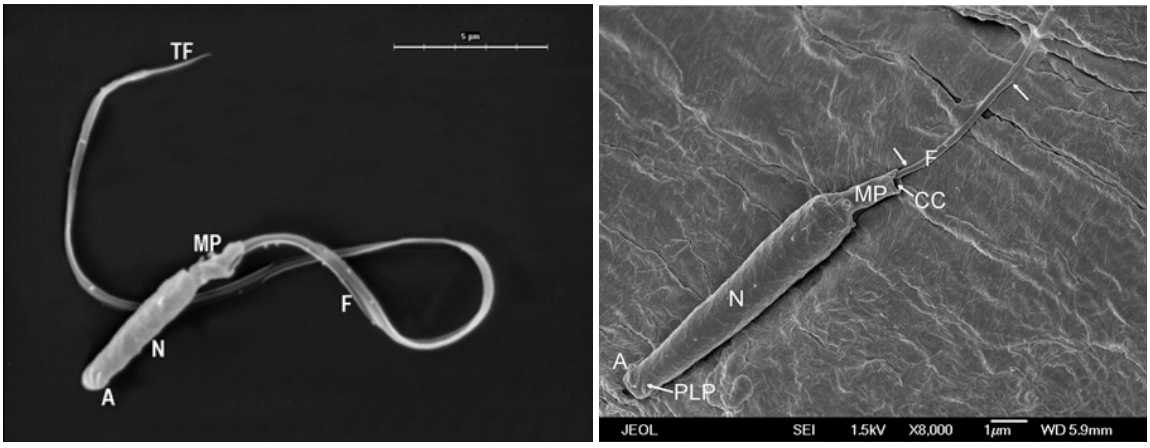
2.1. General morphological parameters

SEM verified that the spermatozoa of Siberian sturgeon are uniflagellated and differentiated into a head with an acrosome, a midpiece and a tail region with fins (Figure 1). The main body of the sperm cell was long, cylindrical and generally radially symmetrical, except for the flagellum (Table 1 and Figure 2).Using PCA (principal components analysis), 53.91% of the total accumulated variance was accounted for by three components. However, the first two components accounted for 41.45% (Figure 3), distinguishing different groups.

The first group comprised all parameters relating to the head of the sperm (acrosome width, anterior nucleus width, acrosome length, central nucleus width and posterior nucleus width).

The remaining variables relating to the midpiece (anterior midpiece width, posterior midpiece width and midpiece length) were grouped together. Interestingly, the length of the flagellum was more isolated. Factor loading on the components extracted by PCA showed a direction of change of variables, for example, between the midpiece length, acrosome width and anterior nucleus width (Figure 3 and Table 2). The strength of each variable in each component (Table 2) showed that the nucleus length had the largest negative coefficient (-0.63) in factor 2, but the midpiece length had the largest positive coefficient (0.72), indicating that the nucleus length had the greatest effect in factor 2, but in the opposite sense to other positive variables, such as midpiece length, anterior midpiece width (0.48) and posterior midpiece width (0.49).

Figure 1 Sperm cell structures observed with SEM



Scanning electron microscopes JSM 6300 (a, left) and JSM 7401 S (b, right) were used. A, acrosome; CC, cytoplasmic channel; F, flagellum; MP, midpiece; N, nucleus; TF, terminal region of flagellum. The arrows indicate the development of fins along the flagellar length. Scale bars are 5 μm (a) and 1 μm (b).

2.2. Acrosome

The acrosomes were 0.94±0.15 μm long and 0.93±0.11 μm wide (Table 1). The head with midpiece and acrosome was 7.01±0.83 μm long, increasing in mean width from 0.87±0.12 μm proximal to the acrosome (anterior) to 1.13±0.16 μm near the midpiece (posterior), whereas its diameter in the median region was 1.12±0.12 μm (Table 1).

Longitudinal sagittal sections showed a subacrosome (Figure 4a), and the cross-sections showed ten right-handed posterolateral projections (Figure 4b) between the nucleus and the acrosome, extending along the anterior sides of the nucleus.

2.3. Nucleus

The nucleus had an elongated trapezoid shape, tapering from the anterior end to the posterior end. It was composed of electron-dense and slightly granular materials surrounded by a nuclear membrane. Three intertwining endonuclear canals (44.59±11.61 nm in diameter), bound by membranes, traversed the nucleus from the junction with the acrosome towards the basal nuclear fossa region (Figure 4). An implantation fossa was present at the posterior end of the nucleus, connecting the axial portion of the nucleus with the midpiece (Figure 5a).

Table 1 SEM data for the ultrastructure of Siberian sturgeon sperm

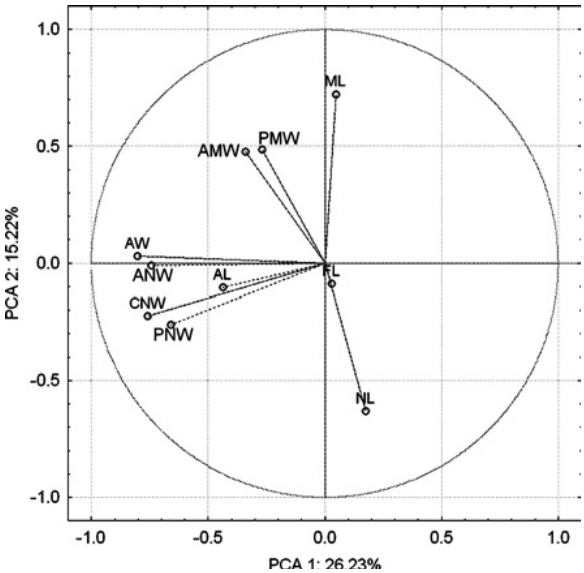
Fish…	male 1				male 2				male 3				male 4				male 5				male 6				male 7				male 8				Total			
	N	mean min	±	SD max	N	mean min	±	SD max	N	mean min	±	SD max	N	mean min	±	SD max	N	mean min	±	SD max	N	mean min	±	SD max	N	mean min	±	SD max	N	mean min	±	SD max	N	mean min	±	SD max
AHML	24	6.92^{abc} 5.62	±	0.90 9.61	20	6.85^{abc} 5.88	±	0.56 7.99	17	7.02^{abc} 5.08	±	0.94 8.35	19	6.13^{c} 5.16	±	0.60 6.99	69	6.92^{a} 4.55	±	0.75 8.99	22	7.13^{ab} 6.29	±	0.58 9.23	28	7.63^{b} 5.49	±	0.77 8.69	22	7.40^{ab} 5.41	±	0.76 8.49	221	7.01 4.55	±	0.83 9.61
HL	26	4.74^{ab} 3.15	±	1.06 7.30	20	4.70^{ab} 3.84	±	0.47 6.00	17	4.97^{abc} 4.86	±	0.70 3.75	19	4.17^{b} 3.26	±	0.44 4.90	69	4.83^{ab} 2.16	±	0.78 6.30	22	5.39^{ac} 4.23	±	0.63 7.52	28	5.44^{ac} 3.46	±	0.75 6.54	25	5.76^{c} 3.47	±	0.85 6.97	226	4.98 2.16	±	0.83 7.52
AL	26	0.89^{abc} 0.66	±	0.17 1.41	20	0.96^{ab} 0.78	±	0.10 1.21	17	0.84^{ac} 0.69	±	0.10 1.08	17	0.78^{c} 0.58	±	0.08 0.89	68	0.99^{b} 0.66	±	0.14 1.33	19	0.88^{abc} 0.65	±	0.15 1.16	28	0.97^{a} 0.83	±	0.12 1.30	25	1.01^{ab} 0.74	±	0.14 1.23	220	0.94 0.58	±	0.15 1.42
AW	25	0.89^{ab} 0.69	±	0.12 1.25	20	0.95^{bc} 0.81	±	0.13 1.44	17	0.91^{ab} 0.79	±	0.08 1.11	17	0.84^{a} 0.72	±	0.08 1.02	69	1.00^{c} 0.80	±	0.09 1.26	22	0.91^{ab} 0.78	±	0.10 1.18	28	0.89^{ab} 0.73	±	0.08 1.06	25	0.90^{ab} 0.76	±	0.08 1.07	223	0.93 0.69	±	0.11 1.44
MHW	25	1.04^{ab} 0.82	±	0.08 1.12	20	1.10^{bc} 1.01	±	0.06 1.20	17	1.01^{a} 0.91	±	0.08 1.22	17	1.05^{abc} 0.99	±	0.04 1.12	69	1.23^{d} 1.04	±	0.09 1.54	22	1.13^{c} 0.99	±	0.08 1.34	28	1.03^{ab} 0.92	±	0.11 1.44	25	1.09^{abc} 0.95	±	0.10 1.27	223	1.12 0.83	±	0.12 1.54
AHW	25	0.87^{acd} 0.66	±	0.13 1.22	20	0.92^{ad} 0.79	±	0.13 1.35	17	0.84^{abc} 0.70	±	0.14 1.29	17	0.83^{abc} 0.74	±	0.07 0.99	69	0.94^{d} 0.76	±	0.80 1.14	22	0.91^{ad} 0.69	±	0.11 1.17	28	0.76^{b} 0.65	±	0.11 1.14	25	0.77^{bc} 0.63	±	0.09 0.96	223	0.87 0.63	±	0.12 1.36
PHW	25	1.02^{a} 0.87	±	0.08 1.18	20	1.12^{a} 0.91	±	0.11 1.31	17	1.03^{a} 0.88	±	0.20 1.76	17	0.99^{a} 0.91	±	0.07 1.12	69	1.27^{b} 0.94	±	0.13 1.73	21	1.02^{a} 0.67	±	0.15 1.31	27	1.09^{a} 0.96	±	0.10 1.31	24	1.11^{a} 0.80	±	0.14 1.38	221	1.13 0.67	±	0.16 1.76
ML	25	1.12^{ab} 0.37	±	0.54 2.21	20	1.06^{ab} 0.55	±	0.36 1.74	17	1.35^{b} 0.61	±	0.47 1.92	15	1.23^{ab} 0.57	±	0.38 1.85	68	1.05^{ab} 0.39	±	0.34 2.12	21	0.83^{a} 0.41	±	0.32 1.62	26	1.09^{ab} 0.56	±	0.44 2.21	24	0.91^{ab} 0.41	±	0.29 1.47	216	1.09 0.37	±	0.42 2.21
AMW	24	0.69^{ab} 0.34	±	0.19 1.08	20	0.90^{ab} 0.47	±	0.31 1.47	17	0.64^{b} 0.42	±	0.12 0.83	15	0.71^{ab} 0.46	±	0.16 0.96	67	0.86^{a} 0.46	±	0.26 1.47	21	0.70^{ab} 0.39	±	0.19 1.19	26	0.92^{a} 0.46	±	0.22 1.33	24	0.83^{ab} 0.46	±	0.29 1.65	214	0.8 0.34	±	0.25 1.65
PMW	24	0.50^{a} 0.33	±	0.13 0.78	20	0.72^{b} 0.30	±	0.27 1.42	16	0.49^{a} 0.28	±	0.12 0.66	15	0.46^{a} 0.33	±	0.11 0.72	67	0.56^{a} 0.28	±	0.18 1.05	22	0.57^{ab} 0.33	±	0.14 0.90	27	0.60^{ab} 0.35	±	0.16 0.95	24	0.54^{ab} 0.33	±	0.23 1.23	215	0.56 0.28	±	0.19 1.41
FL	21	47.14^{ac} 34.72	±	3.66 55.07	16	47.50^{ac} 40.62	±	4.62 57.40	14	50.34^{c} 41.77	±	6.84 62.22	18	39.94^{b} 33.03	±	3.50 44.42	57	44.00^{a} 35.46	±	4.58 55.20	18	43.66^{ab} 39.39	±	2.64 49.48	16	44.69^{ac} 39.20	±	2.07 47.57	11	43.90^{ab} 39.10	±	2.32 46.76	171	44.8 33.03	±	4.93 62.22

Measurements are N, means (µm) ± S.D. for n and the range of measurements. Values with the same superscript letters are not significantly different (ANOVA, P<0.05). AHML, acrosome, head and midpiece length; AHW, anterior head width; AL, acrosome length; AMW, anterior midpiece width; AW, acrosome width; FL, flagellum length; HL, head length without midpiece or acrosome; MHW, median head width; ML, midpiece length; PHW, posterior head width; PMW, posterior midpiece width.

Table 2 Factor loading on the components extracted by PCA of all sperm parameters

Parameter	abbreviation	Factor 1	Factor 2	Factor 3
Nucleus length	NL	0.17	-0.63	0.52
Acrosom length	AL	-0.44	-0.1	0.27
Acrosom width	AW	-0.8	0.03	0.03
Central nucleus width	CNW	-0.76	-0.22	-0.26
Anterior nucleus width	ANW	-0.74	-0.01	-0.19
Posterior nucleus width	PNW	-0.66	-0.26	-0.05
Midpiece length	ML	0.05	0.72	-0.27
Anterior midpiece width	AMW	-0.34	0.48	0.47
Posterior midpiece width	PMW	-0.27	0.49	0.58
Flagellum length	FL	0.03	-0.09	0.4

Figure 2 Ordination diagram of PCA of sperm morphological parameters of Siberian sturgeon



AL, acrosome length; AMW, anterior midpiece width; ANW, anterior nucleus width; AW, acrosome width; CNW, central nucleus width; FL flagellum length; ML, midpiece length; NL, nucleus length; PMW, posterior midpiece width; PNW, posterior nucleus width.

2.4. Midpiece

The midpiece (Figure 5) was 1.09±0.42 μm long, with an approximately cylindrical shape and an elongated caudal base. Three to six mitochondria were observed in a peripheral section of the midpiece, sometimes surrounding the nucleus. In addition to mitochondria, there were several organelles in the midpiece. There were two ovoidal vacuoles or lipid droplets in the midpiece, but these structures could be artefacts. The ovoid proximal centriole (185.28 nm×147.42 nm) was located in an implantation fossa.The distal centriole was observed near the flagellar origin, as it functions as a basal body for the flagellum. The

proximal centriole was connected to the implantation fossa by a structure resembling the fibrous body. Both centrioles were formed of nine peripheral triplets of microtubules in the shape of a cylinder. The axoneme was located immediately posterior to the distal centriole. The electron-dense structures and/or the striated rootlets were located around the centrioles. Around the flagellum there was a cytoplasmic channel, formed by an invagination at the plasmalemma. There was an extracellular space between the cytoplasmic sheath and the flagellum.

Figure 3 Individual variations of sperm morphology within samples from Siberian sturgeon using PCA

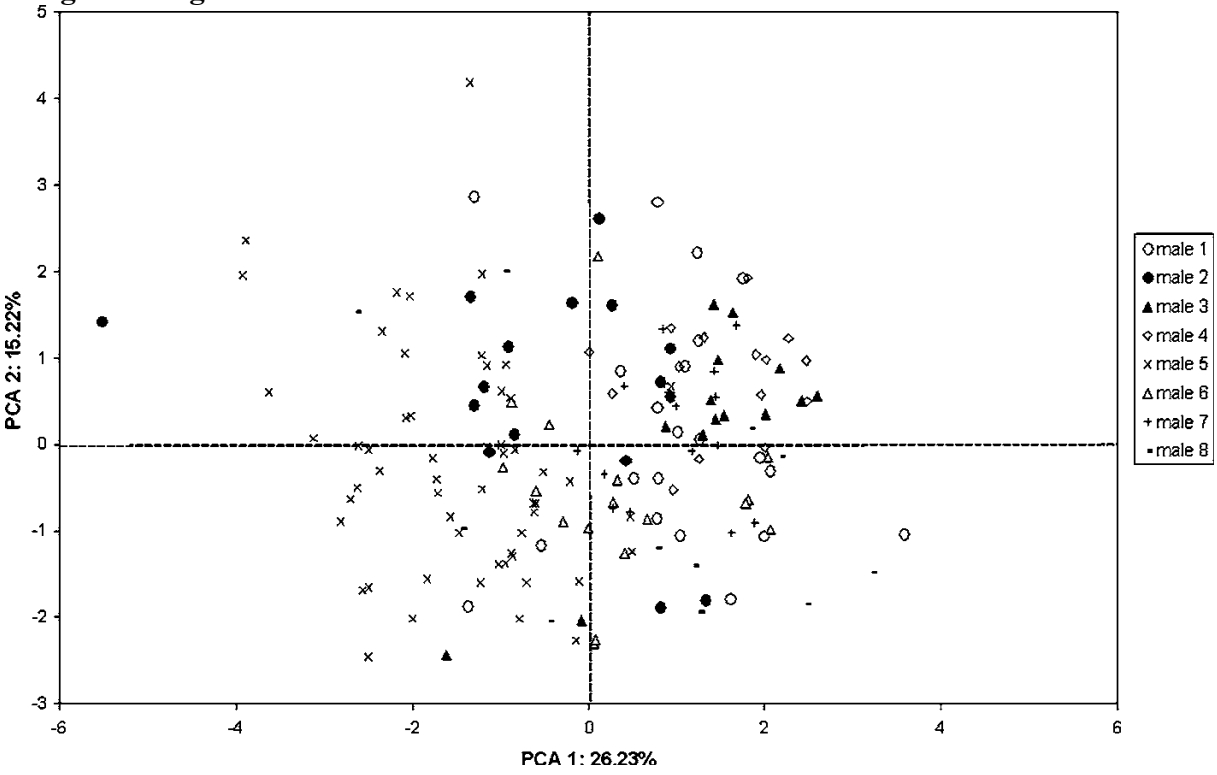


Table 3 TEM data for the ultrastructure of Siberian sturgeon sperm

Fish No.	male 1		male 2		male 3		male 4		Total	
	N	mean ± SD min / max	N	mean ± SD min / max	N	mean ± SD min / max	N	mean ± SD min / max	N	mean ± SD min / max
FD	9	217.10^{ab} ± 12.55 199.26 / 243.12	7	227.31^{b} ± 18.70 193.78 / 245.66	6	216.95^{ab} ± 16.32 198.24 / 228.29	5	197.45^{a} ± 14.11 182.16 / 224.02	27	213.7 ± 17.87 182.16 / 245.66
FW	10	769.93^{a} ± 311.58 489.89 / 1411.81	5	669.70^{a} ± 122.66 553.57 / 820.00	8	633.35^{a} ± 185.04 489.89 / 1020.65	8	720.93^{a} ± 163.65 530.63 / 914.59	31	705.9 ± 220.04 489.89 / 1411.81
MD	41	18.23^{a} ± 4.14 10.19 / 29.32	45	20.34^{a} ± 3.54 14.06 / 29.34	36	25.16^{b} ± 2.37 20.64 / 30.23	55	22.19^{b} ± 5.57 11.45 / 31.48	177	21.41 ± 4.83 10.19 / 31.48
CDMW	9	53.76^{a} ± 4.32 47.43 / 60.28	7	51.78^{a} ± 6.63 43.51 / 61.77	4	58.07^{a} ± 1.21 56.95 / 59.48	10	50.96^{a} ± 5.76 41.95 / 58.11	30	52.94 ± 5.50 41.95 / 61.58
PDMW	50	33.27^{a} ± 4.64 23.68 / 42.12	34	34.47^{a} ± 5.98 22.83 / 47.77	33	41.02^{a} ± 2.77 36.16 / 45.46	41	34.76^{a} ± 6.57 21.54 / 44.88	158	35.53 ± 5.93 21.34 / 47.77
ECD	14	41.65^{a} ± 10.97 27.34 / 58.34	3	64.42^{b} ± 4.55 59.17 / 67.30	6	44.24^{ab} ± 4.24 40.10 / 50.61	8	39.57^{a} ± 11.15 28.43 / 55.88	31	44.59 ± 11.61 27.34 / 67.30

Measurements are N, means (nm) ± S.D. and the range of measurements. Values with the same superscript letters are not significantly different (ANOVA, P<0.05). CDMW, width of the central doublet of microtubules; ECD, endonuclear canal diameter; FD, flagellum

diameter (perpendicular to the plane of the central microtubules); FW, fin width; MD, diameter of one microtubule; PDMW, width of the peripheral doublet of microtubules.

Table 4 Morphological comparisons of sperm cells in sturgeon species

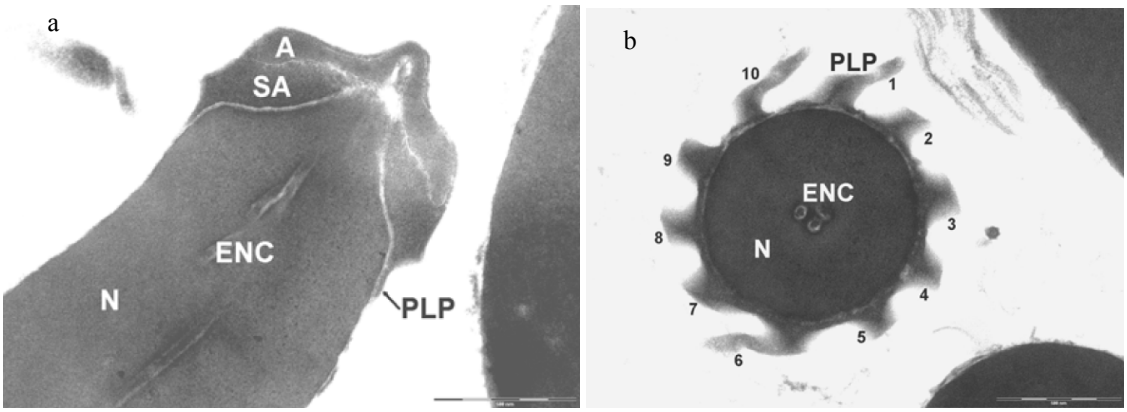
Species	AL	AW	NL	ANW	PNW	ML	MW	TL	FL	*n*	Authors
Stellate sturgeon	0.97	1.22	6.66	0.98	1.49	3.43	1.38	11.05	4.70	1	Ginsburg,1977
White sturgeon	1.31	1.34	9.21	1.25	1.44	2.13	1.08	11.82	30-40	1	Cherr and Clark 1985
Atlantic sturgeon	0.83(0.11)	1.00(0.07)	3.15(0.36)	0.92(0.06)	0.55(0.08)	1.37(0.16)	0.51(0.07)	5.66(0.37)	37.08	12	DiLauro et al., 1998
Shortnose sturgeon	0.78(0.08)	0.91(0.06)	6.99(0.83)	0.75(0.11)	1.21(0.12)	1.91(0.35)	0.81(0.09)	9.71(0.73)	36.70	15	DiLauro et al., 1999
Lake sturgeon	0.73(0.14)	0.81(0.07)	5.69(0.43)	0.68(0.07)	1.04(0.08)	2.68(0.43)	0.70(0.08)	9.10(0.53)	47.53	14	DiLauro et al., 2000
Pallid sturgeon	1.07(0.10)	0.82(0.06)	3.78(0.33)	0.68(0.04)	0.89(0.06)	1.23(0.16)	0.67(0.08)	6.07(0.48)	37.16	16	DiLauro et al., 2001
Siberian sturgeon	0.95(0.17)	0.93(0.12)	4.98(0.83)	0.87(0.13)	1.14(0.18)	1.09(0.43)	0.81(A), 0.57(P)	7.01(0.83)	44.75(4.93)	8	Present study

Data are shown as mean (μm) ± S.D. AHML, acrosome, head and midpiece length; AL, acrosome length; ANW, anterior nucleus width; AW, acrosome width; FL, flagellum length; ML, midpiece length; MW, midpiece width (A, anterior; P, posterior); NL, nuclear length; PNW, posterior nucleus width; TL, total length.

2.5. Flagellum

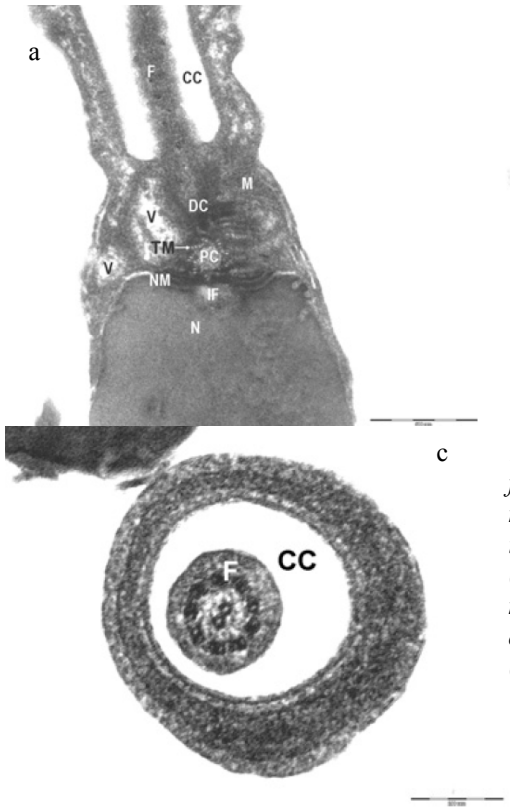
The mean flagellum length was 44.75±4.93 μm (Table 1), with the typical eukaryotic organization of one central pair of singlets and nine peripheral doublets of microtubules (Figure 6). The average diameter of one microtubule was 21.41±4.83 nm (Table 3). The mean widths of the peripheral pair and the central doublet were 35.53±5.93 and 52.94±5.50 nm respectively. The differences resulted from the structure of the attachments of the microtubular doublets or pair, because the two central singlets were separated tubules. The junctions between the peripheral doublets consisted of external and internal dynein arms, which represent the main elements of the propelling machinery. The pair of central microtubules was linked by bridges and they were encased in a central sheath. Nine radial spokes or joins linked the central and peripheral doublets (Figure 6b). The mean diameter of the flagellum was 213.66±17.87 nm when measured perpendicular to the plane of the doublet of central microtubules. Upon its distal exit from the end of the cytoplasmatic sheath of the midpiece to the terminal region of the flagellum, the flagellum gradually develops two independent lateral extensions of the flagellar plasma membrane or fins (the first fin appears prior to the second) (Figures 1b and 6a). These fins were parallel to the plane of the two central microtubules of the flagellum and extended distally up to 705.87±220.04 nm in the micrographs (Table 3). The fins were flat, indicating no rotation. The first and second fins started 0.7 and 5.3 μm post midpiece respectively, and continued along the flagellum. The first and second fins ended 3.4 and 5.1 μm from the end of the flagellum respectively (Figure 7).

Figure 4 Longitudinal sagittal section (a) and cross-section (b) at a region between the nucleus and acrosome with TEM



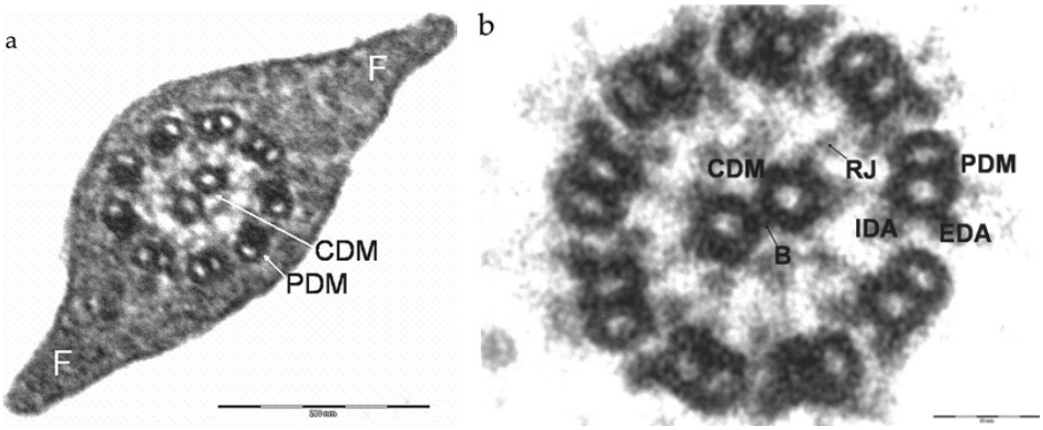
The cross-section shows the acrosome (A) and the subacrosome (SA), with the PLPs and the endonuclear canals (ENC) traversing the nucleus (N). Scale bar, 500 nm.

Figure 5 Longitudinal sagittal section (a) and cross-section (b , c) of the midpiece with TEM



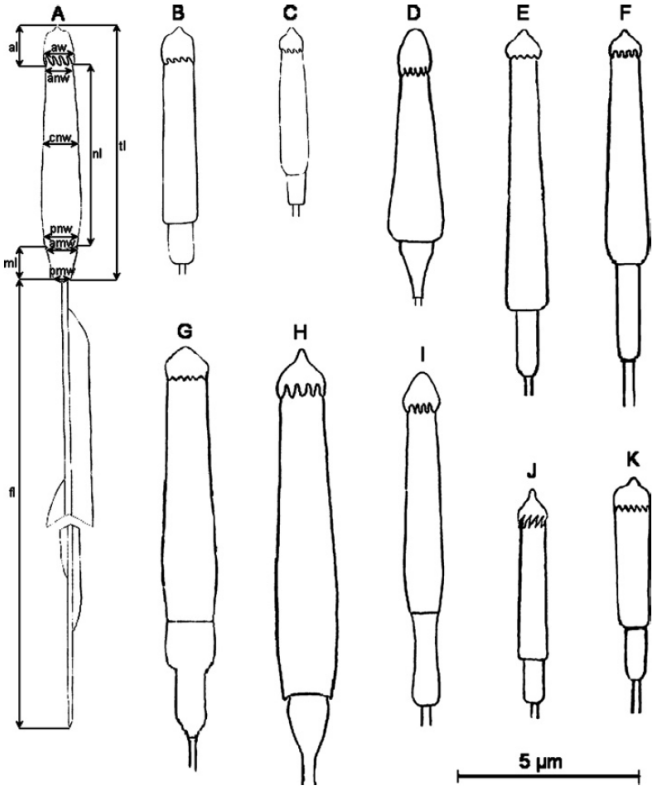
The distal (DC) and proximal (PC) centrioles in the implantation fossa (IF), with triplets of microtubules (TM) and the striated rootlets (SR), are indicated. Numerous mitochondria (M) were irregularly dispersed in the cytoplasm. Vesicles or lipid droplets (V) are present. The nucleus (N) is surrounded by the nuclear membrane (NM). The flagellum (F) is separated by the cytoplasmic channel (CC). Scale bars are 500 nm (a) and 200 nm (b, c).

Figure 6 Cross-section of the flagellum (a) and axoneme (b) with TEM



*The peripheral doublets of microtubules (PDM) and central doublets of microtubules (CDM) with radial joins (RJ) are observed. The CDM joins with a bridge (B). The propelling machinery is the internal (IDA) and external (EDA) dynein arms. The fins (F) make the flagellum more effective. Scale bars are 200 nm (**a**) and 50 nm (**b**).*

Figure 7 Drawings, to scale, of the various described sperm cells of several different sturgeon species



(**A**) *A. baerii* (present study), (**B**) *Polyodon spathula* (unpublished data), (**C**) *A. ruthenus* (unpublished data), (**D**) *A. sinensis* (Xu and Xiong, 1988), (**E**) *A. brevirostrum* (DiLauro et al., 1999), (**F**) *A. fulvescens* (DiLauro et al., 2000), (**G**) *A. stellatus* (Ginsburg, 1977), (**H**) *A. transmontanus* (Cherr and Clark, 1984; 1985), (**I**) *A. gueldenstaedti colchicus* (Ginsburg, 1968), (**J**) *Scaphirhynchus albus* (DiLauro et al., 2001), (**K**) *A. oxyrchinchus* (DiLauro et al., 1998).

3. Discussion

Understanding the morphology and ultrastructure of sperm cells is a prerequisite to evaluating and establishing methods for analysing sperm motility, fertilizing ability and cryopreservation conditions. In addition, the data are valuable to ichthyologists for determining taxonomic and phylogenetic relationships at either inter- or intra-specific levels.

3.1. General morphological characteristics

Fish spermatozoa are widely divergent in both morphology and ultrastructure (Ginsburg, 1968; Jamieson, 1991). The ultrastructure of sturgeon sperm shares some properties with that of sperm of modern fish (DiLauro et al., 1998), although sturgeons are considered and classified as primitive fishes (Bemis, 1997a, 1997b). The present study showed that the sperm cell differentiated into a head with an acrosome, a midpiece and a flagellar region with 9+2 structure of microtubules. The head morphology of Siberian sturgeon sperm is elongated, cylindrical and radially symmetrical, as found in other sturgeon species (Cherr and Clark, 1984; DiLauro et al., 1998, 1999, 2000, 2001; Ginsburg, 1968, 1977), and the tail is differentiated with a narrow part at the end (Figure 1). These results support the suggestion of many researchers (Baccetti et al., 1984; DiLauro et al., 2001; Jamieson, 1991) that spermatozoan ultrastructure is of taxonomic use and that the spermatozoa of sturgeon species are primitive in their general organization, because the cells have an almost radial symmetry, are discharged directly into the water and their duration of motility is long (Anderson and Personne, 1975; Baccetti, 1986). Comparisons of morphological parameters in different species of sturgeon (Table 4 and Figure 7) reveal interspecies variation.

3.2. Acrosome

Spermatozoa of most teleost fishes are devoid of an acrosome (Afzelius, 1978, Linhart et al., 1991; Lahnsteiner et al., 1995; Psenicka et al., 2006); the lack of an acrosome in teleosts is compensated for by the presence of a micropyle in the egg which can be penetrated by the spermatozoon (Ginsburg, 1968; Kudo et al., 1994; Lahnsteiner and Patzner, 1999; Linhart et al., 1995; Riehl and Patzner, 1998; Yamamoto, 1960).

Interestingly, sturgeons and paddlefish have an acrosome, whereas their corresponding eggs possess several micropyles (Debus et al., 2002; Linhart and Kudo, 1997; Markov, 1975; Podushka 1993a, 1993b). The acrosome, possibly functioning to improve penetration into the egg during fertilization, is considered to be derived from the Golgi apparatus and commonly cap-shaped (Nicander, 1970). The present study showed that the acrosomal dimensions of the Siberian sturgeon sperm are generally similar to those found in other sturgeon species, with differences in length and width (Table 4), reflecting an inter-specific diversity in size of the acrosome. In all described sturgeon sperm cells, the acrosome is terminated by PLPs (posterolateral projections) that are lobes radially distributed (DiLauro et al., 1998, 1999, 2000, 2001). Numbers and sizes of PLPs vary between species. Ten PLPs are present in Siberian sturgeon, which differs from pallid sturgeon which have eight PLPs (DiLauro et al., 2001), but there are no data for other species. The lengths of the PLPs in the lake sturgeon (*Acipenser fulvescens* Rafinesque, 1817), in the Atlantic (*Acipenser oxyrinchus* Mitchill, 1815), in the shortnose (*Acipenser brevirostrum* Lesueur, 1818), and the pallid sturgeon species have been reported as 324, 233, 246 and 760 nm respectively. The present study showed that the PLPs of Siberian sturgeon (600 nm) have a size similar to those of the pallid sturgeon.

Therefore, there is high inter-specific variation between the sizes of PLPs. The function of PLPs is not well understood, so more studies are needed to determine the role of PLP structure and size during fertilization. Could the length of PLPs play a role in sperm fertility? In addition, are there any biochemical interactions that occur between sperm and eggs when PLPs adhere to the egg-jelly surface? DiLauro et al. (2000) mentioned briefly that the longer PLP may be an adaptation which provides a more secure anchorage to the egg in fast-flowing water during spawning and fertilization.

3.3. Nucleus

The sperm nucleus is an elongated trapezoid, but slight differences in the shapes and sizes of sturgeon sperm nuclei are noticeable (Table 4). Differences between the posterior and anterior nucleus widths vary among species, possibly reflecting differences in their components. Inter-specific diversity in size and shape of sturgeon sperm cell nuclei has evolutionary and genetic implications (DiLauro et al., 1999). Increase of the size of the nucleus allows the sperm cells to have loosely packed chromatin and nucleic acids. This feature has been reported in Atlantic sturgeon, the species with the smallest sperm nucleus (DiLauro et al., 1998, 2001). Previously, Baccetti and Afzelius (1976) reported a common variation in the relative density of chromatin and nucleic acids between species. The three endonuclear canals found in the present study have been reported by Ginsburg (1977), Cherr and Clark (1984), DiLauro et al. (2000, 2001) in stellate (*Acipenser stellatus* Pallas, 1771), white (*Acipenser transmontanus* Richardson, 1836), lake and pallid sturgeons respectively. Atlantic sturgeon has only two endonuclear canals (DiLauro et al., 1998). The membrane-lined endonuclear canals traversed the nucleus from the acrosomal end to the basal nuclear fossa region. Nevertheless, there are differences in the diameters of canals between sturgeon species, with values of 35, 97, 49, 57.41 and 44.59 nm in Atlantic, shortnose, lake, pallid and Siberian sturgeons respectively. The role of the endonuclear canals is not well understood. They may assist in transferring the centriole into the egg after sperm–egg interaction during fertilization (DiLauro et al., 1999). Cherr and Clark (1984) and Jamieson (1991) suggested that the material in the nuclear canals plays a perforatorial role during penetration of the sperm into the egg. There is an implantation fossa at the posterior end of the nucleus in Siberian sturgeon, as observed in other sturgeon species (Cherr and Clark, 1984; DiLauro et al., 1998, 1999, 2000, 2001; Ginsburg, 1977). It seems that its function is to connect the endonuclear canals with the centrioles of the midpiece.

3.4. Midpiece

The midpiece of sturgeon sperm cells contains the mitochondria, the proximal and distal centrioles, and the ovoidal vacuoles (Ginsburg, 1968; Cherr and Clark, 1984; DiLauro et al., 1998, 1999, 2000, 2001). The number of mitochondrial components may differ between sturgeon species (DiLauro et al., 1999), as in teleosts (Linhart et al., 1991); they are the source for ATP generation during sperm motility (Billard et al., 1995, 2000; Tsvetkova et al., 1996). Changes of intracellular ATP after activation of sperm motility in Siberian sturgeon have been studied both in fresh and cryopreserved sperm (Billard et al., 2000; Tsvetkova et al., 1996). In spite of evidence of a decrease in ATP level after initiation of sperm motility, there is no information about ultrastructural changes of the mitochondrial components after activation of sperm either in fresh or cryopreserved sperm. To determine developmental changes of structure and behaviour of sperm in *Acipenseriformes*, the study and comparison of the number of mitochondria, their physiological functions during sperm motility and ATP generation, and the effects of storage procedures, both short-term and long-term

(cryopreservation), between species is required. In all sturgeon species (Cherr and Clark, 1984; DiLauro et al., 1998, 1999, 2000, 2001 Ginsburg, 1977; and the present study) both proximal and distal centrioles have been observed in the midpiece. These centrioles differ in size within and between species. In general and common to all sturgeon species, the proximal centriole is apparently attached to the nucleus by several thin strands in the centre of the nuclear fossa, called the fibrous body.
A similar fibrous body is detected in Atlantic (DiLauro et al., 1998) and Siberian (present study) sturgeon. This structure appears to connect the nuclear fossa with the proximal centriole.
The distal centriole is located adjacent to the proximal centriole and posterior to the distal centriole is the axoneme. The electron-dense striated rootlet structures (Figure 3) are considered to be stabilization structures, as microfilaments are inserted there, connecting the centrioles with each other or with the nucleus. Similar electron-dense structures have been reported in *Sparinae* and *Pagrinae*, whose basal foot and the rootlet are located laterally and opposite to each other at the distal centriole (Gwo et al., 1993; Gwo, 1995). In *Boopsinae* (Assem, 2003) and *Diploinae* (Taddei et al., 2001), this structure has not been observed, but an approximately square plate of electron-dense material is found adjacent to the side of the proximal centriole facing the distal centriole. Vacuoles, composed of lipid droplets, are often observed in the midpiece region of the sperm cells in various fish species (Afzelius, 1978), and have been detected in sturgeon (DiLauro et al., 1999), but not in the lake sturgeon (DiLauro et al., 2000).

3.5. Flagellum

The flagellum is separated from the midpiece by the cytoplasmic channel, as in other fish spermatozoa. The axoneme emanates from the posterior end of the distal centriole in the classical 9+2 structure of microtubules (DeRobertis and DeRobertis, 1980; Threadgold, 1976), giving rise to the flagellum in all described sturgeon sperm cells.
The flagella of Siberian (present study), shortnose (DiLauro et al., 1999), stellate (Ginsburg, 1977), white (Cherr and Clark, 1984) and Atlantic (DiLauro et al., 1998) sturgeons gradually assume a distal paddle- or fin-like shape upon adaptation for locomotion during external fertilization.

One possible role of these fin-like flagella could be to contribute to the efficiency of the thrust generated by waves, by increasing the flagellar surface used for friction on the surrounding medium. A second role could also be to contribute to the large increase in the membrane surface, due to the presence of such fins, compared with that of a simple cylindrical axoneme.

It is estimated that the surface area of the flagellum, whose shape is that of a simple cylinder (34×10^6 nm^2), is approx. one quarter of that of the same cylinder comprising fins (160×10^6 nm^2). This has large implications for water exchange/osmotic regulation at activation. At initiation of movement of fish spermatozoa in fresh water species, such as sturgeon, the first signal perceived by the membrane is osmotic; it is followed by a water flux in either direction which provokes local membrane distortions due to osmotic constraints. As the sperm flagellar membrane in fishes has these unusual fin-shaped folds (Cosson et al., 2000; present study), these significantly increase the membrane surface area, thus contributing to an apparent membrane excess favouring water exchange, but also can be easily distorted, eventually leading to blebs on exposure to extreme osmotic situations (Perchec et al., 1996; Cosson et al., 2000).

4. Conclusions

In fish sperm cell, structure is related to function through adaptations (Anderson and Personne, 1975; Baccetti and Afzelius, 1976). Although sturgeons are considered primitive chondrostean fishes (living fossils), the sturgeon sperm cells share some ultrastructural properties with those of modern fishes and mammals (Jamieson, 1999). The present study showed high individual variations in morphological parameters. In addition, comparison of the present results with the current literature on other sturgeon species reveals clear differences which could be used to determine phylogenetic and taxonomic relationships using discriminant factor analysis or PCA in further studies, as carried out in teleosts (Jamieson, 1999).

5. Material and methods

The study was carried out in the Department of Fish Genetics and Breeding, Research Institute of Fish Culture and Hydrobiology, University of South Bohemia, Vodnany, Czech Republic.

Semen samples were collected from broodfish reared at the fish farm of the Research Institute of Fish Culture and Hydrobiology. Electron microscopic studies were performed at the Laboratory of Electron Microscopy, Institute of Parasitology, Academy of Sciences of the Czech Republic, Ceske Budejovice, Czech Republic.

5.1. Broodfish and gamete collection

Eight individual diploid males, 5–7 years of age and averaging 3–6 kg, were transferred from a pond into a 4000 litre broodfish tank with recirculated water (0.2l s^{-1}) in the hatchery, 5 days before spawning induction in March 2004. Water temperature in the tank was stabilized at 13°C. Spermiation was induced by a single intramuscular injection of carp pituitary homogenized extract at a dose of 4 mg·(kg of body weight)$^{-1}$. Sperm was collected 48 h after hormonal injection by catheter from the urogenital papilla of each male, transferred into a separate cell culture container (250 ml) and stored at 4°C until the samples were fixed.

5.2. Method of sperm fixation

The samples were diluted in a solution containing 50 mM sucrose, 20 mM Tris/HCl, pH 7.6, with an osmolality of 70 mOsmol·kg^{-1}, using an Eppendorf micropipette. The dilution ratios were 1:50 and 1:20 for SEM and TEM respectively. Then the sperm were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 days at 4°C, and post-fixed and washed repeatedly for 2 h at 4°C in 4% osmium tetroxide and dehydrated through an acetone series (30, 50, 70, 90, 95 and 100% acetone for 15 min). Samples for SEM were dehydrated with the Pelco CPD 2 critical point dryer. The samples were coated with gold under vacuum with an SEM coating unit E5100 (Polaron Equipment) and studied using a JSM 6300 scanning electron microscope (JEOL) for morphological parameters and JSM 7401 S (JEOL) in cryoregime for finer details. Samples for TEM were embedded in resin (Polybed 812). A series of ultra-thin sections were cut using a UCT ultramicrotome (Leica), double-stained with uranyl acetate and lead citrate, and observed with a 1010 transmission electron microscope (JEOL) operated at 80 kV. To measure spermatozoa morphological parameters, micrographs were evaluated using Olympus MicroImage software (version 4.0.1 for Windows).

5.3. Data analysis

Means±S.D. values were calculated for each male. Statistical significance was assessed by ANOVA after testing normality by the Kolmogorov–Smirnov test followed by the Tukey HSD (honestly significantly different) test for comparisons ($P<0.05$ was considered as significant). Multiple variation and PCA were used to define the most important parameters, which could be used as key factors for individual variations using Statistic 6.

6. Acknowledgements

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Chapter 3

Fine structure and morphology of sterlet (*Acipenser ruthenus* L. 1758) spermatozoa and acrosin localization

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Fine structure and morphology of sterlet (*Acipenser ruthenus* L. 1758) spermatozoa and acrosin localization

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Abstract

Ultrastructure of sterlet *Acipenser ruthenus* L. 1758 sperm was examined by scanning and transmission electron microscopy, which allowed us to use various methods for visualizations of different parts of sterlet spermatozoa. Sperm cells possess a head with a distinct acrosome, a midpiece and a single flagellum surrounded by the flagellar plasma membrane. The average length of the head including the acrosome and the midpiece was estimated as 5.14 ± 0.42 μm. Nine to 10 posterolateral projections were derived from the acrosome. Three inter-twining endonuclear canals bounded by membranes traversed the nucleus in its whole length from the acrosome to the implantation fossa. Acrosin was located in all the three parts (acrosome, endonuclear canals and implantation fossa). The proximal and distal centrioles located in the midpiece compacted of nine peripheral triplets of microtubules. One cut of the midpiece contained from two to six mitochondria with area of 215 ± 85 nm^2 in average. The flagellum was 42.47 ± 1.89 μm in length with typical eukaryotic organization of one central pair and nine peripheral pairs of microtubules. It passed through a cytoplasmic channel in the midpiece, which was formed by an invagination at the plasmalemma. The flagellum gradually developed two lateral extensions of its plasma membrane, so-called "fins". Detected morphological variation can be described by four principal component axes corresponding to groups of individual morphometric characters defined on the sperm structures. Correlations among the characters indicate that the sperms are variable in their shape rather than size. Significant variation among examined fish individuals was found only in flagellum and nucleus length. Comparison between the present and previous studies of morphology of sturgeon spermatozoa confirmed large inter- and/or intra-specific differences that could be of substantial taxonomic value.

Keywords: Acipenser ruthenus; Acrosome; Acrosin; Morphometric analysis; SEM; TEM

1. Introduction

Spermatozoa of sturgeon fishes show characteristic differences compared to those of teleost fishes (Jamieson, 1991) in terms of morphology, presence of acrosome (Cherr and Clark, 1984), (Psenicka et al., 2007) and (Wei et al., 2007), sperm behavior (Cosson et al., 2000) and biochemistry with the presence of acrosin- and trypsin-like activities (Ciereszko et al., 1994) and (Ciereszko et al., 1996). Comparative spermatology could provide fish

biologists and aquaculturists with information on fish phylogeny and fish sperm quality evaluation. In this regard, morphology and fine structure are considered to be the major sources of information in comparative spermatology (Baccetti, 1986), (Jamieson, 1991), (Jamieson, 1999) and (Lahnsteiner and Patzner, 2007). The present set of results creates the first study about the fine structure and morphology of sterlet spermatozoa.
The defining structure of the sturgeon sperm cell constituents composed of three major parts; (a) an elongated head, (b) a cylindrical midpiece and (c) a flagellum (see Dettlaff et al., 1993 for general information). In the head, the nucleus occupies the major part and usually contains three endonuclear canals (ECs) leading from the implantation fossa to the acrosome. The acrosome often forms several posterolateral projections (PLPs) which have been also observed in pallid (DiLauro et al., 2001), Siberian (Psenicka et al., 2007) and in Chinese sturgeon (Wei et al., 2007). Mitochondria, implantation fossa and centriolar complex are located in the midpiece. The flagellum consists of nine peripheral double and two central single microtubules. The plasma membrane often forms lateral fin along the flagellum of sperm of sturgeons such as in pallid (DiLauro et al., 2001), Siberian (Psenicka et al., 2007) and Chinese sturgeon (Wei et al., 2007). These structures are preferentially oriented along the horizontal plane of central microtubules (Billard, 1970) and it was suggested that they could contribute to the efficiency increase of propelling during wave propagation (Cosson et al., 2000).
The principal aim of this study was to describe the fine structure and morphology of sterlet spermatozoa using transmission electron microscopy (TEM) and scanning electron microscopy (SEM).
Examination of multiple sperm specimens allowed us to perform not only a simple description but also a morphometric analysis of characters defined on sperm structures. Consequently, we present a detailed mathematical description of variation in individual traits as well as relationships among them.

2. Materials and methods

2.1. Samples preparation

Sperm of three males were fixed with 2.5% glutaraldehyde for 2 days at 4 °C. Samples for TEM were postfixed in osmium tetroxide for 2 h, at 4 °C, washed, dehydrated through an acetone series and embedded in resin (Polybed 812; Polysciences, Inc.). Series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Microsystems), counterstained with uranyl acetate and lead citrate and examined in a TEM JEOL 1010 operated at 80 kV. Sperm samples intended for TEM visualization of mitochondria were briefly fixed in glutaraldehyde and incubated at room temperature (RT) for 15 min in the medium prepared by mixing of 0.2 M Na_2HPO_3 (6.5 ml), 0.2 M succinic acid (0.2 ml), 0.2 M potassium sodium tartrate (5 ml), distilled water (4 ml), polyvinylpyrrolidone (PVP; 1.5 g), 0.1 M $CuSO_4$ (1 ml) and 0.02 M potassium ferricyanide (1.5 ml). After the incubation, sperm samples were washed in 20 ml of the solution containing 0.2 M NaH_2PO_4, 2.5 M Na_2HPO_4 and PVP (1.5 g) and finally processed for TEM as mentioned above. The sperm axonemes were harvested using centrifugation at 200 × *g* and filtration, then rinsed in 0.1 M Na-phosphate buffer (pH 7.0) containing 8% tannic acid and finally fixed in glutaraldehyde (1–3%; pH 7.2). Within 6 h, fixative solution was replaced with a fresh solution and processed for TEM as described.
The samples meant for acrosin localization in sperm were fixed with 4% formaldehyde at RT for 2 h. Fixed cells were washed in PBS/0.02 M glycine, embedded in 10% gelatin (Serva) at 37 °C. Samples were infused with 2.3 M sucrose at 4 °C overnight and frozen by plunging into liquid nitrogen. The cryosections were obtained using a microtome Ultracut UCT with

FCS cryochamber (Leica Microsystems). The sections were picked up in a solution containing 1.05 M sucrose and 1% methylcellulose (25 cP; Sigma) and then transferred onto formwar/carbon-coated nickel grids. Thawed cryosections were blocked in solution containing 1% fish skin gelatin (FSG, Sigma), 10% goat serum and 0.05% Tween 20 in PBS and then labeled with mouse anti-acrosin antibody ACR 2 (Peknicova and Moos, 1990) and (Peknicova et al., 1994). The antibody was detected with goat anti-mouse IgG conjugated with 10 nm particles (Aurion). Finally, grids with cryosections were contrasted and dried in a mixture of 2% methylcellulose and 3% aqueous uranyl acetate according Tokuyasu (1980).
Samples for conventional SEM were fixed, dehydrated in a graded concentration of acetone and dried using the critical point dryer CPD 2 (Pelco TM). The samples were coated with gold under vacuum using SEM Coating Unit E5100 (Polaron Equipment Ltd.) and examined with SEM JEOL 6300, equipped with Sony CCD camera for morphological parameters determinations.
The samples for observation in a cryo field-emission SEM JSM 7401-F (JEOL Ltd., Tokyo, Japan) were frozen by plunging into liquid nitrogen and immediately transferred into cryochamber of the microscope (CryoALTO 2500, Gatan). The samples were fractured at −140 °C, freeze etched at −90 °C for 10 min and finally coated by platinum/palladium at −140 °C for 2 min.

2.2. Statistical analysis

Mean values of the measured parameters and standard deviation (S.D.) were calculated per male and summarized in a table. Variance components were calculated using the expected mean square (EMS; Quinn and Keough, 2002) estimation for all morphological characters together as well as for each character separately in order to quantify the distribution of variance on levels within-fish individuals and among them. Relationships among the characters were examined by a partial principal component analysis (PCA; Leps and Smilauer, 2003) in which categorical covariates were employed to filter out variation corresponding to differences among fish individuals. Furthermore, partial Pearson correlation coefficients (only within-fish variation was taken into account) were calculated to quantify these relationships precisely.

Multivariate statistical analyses were performed using Canoco for Windows, Version 4.52 (ter Braak and Smilauer, 2002). Statistica for Windows, Version 7.1 (StatSoft, 2006) was employed to calculate variance components, partial correlations and descriptive statistics.

3. Results

3.1. General morphology

Our results by SEM clarified that a spermatozoan of sterlet is uniflagellated and is differentiated into a head with acrosome, a midpiece and a tail region (Fig. 1a–c). The main body of the sperm cell is long, cylindrical, and in general radially symmetrical. Detailed descriptions of the individual sperm structures are presented in the following paragraphs. Mean values and standard deviations of individual morphological characters are summarized in Table 1. Within-fish individual variation accounted for 3.89% of total variability in all morphometric parameters. However, the distribution of variance markedly differed across the characters (Table 2). Our calculations revealed insignificant among-fish variance component in the major part of the variables except for nucleus length (NL) and flagellum length (FL). The first four axes of the partial principal component analysis (PCA) explained 66.8% of total variance. Several groups of inter-correlated morphometric variables can be distinguished on

the ordination plots (Fig. 2a and b). The first group comprises sperm head parameters CNW and PNW, the second group characters AMW, AW and PMW related to midpiece and acrosome width. AN and ANW form a third group positioned approximately between the first two. The remaining characters (ML, FL and NL) appear independent of these groups and correspond to the third and fourth principal components, respectively (Fig. 2b). The observed relationships among the characters are furthermore confirmed and quantified by the table of correlations (Table 3).

3.2. Acrosome and PLPs

Mean acrosomal length and width were 0.79 ± 0.07 μm and 0.75 ± 0.05 μm, respectively (Table 1). Longitudinal, sagittal (Fig. 3a) and cross-section (Fig. 3b) showed a subacrosome region between nucleus and the acrosome and 9–10 PLPs are present. The presence of immunolabeled acrosin specifically in the acrosome region was visualized using TEM (Fig. 3c).

3.3. Nucleus

The nucleus was an elongated trapezoid, tapering from the posterior end to the anterior end of the head (Fig. 1b). The head with midpiece and acrosome had a length of 5.14 ± 0.42 μm, increasing in mean width from 0.67 ± 0.07 μm close to the acrosome border (anterior) to 0.85 ± 0.08 μm at the midpiece border (posterior) while its diameter in median region was 0.82 ± 0.04 μm (Table 1). It is composed of electron dense and slightly granular materials surrounded by a nuclear membrane. The shape of anterior nucleus after release is shown in Fig. 4a, where three ECs (40.13 ± 4.61 nm in diameter) (Fig. 3a–c) are clearly visible. Positions of implantation fossa and ECs are shown in Fig. 4b. Taken together, these observations show that three ECs bounded by membranes traverse the nucleus from the acrosomal to the nuclear fossa region. The connection between acrosome and nuclear fossa supports the presence of acrosin, which is found in both acrosome and nuclear fossa by means of immunolabeling method (see Fig. 3 and Fig. 4, respectively). An implantation fossa is present at the posterior end of the nucleus, connecting the axial portion of the nucleus with the midpiece (Fig. 4 and Fig. 5). In addition Fig. 5b shows the proximal centriole, which was found under implantation fossa (IF), after release of nucleus including IF.

3.4. Midpiece

The mean length of midpiece was 0.97 ± 0.23 μm and had approximately a cylindrical shape with elongated part at its caudal base (Fig. 1a and b). Two to six mitochondria, with area in one cut 215 ± 85 nm in average, containing several cristae, were observed in peripheral section of the midpiece and sometimes around the nucleus. In addition to mitochondria, several organelles were also located in the midpiece. Two ovoidal vacuoles or lipid droplets were observed in the midpiece, but it may be an artifact. An ovoid proximal centriole ($163.82 \pm 12.67 \times 173.36 \pm 16.76$ μm) was located at the “implantation fossa”. A distal centriole was observed near to flagellar base playing the role of a basal body for the flagellum (Fig. 4). Both centrioles were formed of nine peripheral triplets of microtubules. Axoneme was immediately linked to the posterior of the distal centriole. A cytoplasmic channel was observed around the flagellum, which was formed by an invagination of the plasmalemma (Fig. 4 and Fig. 5). Extracellular space was found between the cytoplasmic sheath and the flagellum (Fig. 5a).

3.5. Flagellum

The mean flagellum length was 42.47 ± 1.89 μm (Table 1) with typical eukaryotic organization: two central single microtubules and nine peripheral doublets of microtubules (Fig. 6a–c). Average diameter of one central microtubule was 16.40 ± 2.84 nm. Mean widths of peripheral doublets of microtubules and the central pair of microtubules (long axis of doublets or pairs) were 31.75 ± 3.74 and 45.41 ± 6.14 nm, respectively. The junctions between two adjacent peripheral doublets consist of outer and inner dynein arms (Fig. 6c). The central doublets of microtubules were linked with bridges (Fig. 6c). Nine radial spokes or joins linked the central structure with peripheral doublets. The diameter of the flagellum was 151.39 ± 20.06 nm when measured orthogonal to the plane of the two central microtubules. The flagellum gradually developed two lateral extensions of the flagellar plasma membrane, so-called "fins", which were flat without twist and were located parallel to the plane of the two central microtubules of the flagellum; they extended distally up to 568.46 ± 170.08 μm in length as measured in micrographs. The first and second fins initiated 0.37 ± 0.21 and 5.37 ± 2.10 μm distally from the midpiece, respectively, then were all present along the flagellum and ended at 7.24 ± 3.99 and 10.67 ± 3.99 μm from the end of flagellum, respectively.

4. Discussion

4.1. General morphology

Similarly to other sturgeon species (Cherr and Clark, 1984; DiLauro et al., 1998, 1999, 2000, 2001; Ginsburg, 1968, 1977; Psenicka et al., 2007; Wei et al., 2007), sterlet spermatozoan can be differentiated into a head with an acrosome, a midpiece and a flagellar region with 9 + 2 microtubular structure. The head is elongated, cylindrical and radially symmetrical. Comparison of results of the present study with other sturgeon species confirms that spermatozoan ultrastructure is of taxonomic use.

Independency or negative dependency observed among morphometric characters demonstrates that examined sperms and their structures were variable primarily in their shape (if shape is defined as a feature of an object independent of its size and orientation; see e.g. Zelditsch et al., 2004) rather than size. This applies especially for the midpiece the length of which (ML) was rather negatively correlated with its width (significantly with AMW, insignificantly with PMW) but also for the nucleus displaying similar negative (although insignificant) relationship between its width (ANW, CNW and PNW) and length (NL). Magnitude and significance of NL and FL variation components among fish individuals indicates that these parameters can have a distinctly higher biological importance in comparison with other morphological characters. This study confirms our recently published results on Siberian sturgeon sperm (Psenicka et al., 2007); showing FL and NL explains individual variations.

Table 1. Statistical data on ultrastructure of sterlet sperm

Fish No.	male 1		male 2		male 3		Total	
dimension (µm)	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
Total lenght	30	5.03^{a} ± 0.50	24	5.34^{b} ± 0.26	23	5.09^{ab} ± 0.40	77	5.14 ± 0.42
nucleus length	30	3.28^{ab} ± 0.33	24	3.44^{b} ± 0.22	23	3.18^{a} ± 0.33	77	3.30 ± 0.31
acrosom length	30	0.78a ± 0.07	24	0.79^{a} ± 0.06	23	0.79^{a} ± 0.07	77	0.79 ± 0.07
acrosom width	30	0.74^{a} ± 0.06	24	0.76^{a} ± 0.05	23	0.75^{a} ± 0.06	77	0.75 ± 0.05
anterior nucleus width	30	0.66^{a} ± 0.04	24	0.69^{a} ± 0.08	23	0.69^{a} ± 0.09	77	0.67 ± 0.07
central nucleus width	30	0.82^{a} ± 0.03	24	0.82^{a} ± 0.05	23	0.84^{a} ± 0.03	77	0.82 ± 0.04
posterior nucleus width	30	0.85^{a} ± 0.07	24	0.85^{a} ± 0.09	23	0.87^{a} 0.09	77	0.85 ± 0.08
midpiece length	30	0.93^{a} ± 0.22	24	0.98^{a} ± 0.22	23	1.01^{a} ± 0.25	77	0.97 ± 0.23
anterior midpiece width	30	0.63^{a} ± 0.12	24	0.66^{a} ± 0.12	23	0.62^{a} ± 0.12	77	0.64 ± 0.12
posterior midpiece width	30	0.48^{a} ± 0.10	24	0.47^{a} ± 0.12	23	0.49^{a} ± 0.12	77	0.48 ± 0.11
mitochondrial area (nm^2)	26	215^{a} ± 90	20	232^{a} ± 92	21	200^{a} ± 72	67	215 ± 85
flagellum length	30	42.63^{a} ± 1.97	24	41.43^{a} ± 1.68	23	43.63^{b} ± 1.27	77	42.47 ± 1.89

Data are shown as number of measurements (*n*), mean, standard deviation (S.D.) and range (min./max.). Values with the same letters are not significantly different (ANOVA, $P < 0.05$).

Fig. 1. SEM showing sperm cell morphology, with arrows pointing to the origin and the end of the fins along the flagellum.
Acrosome (A), nucleus (N), midpiece (MP) and flagellum (F). (1a) SEM (JSM 6300) scale bar 10 µm, (1b) SEM (JSM 6300) scale bar 5 µm and (1c) (JSM 7401 S) scale bar 100 nm.

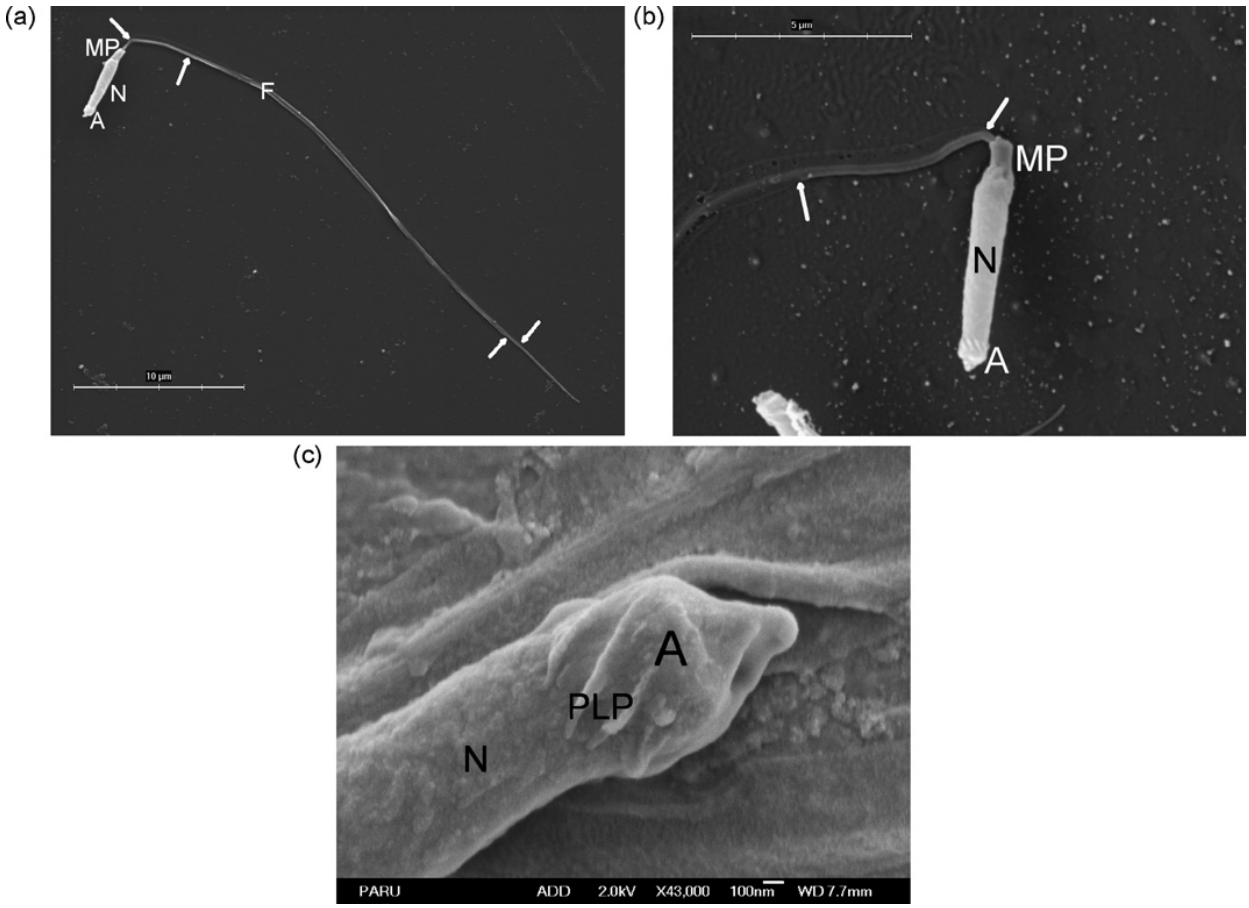


Table 2. Variance components of individual characters corresponding to the among- and within-fish individual levels

Morphological character	% of variation among individuals	% of variation within individuals	Significance of among individual variance component
NL	**11.60%**	**88.40%**	**P < 0.05**
AL	0.00%	100.00%	n.s.
AW	0.00%	100.00%	n.s.
ANW	3.12%	96.88%	n.s.
CNW	4.74%	95.26%	n.s.
PNW	0.00%	100.00%	n.s.
ML	0.00%	100.00%	n.s.
AMW	0.00%	100.00%	n.s.
PMW	0.00%	100.00%	n.s.
FL	**25.80%**	**74.20%**	**P < 0.001**

The variance components are based on a random effect extraction from a linear mixed effect model using an expected mean square estimation. Significance of the among individual variance component is also displayed.

Table 3. Table displaying Pearson correlation coefficients between pairs of morphological characters

AL	0.02								
AW	0.15	0.21							
ANW	-0.09	**0.26**	**0.37**						
CNW	-0.11	**0.33**	0.03	**0.39**					
PNW	-0.03	0.11	-0.05	**0.40**	**0.39**				
ML	0.01	0.18	-0.11	-0.20	0.05	**-0.25**			
AMW	-0.03	0.11	**0.33**	0.21	0.01	-0.01	**-0.31**		
PMW	0.06	**0.44**	**0.43**	**0.45**	0.06	0.10	-0.02	**0.34**	
FL	0.14	-0.14	-0.04	-0.16	-0.07	-0.01	0.05	-0.19	-0.20

Statistically significant correlations ($P < 0.05$) are displayed in bold.

Fig. 2. Partial PCA plot depicting correlations among individual morphological characters describing sperm morphology of *A. baerii*. The first and second ordination axes are displayed on the first diagram accounting for 25.1% and 14.8% of total variation in data. The third and fourth principal components are displayed on the second diagram accounting for 13.4% and 10.1% of variation.

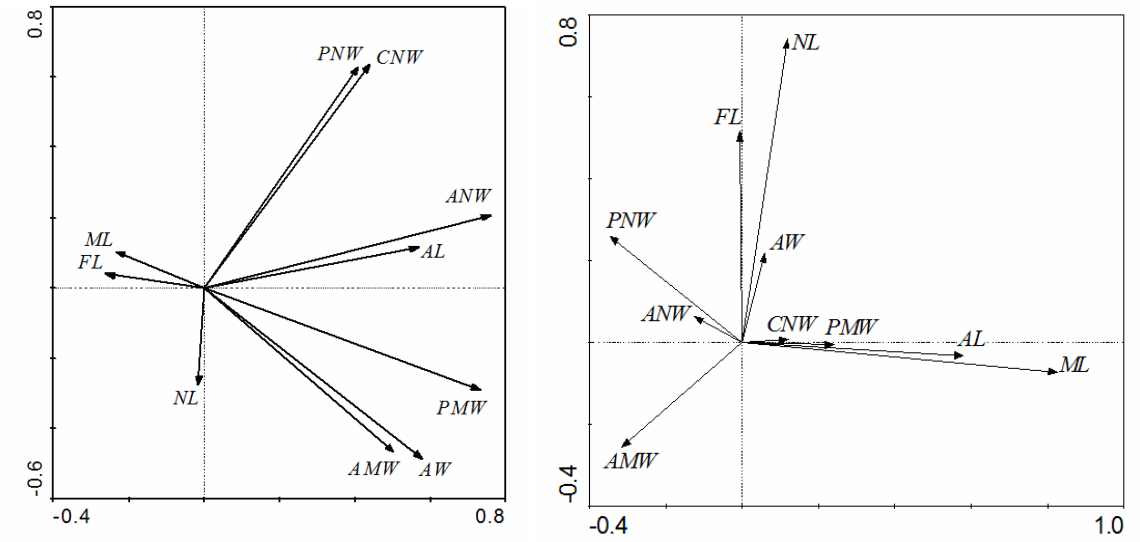


Fig. 3. (a) Longitudinal sagittal section showing acrosome (A) and subacrosomal region (SA) with the posterolateral projections (PLP) using transmission electron micrographs. The endonuclear canals (EC) traverse the nucleus (N) (scale bar 0.2 µm). (b) Cross-section at a region between nucleus (N) and acrosome shows nine posterolateral projections and endonuclear canals (arrow). (c) Immunolabeling of this longitudinal sagittal section shows localization of acrosin in the acrosome region (A) and in the endonuclear canals (EC) by means of gold particles, but no labeling was found in nucleus (N) (scale bar 0.2 µm).

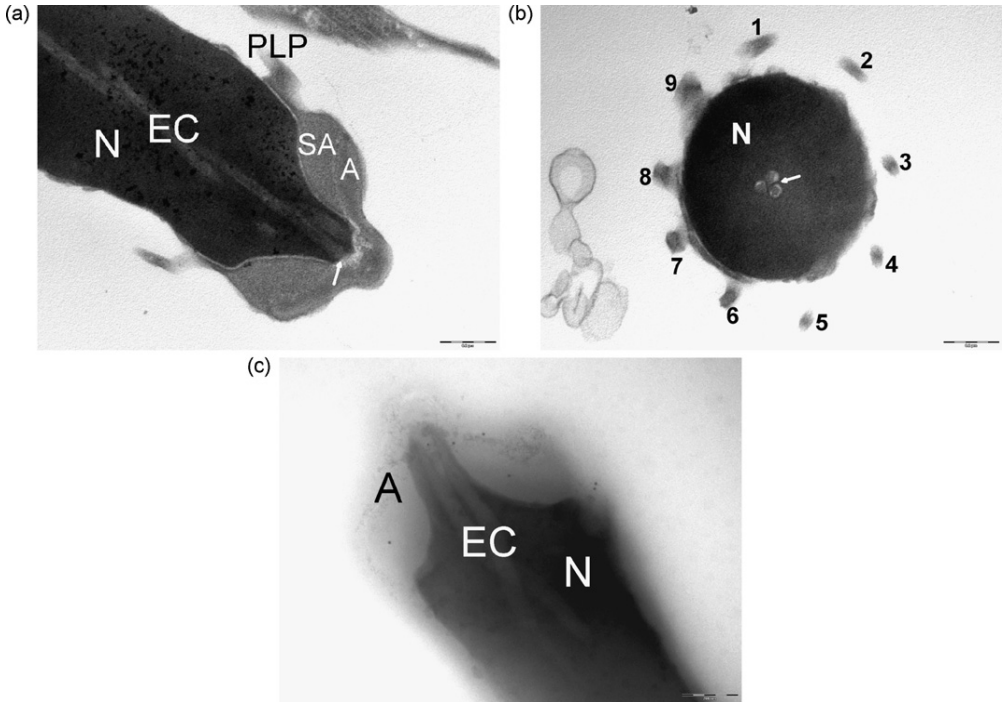


Fig. 4. (a) The anterior surface of nucleus after release of the acrosome. (b) The surface of implantation fossa after release of the nucleus observed by means of SEM (JSM 7401 S) (scale bar 100 nm). The arrow shows opening of an endonuclear canals. (c) The immunolabeling of a longitudinal sagittal section of midpiece shows presence of acrosin by means of gold particles localization in the implantation fossa (scale bar 200 nm).

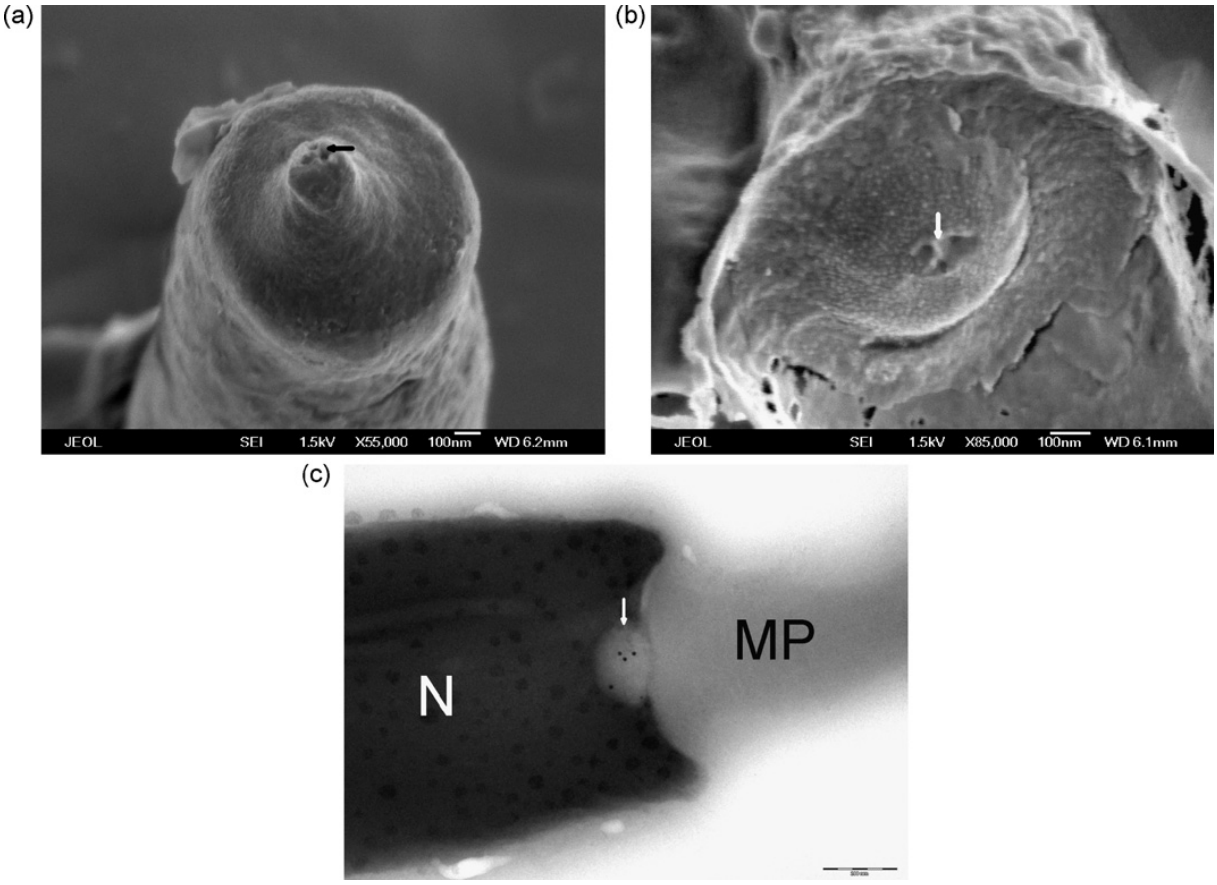


Fig. 5. (a) Longitudinal sagittal section shows nucleus (N) implantation fossa (IF) with connection to endonuclear canals (EC), distal (DC) and proximal centriole (PC). Numerous mitochondria (M) appear irregularly dispersed in the cytoplasm. The flagellum (F) passed through cytoplasmic channel (CC) (scale bar 0.2 μm). (b) SEM (JSM 7401 S) of the midpiece after release of nucleus including the implantation fossa and nuclear membrane. The arrow shows the proximal centriole (scale bar 100 nm).

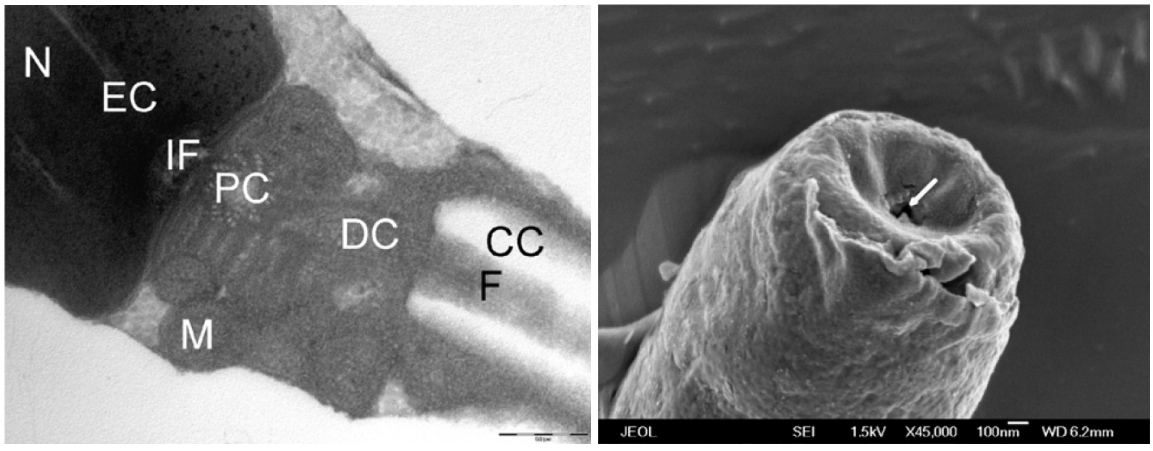
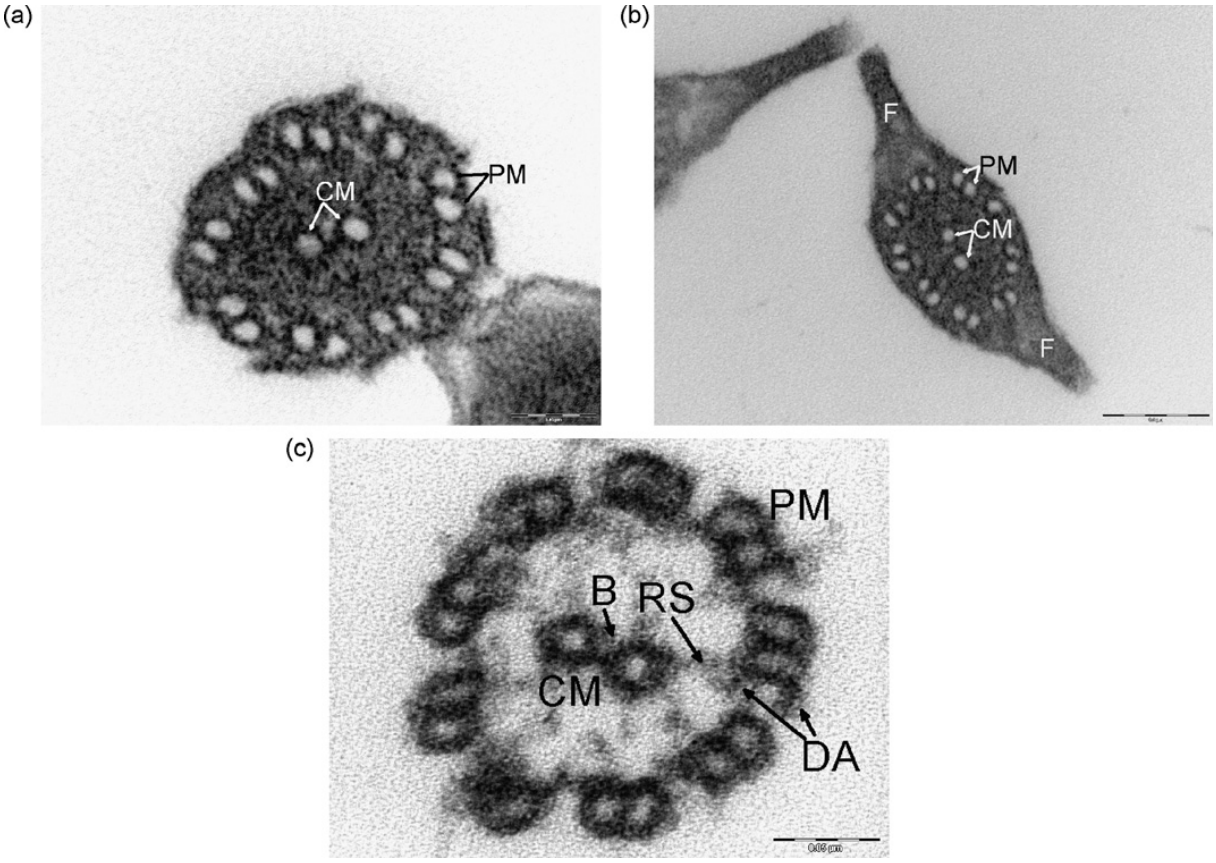


Fig. 6. Cross-sections of the axoneme shows (a) the flagellum between the midpiece and the extension of fin (scale bar 0.05 μm), (b) the flagellum more distally from head with extension of fin (scale bar 0.1 μm) and (c) the flagellar axoneme (scale bar 0.05 μm). The peripheral doublets of microtubules (PM) and central singlet of microtubules (CM) with radial spokes (RS), the 2 CM being joined by a bridge (B). Adjacent peripheral doublets are linked by inner and outer dynein arms (DA).



4.2. Acrosome and PLPs

The lack of acrosome in teleost fishes is compensated by the presence of a micropyle, through which the sperm penetrates into the egg (Lahnsteiner and Patzner, 1999). Interestingly, *Acipenseriformes* sperm posses an acrosome while the corresponding eggs have several micropyles (Linhart and Kudo, 1997) and (Debus et al., 2002). The acrosome, possibly functioning to expedite egg penetration during fertilization, is considered to be derived from the Golgi apparatus and commonly cap-shaped (Nicander, 1970). Using immunolabeling method was shown that acrosin is present in sterlet spermatozoa and localized in the acrosome and the nuclear fossa region. Further studies are needed for better understanding of the possible role of ECs in localization of acrosin. Acrosin appears to be a widely distributed conserved protein (Baccetti et al., 1989), which had taken place a billion years ago during the early period of eukaryote evolution (Klemm et al., 1991). For this reason, the presence of acrosin in sturgeon sperm might be expected. Ciereszko et al. (1996) found the trypsin-like activity in sturgeon spermatozoa shares many properties with mammalian acrosin.

In sturgeon spermatozoa, the acrosome terminates with PLPs, however, their numbers and specially their sizes vary among species (DiLauro et al., 2001), (Psenicka et al., 2007) and (Wei et al., 2007). Sterlet spermatozoa present 9–10 PLPs number of which is different from that observed in pallid sturgeon ($n = 8$, DiLauro et al., 2001), but similar to that observed in Siberian and Chinese sturgeons ($n = 10$, Psenicka et al., 2007) and (Wei et al., 2007). The length of PLPs in lake, Atlantic, shortnose, pallid, Siberian and Chinese sturgeons has been reported with values of 324, 233, 246, 760, 0.94 and 0.37 μm, respectively. The length of PLPs in sterlet spermatozoa (295.72 ± 34.92 μm) is close to lake sturgeon. DiLauro et al. (2000) mentioned briefly the longer PLPs may be an adaptation providing more secure

anchorage of sperm cell to the egg in fast flowing water during spawning and fertilization.

4.3. Nucleus

In sturgeon spermatozoa, the nucleus usually appears as elongated trapezoids, but differences in its shape and size are evident, for example between nucleus length of sterlet (3.30 μm) and white sturgeon (9.21 μm). Inter-specific diversity in size and shape of sturgeon sperm cell nuclei has evolutionary and genetic implications (DiLauro et al., 1999). Except for the Atlantic sturgeon (DiLauro et al., 1998) that has two ECs, all other sturgeon species have three ECs in spermatozoa (Cherr and Clark, 1984; DiLauro et al., 2000, 2001; Ginsburg, 1977; Psenicka et al., 2007; Wei et al., 2007). The membrane-lined endonuclear canals traverse the nucleus from the acrosomal end to the basal nuclear fossa region. Nevertheless, there are differences between diameter of ECs among sturgeon species: diameters of 35, 97, 49, 57.41, 44.59, 80.00 and 40.13 ± 4.61 nm have been measured in Atlantic, shortnose, lake, pallid, Siberian, Chinese and sterlet sturgeons, respectively. The role of endonuclear canals is unclear: they may assist the transfer of sperm centriole into the egg after sperm–egg interaction upon fertilization. DiLauro et al. (1999), Cherr and Clark (1984) and Jamieson (1991) believed that the materials in nuclear canals would play a perforatorial role during penetration of sperm into the egg. The observed implantation fossa in sturgeon spermatozoa seems to play a connection role between ECs and centrioles of the midpiece.

4.4. Midpiece

The number of mitochondrial component may be different among sturgeon species as it was observed in teleosts (Linhart et al., 1991). Three to six and three to eight mitochondria have been reported in Siberian (Psenicka et al., 2007) and Chinese (Wei et al., 2007) sturgeons, respectively. Mitochondria are the main source for ATP-generation during sperm motility (Billard et al., 1999). Further research is needed to investigate possible correlation between the number of mitochondrion, ATP production/consumption and motility of sperm.

Data show that the size is different between centrioles, in an inter- and intra-species way. Distal centriole is located adjacent to the proximal centriole and the axoneme rises posteriorly to the distal centriole. As in teleost fishes, the flagellum of sturgeon spermatozoan was separated from the midpiece by the cytoplasmic channel.

4.5. Flagellum

The flagellum length showed to be different within sturgeon species (42.47 and 33.26 μm in sterlet and Chinese sturgeon, respectively) and the size of flagellum fin as well (568.46 ± 170.08 and 705.87 ± 220.04 μm in sterlet (this study) and Siberian sturgeon, respectively (Psenicka et al., 2007)). This increase of the surface area may have large implications for water exchange/osmotic regulation during sperm activation. More studies are needed to reveal if there is any correlation between the flagellar length and the size of the flagellar fin and swimming pattern of flagella within motility duration and velocity as well.

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Chapter 4

Staining of sturgeon spermatozoa with trypsin inhibitor from soybean, Alexa Fluor® 488 conjugate for visualization of sturgeon acrosome.

Psenicka, M., Cosson, J., Alavi, S.M.H., Rodina, M., Kaspar, V., Gela, D., Linhart, O., Ciereszko, A. 2008. Staining of sturgeon spermatozoa with trypsin inhibitor from soybean, Alexa Fluor® 488 conjugate for visualization of sturgeon acrosome. J. Appl. Ichthyol. 24, 514–516.

Staining of sturgeon spermatozoa with trypsin inhibitor from soybean, Alexa Fluor® 488 conjugate for visualization of sturgeon acrosome.

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Summary

Trypsin-like activity, similar to acrosin, is present in sturgeon spermatozoa and can be a potential target for trypsin
inhibitors. The objective of this work was to use a fluorescent soybean trypsin inhibitor (SBTI) conjugate with the Alexa Fluor_ 488 dye for visualization of the sturgeon acrosome. After incubation with SBTI-Alexa, a strong signal was observed both in the acrosome and midpiece or implantation fossa region. We have also found that SBTI-Alexa staining can be combined with PI viability test. Detailed examination of staining pattern revealed that SBTI-Alexa can stain ether acrosome or whole sperm. Staining of whole sperm correlated with dead staining ($r2 = 0.94$, $P < 0.01$). However, in fresh semen most cells (93–97%) were not stained with SBTI-Alexa, probably due to intact acrosomes. Further studies should test if SBTI-Alexa can be applied to monitor the acrosome status during the acrosome reaction and cryopreservation.

1. Introduction

Chondrostean spermatozoa differ from those of teleost fish in many aspects, including the presence of an acrosome (Ginsburg,1968; Cherr and Clark, 1984; Dettlaff et al., 1993; Psenicka et al., 2007, 2008a; Wei et al., 2007). The sturgeon sperm structure can be differentiated into three major regions; (a) an elongated head with a front part called an acrosome and implantation fossa region at the posterior of the nucleus (the nucleus usually contains three endonuclear canals that lead from the implantation fossa to the acrosome, which often forms several posterolateral projections); (b) a cylindrical midpiece with the centriolar complex and several mitochodria and (c) a flagellum with the 9+2 microtubular structure (Ginsburg, 1968; Cherr and Clark, 1984; DiLauro et al., 1998; Psenicka et al., 2007). The sturgeon acrosome seems to be functional, because the acrosome reaction (AR) has been described for this species; however presence of micropyle in eggs is observed at the same time (Ginsburg, 1968; Cherr and Clark, 1984; Alavi et al., 2008). Trypsin-like activity has been identified and characterized in spermatozoa of the sturgeon fish (Ciereszko et al., 1994, 1996, 2000). This activity is similar to that of acrosin, an acrosomal protease, and therefore is probably located within the acrosome. Acrosin-like activity can be a target for labelled serine proteinase inhibitors. Soybean trypsin inhibitor (SBTI) inhibits catalytic activity of serine proteinases, including acrosin-like activity of sturgeon sperm (Ciereszko et al., 1994). SBTI-Alexa is a fluorescent SBTI conjugate with the Alexa Fluor_ 488 dye produced by Molecular Probes, Inc. This conjugate has successfully been used for real-time observation of the acrosome reaction in mammalian sperm (Tollner et al., 2003) but has not yet been tested for

lower vertebrate spermatozoa. Until now, no specific method for staining of the sturgeon acrosome has been available. Development of such staining is a prerequisite to study acrosome reaction and damage to sturgeon spermatozoa. The objective of this work was therefore to use of SBTI-Alexa for visualization of sturgeon acrosome.

2. Materials and methods

Semen of sterlet (Acipenser ruthenus) was obtained from males(5–7 year old) with an average weight of 2–5 kg raised in an aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Spermiation of males was induced by a single intramuscular injection of carp pituitary homogenized extract with dosage 4 mg kg)1 b.w. Males were kept in a recirculated tank (4000 L; temperature 13_C, flow rate 0.2 L s)1) until stripping. Sperm was collected 48 h after hormonal injection by catetherisation from urogenital papilla of each male (in order to avoid contamination of milt with urine) separately and collected in 250 ml cell culture containers. No blood contamination was recorded. The collected sperm samples were kept on ice (0–4_C) during the experiments. SBTI-Alexa Fluor_ 488 conjugate was obtained from Molecular Probes, Inc. Eugene, OR. Stock solution was diluted with PBS to a concentration of 10 lg ml)1 and stored at)70_C. For staining of spermatozoa, final concentration was 1 lg ml)1 in PBS. Slides were observed and recorded under Olympus BX50 fluorescence microscope with 100 W Ushio mercury lamp at 1000· magnification using a blue filter. Live/dead staining with propidium iodide (PI viability test) use only (PI) (final concentration of PI was 5 1) was performed as described by Flajshans et al. (2004). Acrosome reaction was induced by incubation of spermatozoa in a solution composed of 10 mM Tris–HCl, pH 10.5, containing 10 mM NaCl, 2% DMSO and 1 mM CaCl2 (higher concentrations of calcium caused precipitation of SBTI-Alexa).

3. Results and discussion

After incubation with SBTI-Alexa, a strong signal was observed in the acrosome region while a weaker fluorescence was also detected at midpiece or implantation fossa region (Fig. 1). The staining pattern with the use of SBTI-Alexa was similar to immunostaining using anti-acrosin monoclonal antibodies (Psenicka et al., 2008b). We have also found that SBTI-Alexa staining can be combined with PI staining (Figs 1a,b). Close examination of staining pattern revealed that SBTI-Alexa can either stain acrosome or whole sperm head (Figs 2a,b). SBTI-Alexa staining of whole sperm cells correlated with PI staining ($r2 = 0.94$, $P < 0.01$; Fig. 3). However, in preparations of fresh (not activated) spermatozoa, most cells (93–97%, $n = 5$) were not stained with SBTI-Alexa. The results strongly suggest that the target molecules for SBTI Alexa (presumable acrosin-like enzymes) are localized within acrosome. Therefore, the binding of SBTI-Alexa with sperm trypsin-like activity is possible after permeabilization of membranes or their damage. We obtained preliminary results suggesting an increased staining of the acrosome after incubation of spermatozoa in acrosome reaction-inducing solution, as demonstrated by an increase in the number of SBTI-Alexa stained spermatozoa. In addition to the acrosome region, our results suggest the presence of trypsin-like activity at midpiece or the implantation fossa region. The presence of such an activity has been confirmed with the use of anti-acrosin antibodies (Psenicka et al., 2008b). These results are in agreement with data indicating the involvement of proteolytic activity in sperm motility (Cosson and Gagnon, 1988; Inaba and Morisawa, 1991; Inaba et al., 1998). Therefore further studies are warranted concerning the identification of trypsin-like aktivity in the midpiece of sturgeon spermatozoa. In summary, in this study we found that SBTI-Alexa

can be useful for the visualization of sturgeon acrosome and the acrosome reaction, because this reaction cannot be observed with classical light microscopy. It seems that the staining is possible after exposure of acrosome structure either to damaging or to acrosome reaction triggering conditions. It can be suggested that acrosin-like activity is a target for SBTIAlexa and that this activity is localized within the acrosome. Further studies should test if SBTI-Alexa can be applied to monitor the acrosome reaction and to evaluate damage to acrosome caused by cryopreservation.

Fig. 1. Fluorescence staining of sterlet spermatozoa with SBTI-Alexa (a) and SBTI-Alexa and PI (b) (scale bar = 1 lm). Spermatozoa are stained green and red with SBTI-Alexa and PI, respectively

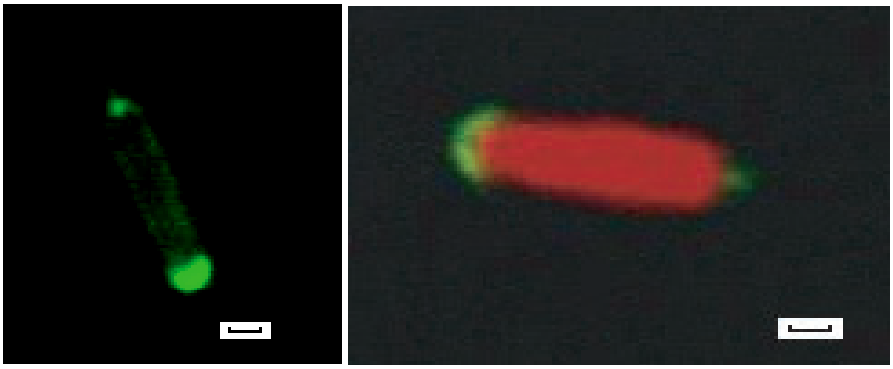


Fig. 2. Fluorescent micrograph of sterlet spermatozoa stained with SBTI-Alexa and PI (a) and light microscopy micrograph of the same preparation (b)

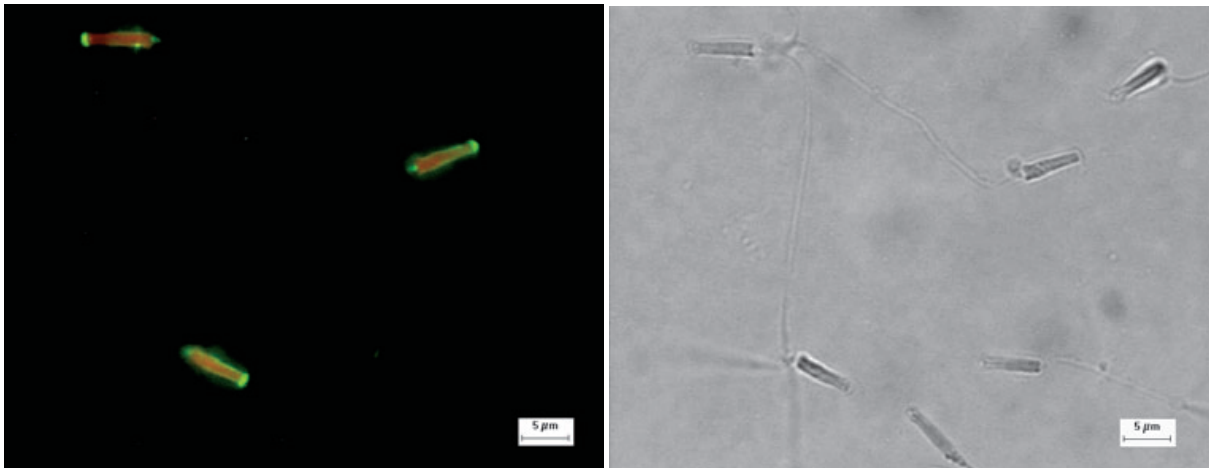
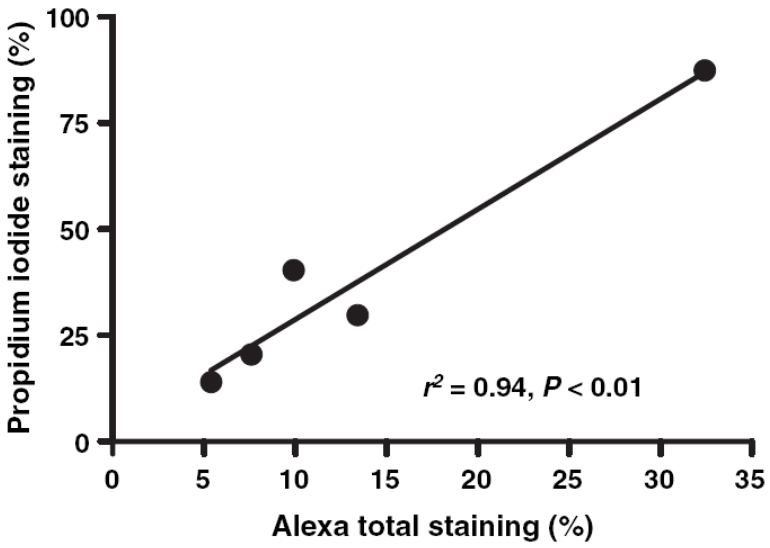


Fig. 3. Linear regression between SBTI-Alexa total staining and propidium iodide staining of sterlet spermatozoa



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Chapter 5

Acrosome staining and motility characteristics of sterlet spermatozoa after cryopreservation with use of methanol and DMSO

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Acrosome staining and motility characteristics of sterlet spermatozoa after cryopreservation with use of methanol and DMSO

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Abstract

In this study we describe acrosome staining and motility characteristics of fresh and cryopreserved sterlet (*Acipenser ruthenus* L.) spermatozoa using soybean trypsin inhibitor-Alexa conjugate fluorescent staining and computer-aided sperm analysis (CASA), respectively. Methanol or dimethylsulfoxide (DMSO) were used as cryoprotectants. After cryopreservation a decline in sperm motility characteristics occurred, but no differential effect between cryoprotectant was observed. Cryopreservation caused a significant increase in the percentage of spermatozoa with acrosome stained by SBTI-Alexa for samples cryopreserved using DMSO compared to methanol. These data suggest that the low usefulness of DMSO for cryopreservation of sturgeon spermatozoa is related to its harmful specific effect towards the acrosome, probably by causing its precocious triggering, much before any egg contact.

Keywords: *Sturgeon; Spermatozoa; Acrosome; Cryopreservation*

Successful cryopreservation of fish spermatozoa is well established for many species and DMSO is the most commonly used cryoprotectant (Ciereszko et al., 1996). Earlier works indicated that the cryopreservation of sturgeon spermatozoa using DMSO-sucrose extender resulted in recovery of motile spermatozoa with basic motility characteristics similar to those of fresh semen (Ciereszko et al., 1996). Unfortunately, despite good and sometimes excellent post-thaw motility performances, the fertilizing ability of such spermatozoa was poor and therefore DMSO-cryopreserved sturgeon sperm was unsuitable for practical applications. High post-thaw motility and low or non-existent fertilization rates of sturgeon and paddlefish spermatozoa cryopreserved with the use of DMSO has also been confirmed by Lahnsteiner et al. (Lahnsteiner et al., 2004), Horvath et al. (2005) and Linhart et al. (2006). Horvath and Urbanyi (2000) and Linhart et al. (2006) introduced methanol as a cryoprotectant for the cryopreservation of sturgeon and paddlefish spermatozoa. Methanol as a cryoprotectant secures both high values of sperm motility and velocity (although sometimes lower motility than DMSO (Lahnsteiner et al., 2004) is observed), and fertilization rates of frozen-thawed spermatozoa (Glogowski et al., 2002). Usefulness of methanol for sperm cryopreservation has also been confirmed for sterlet (*Acipenser ruthenus* L.). Lahnsteiner et al. (2004) have recorded a significant reduction in fertilizing ability of sterlet semen frozen in DMSO while with methanol the fertility was comparable with the controls.

The reason for the reduced fertilization capability of sturgeon spermatozoa post-cryopreservation with DMSO is unknown. Since motility of cryopreserved sperm is well preserved, DMSO may specifically cause damage to sperm structure other than motility apparatus. The acrosome, a specific sperm structure responsible for penetrating the egg, is a

potential target for such damages caused by DMSO. If this assumption is correct, one may expect higher disruption to acrosome of spermatozoa cryopreserved with the use of DMSO as compared to methanol counterpart. Recently, we have described a fluorescent technique for the visualization of sturgeon acrosome which uses soybean trypsin inhibitor conjugated with Alexa Fluor® 488 (Psenicka et al., 2008). In the present study, using this technique we describe and compare acrosome staining of fresh and cryopreserved sterlet (*A. ruthenus* L.) spermatozoa. Methanol or DMSO were used as cryoprotectants. Sperm motility evaluations were monitored on the same samples to verify cryopreservation success according to this parameter.

Semen of sterlet was obtained from five different males (5 to 7 year old) with average weight of 2–5 kg raised in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Spermiation of males was induced by a single intramuscular injection of carp pituitary homogenized extract with a dosage of 4 mg kg^{-1} b.w. Sperm was collected 48 h after hormonal injection by catheterization from urogenital papilla of each male separately and collected in 250 ml cell culture containers. The collected undiluted sperm samples were shipped on ice (0–4 °C) to Olsztyn, Poland where they arrived and were used two days latter.

The cryopreservation procedure followed that is described by Glogowski et al. (2002). Milt was diluted with an extender consisting of 30 mM Tris, 23.4 mM sucrose and 5.0 mM KCl (pH 8.0) supplemented either with 10% methanol or 10% DMSO. The milt and extender were stored on ice. Milt was diluted with the extender at a ratio of 1:1 and immediately drawn into 0.25 ml plastic straws (IMV Technologies, L'Agile, France). The straws were placed on a 3-cm-high frame made of Styrofoam floating on liquid nitrogen. Straws were thawed by immersion in a water bath at 40 °C for 6 s. For both fresh and cryopreserved semen, SBTI-Alexa staining and motility analysis were performed. Evaluations were made in duplicate.

SBTI-Alexa Fluor® 488 conjugate was obtained from Molecular Probes, Inc. Eugene, OR, USA. Stock solution was diluted in PBS to a concentration of 10 μg/ml and stored at −70 °C. For staining spermatozoa, the final concentration was 1 μg/ml in PBS. Slides were observed and recorded under Olympus BX50 fluorescence microscope with 100 W Ushio mercury lamp at 1000× magnification using a blue filter. Our previous study (Psenicka et al., 2008) revealed that SBTI-Alexa can either stain the acrosome (specific staining) or the whole sperm head (total staining identifying dead spermatozoa). SBTI-Alexa does not stain acrosome filaments; these filaments can be observed by light microscopy. However, in preparations of fresh spermatozoa, most cells are not stained with SBTI-Alexa, probably because the target molecules for SBTI-Alexa (presumable acrosin-like enzymes, (Ciereszko et al., 1996)) are localized within acrosome and not accessible to the dye.

Video recordings were made using a microscope with a 10× negative phase objective and a Sony CCD black and white video camera. About 1 μl of milt was added to a small polyethylene tube containing 299 μl of sperm activating solution (10 mM Tris, 20 mM NaCl, 2 mM $CaCl_2$, pH 8.5, at 12 °C) supplemented with 0.2% bovine serum albumin to prevent sticking of spermatozoa to the glass. After rapid mixing, just before registration, 0.7 μl of this dilution was immediately placed into a well of 12-well multitest glass slide (ICN Biomedicals Inc., OH, USA) and covered with a coverslip. Sperm motility parameters were measured over 30 s period, between 5 and 35 s post-activation time. Video recordings were analyzed using Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, UK). The sperm tracker simultaneously assessed 15 sperm motility parameters, but for simplification only straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN = 100 × VSL/VCL), amplitude of lateral head displacement (ALH) and percentage of motile sperm (MOT) were chosen for further analysis.

All values are expressed as mean values ± SD. Data were analyzed using ANOVA

followed by Tukey's post hoc test. Data were tested for normal distribution and log-transformed when necessary. Percentage data were subjected to arcsine transformation. The level of significance was set at 0.05.

Specific staining of acrosome of fresh spermatozoa was low (about 5%) and slightly increased to about 6% in samples cryopreserved using methanol (Fig. 1). However, this increase was not significant ($P = 0.24$). On the other hand, about 100% increase in specific staining was observed in samples cryopreserved with the use of DMSO. Total SBTI-Alexa staining of fresh sperm (Fig. 2) was $12.7 \pm 4.4\%$ and was significantly lower than for cryopreserved sperm ($21.0 \pm 3.8\%$ and $26.2 \pm 6.1\%$, for DMSO and methanol, respectively). Most spermatozoa were not stained with SBTI-Alexa, most likely because their membranes were either not permeable to dye or acrosome content was not exposed. Spermatozoa cryopreserved using DMSO compared to methanol did not show any significant difference.

After cryopreservation a decline in sperm motility parameters occurred, but no significant cryoprotectant effects were recorded (Table 1). A decrease in the percentage of motile spermatozoa, VCL, VSL, and ALH was observed, while the linearity index remained unchanged. In this experiment the post-thaw percentage of motile sperm (40–50%) was higher than previously published for lake sturgeon (14–19%, (Ciereszko et al., 1996) but similar to data obtained for sterlet (Lahnsteiner et al., 2004)). These data suggest that semen of sterlet is more resistant to cryopreservation. Similar values of motility parameters in semen cryopreserved using DMSO or methanol are recorded in this study, which confirm previous findings and suggests that damage to sperm motility apparatus was similar for both cryoprotectants.

Our results strongly suggest that the cryopreservation of sturgeon spermatozoa using DMSO induces significant changes in the acrosome region, allowing SBTI-Alexa to react with acrosome localized serine proteinases. The mechanism leading to the exposure of the acrosome interior compartment is unknown at present; it is likely that it includes the acrosome reaction, because we observed acrosome filament (using light microscopy) in spermatozoa specifically stained with SBTI-Alexa (Fig. 3). It is worth mentioning that DMSO has been found to be an efficient inducer of the acrosome reaction for sea urchin spermatozoa (Mikami-Takei et al., 1987). Our data strongly suggest that the reduced fertilization capability of sturgeon spermatozoa post-cryopreservation with DMSO is related to its harmful specific effect towards the acrosome.

Although the difference in acrosome staining between sperm cryopreserved with the use of methanol and DMSO was significant, the percentage of specific staining of spermatozoa cryopreserved with the use of DMSO was only 12%. Such a low percentage of disrupted acrosomes does not fully explain very low or non-existent fertilization rates obtained with sperm cryopreserved with the use of DMSO. Mikami-Takei et al. (1987) suggested that DMSO can induce preliminary steps leading to acrosome reaction of sea urchin spermatozoa. Perhaps a similar phenomenon is true for sturgeon spermatozoa, and therefore DMSO would induce subtle changes to the acrosome of cryopreserved sturgeon spermatozoa but such changes may not be detected by SBTI-Alexa staining. Therefore further studies aiming to better evaluate the acrosome status of sturgeon spermatozoa during cryopreservation are warranted. Such studies should also be aimed towards the evaluation of DMSO and methanol effects on fresh spermatozoa and the acrosome status at the conditions of in vitro fertilization.

Fig. 1. Percentage of specific acrosome staining (mean ± SD) of fresh and cryopreserved sterlet spermatozoa ($n = 5$). Methanol (METH) or dimethylsulfoxide (DMSO) were used as cryoprotectants. Different letters indicate a significant difference ($P < 0.05$).

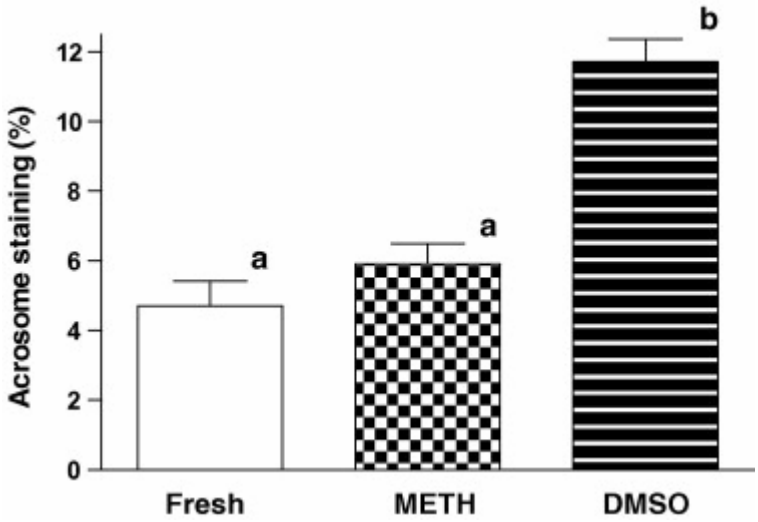


Fig. 2. Fluorescent micrograph of sterlet spermatozoa stained with SBTI-Alexa. Specific staining is indicated by white arrow; total staining is indicated by arrowhead.

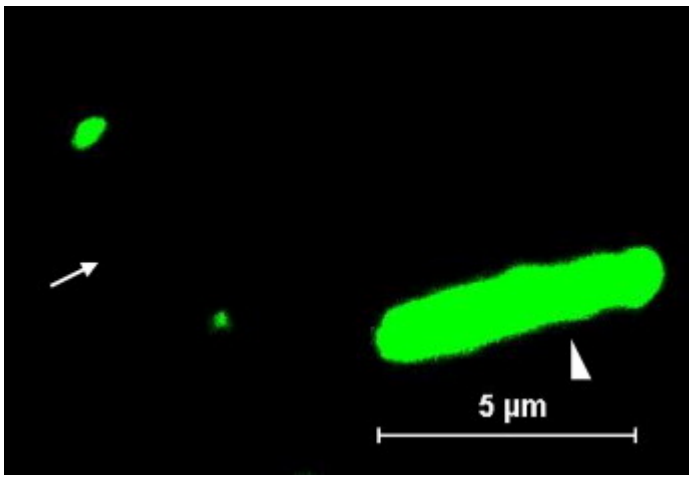
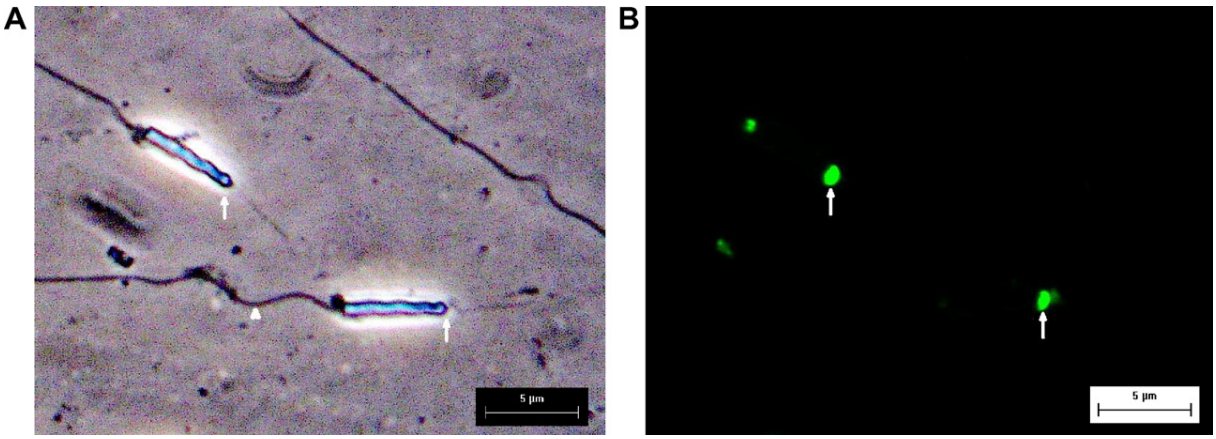


Table 1. Motility parameters (mean ± SD) of fresh and cryopreserved sterlet spermatozoa ($n = 5$)

Motility parameter		Cryopreserved	
	Fresh	Methanol	DMSO
Motile sperm (%)	76.6 ± 6.4[a]	48.0 ± 8.8[b]	39.9 ± 9.3[b]
VCL (µm/s)	136.8 ±13.8[a]	88.2 ± 8.8[b]	94.6 ± 2.2[b]
VSL (µm/s)	80.9 ± 3.4[a]	50.6 ±13.4[b]	63.1 ± 9.3[b]
LIN	54.8 ± 4.4[a]	41.4 ±10.0[a]	51.2 ±10.5[a]
ALH	10.9 ± 3.5[a]	3.5 ± 0.2[b]	3.4 ± 0.7[b]

Different letters indicate significant differences ($P < 0.05$). See the Material and methods paragraph for definition of VCL,VSL, LIN and ALH.

Fig. 3. Light microscopy micrographs (phase contrast) of the cryopreserved sterlet spermatozoa (A) and fluorescent micrograph of the same preparation stained with SBTI-Alexa (B). White arrows indicate acrosomal filament in (A) or acrosome region of spermatozoa in (B). Flagella are indicated by arrow heads in (A).



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Chapter 6

Ultrastructural study on fertilization process in sturgeon (Acipenser), function of acrosome and prevention of polyspermy

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Ultrastructural study on fertilization process in sturgeon (Acipenser), function of acrosome and prevention of polyspermy

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Abstract

Sturgeon gametes differ from other fish in that their spermatozoa possess acrosome with fingers-like posterolateral projections, which undergo exocytosis and filament formation, whereas eggs possess numerous micropyles. Fertilization process in *Acipenser baerii* was investigated by fluorescence and electron microscopy.

A suitable activation solution containing 2.5 mM $CaCl_2$, 15 mM tris, pH 10 was found for detailed description of acrosomal reaction. The acrosome reaction includes formation of a spear-like fertilization filament coming from 3 endonuclear canals and implantation fossa through the acrosomes. It can accelerate the process of polyspermy prevention. Another unique feature of the acrosome was an anchor-like opening of posterolateral projections. Mature eggs of *Acipenser baerii* possessed from 2 to 10 micropyles in the animal pole region. Eggs consisted of three principal layers and an outermost jelly coat blocking micropyle, and a layer of cortical granules in unfertilized eggs. With exposure to freshwater the jellyish layer separated from the egg surface, whereas the cortical granules swelled. No change between layers of fertilized and unfertilized eggs, apart from generation of an increasing perivitelline space by dissolution of the cortical granules, had been observed after fusion of spermatozoon with egg. A fertilization cone blocked a fusion of other spermatozoa with cytoplasmatic projection in the fertilized micropyle.

Key words: sturgeon, sperm, egg, penetration, acrosome, fertilization, ultrastructure

1. Introduction

Species-specific morphological and physiological features have been shown in spermatozoa and eggs of several fish species reflecting differences in functional capabilities (Alavi et al., 2008). Gametes of sturgeon fishes differ from those of teleost fishes by the presence of acrosomes in spermatozoa and multiple micropyles in eggs.

In the Siberian sturgeon *Acipenser baerii* (Brandt 1869) spermatozoon is composed of an elongated head with acrosome, subacrosome and 10 posterolateral projections (PLPs), a cylindrical midpiece with mitochondria, and a centriolar complex and flagellum with the "9+2" structure of axoneme. Three endonuclear canals, bound by membranes, traverse the nucleus from the junction with the acrosome towards the implantation fossa, the opposite end of the nucleus, in front of the midpiece (Psenicka et al., 2007, 2008a,b). Psenicka et al. (2008b) evidenced the presence of acrosin in these two parts (acrosome and implantation fossa).

The acrosome reaction includes exocytosis of acrosomal vesicles, and the formation of a fertilization filament called the acrosomal process (Cherr and Clark, 1984). The biological importance of the acrosomal reaction in sturgeons, given the presence of micropyles, is unclear. The acrosome reaction can be induced by treating spermatozoa with a water-soluble 66 kDa glycoprotein, which is present in the jelly coat of the egg (Cherr and Clark, 1985), with Ca^{2+} at pH 9.5 (Psenicka et al., 2008c), and in Ca ionophore 123187 (Cherr and Clark, 1984; Ginsburg and Nikiforova, 1978). Dettlaff et al. (1993) reported that the presence of Ca^{2+} is necessary for the acrosomal reaction and hence, for fertilization. Nevertheless good control of acrosomal reaction has not been well elaborated in studies mentioned above. Psenicka et al. (2008c,d) describe using Soybean Trypsin Inhibitor-Alexa conjugate fluorescent (STBI-Alexa) staining for screening of acrosomal integrity.

The spermatozoa in most teleosts can enter the egg only through single micropyle. In teleosts a species specific feature of the sperm entry site has been shown (Kudo, 1991). The diameter of the inner aperture of the micropyle enables only a single spermatozoon to enter the egg. Nevertheless, there is some evidence showing the role of cortical granules (CGs) in prevention of supernumerary sperm penetration in teleost fishes (Kudo, 1991).

In sturgeons the number of micropyles varies markedly in different females of a species, and also in the eggs of one female (Ginsburg, 1969; Podushka, 1993a,b). The number of micropyles has been reported as up to

52 micropyles in the Russian sturgeon *A. gueldenstaedti* Brandt and Ratzeburg, 1833 (Ginsburg, 1969) and 3 to 16 in the Siberian sturgeon (Debus et al., 2008).

Sturgeon eggs contain four layers, with total thickness 70 μm in Stellate sturgeon *A. stellatus*. However, the thickness of the layers in the animal pole region is lower than in the rest of the egg surface, with values of about 50 μm (Dettlaff and Ginsburg, 1954). In contrast, most teleost egg layers thickness ranges from 2 μm to 50 μm. One to two rows of CGs are located under the egg layers over the egg surface. The release of CG components upon sperm penetration blocks polyspermy, and creates a single layer called "perivitelline space (PS)" (Cherr and Clarr, 1985; Dettlaff et al., 1993). Mature eggs of several fish species respond to sperm entry by a formation of fertilization cone at the sperm entry site, which blocks polyspermy (Iwamatsu et al., 1991; Kobayashi and Yamamoto, 1981; Kudo, 1980, 1983; Kudo and Sato, 1985; Linhart and Kudo, 1997).

The aim of the present study was to describe the ultrastructure of eggs of the Siberian sturgeon, to examine changes of eggs upon fertilization, and to monitor the sperm fertilization process, using fluorescence, scanning (SEM) and transmission (TEM) electron microscopy.

2. Materials and Methods

2.1. Broodfish and gamete collection

Semen samples were collected from broodfish reared in Fischzucht Groß farm, Gersfeld, Germany. Four individual females and five males, 5 to 7 years old and averaging 4 to 8 kg, were held in the tank with temperature stabilized at 13°C. To induce spermiation, males were injected by a single intramuscular injection of carp pituitary homogenized extract (CPE) at a dose of 4 mg kg^{-1} b.w. Sperm was collected 48 h after hormonal injection. Sperm was collected by use of a catheter from the urogenital papilla of males, transferred to a separate cell culture container (250 ml), and stored at 4°C until sampling. Ovulation was induced with CPE in two steps: the first 0.5 mg kg^{-1} and the second 4.5 mg kg^{-1} body weight, 12 h after first injection. The ovulated eggs were collected 18 – 20 h after the second injection. Proportional mixtures of sperm and eggs from each individual were used for further experiments.

2.2. Sample treatment

For detailed comparison of activated and non-activated spermatozoa a suitable activation solution was selected from solutions containing 0, 1 and 2.5 mM $CaCl_2$, 15 mM tris-HCl adjusted to pH 9.5, 10 and 10.5 in all combinations (see Table 1). Treating by hatching water was used as a control. The process of activation and motility were stopped after 90 sec by addition of KCl (3 mM final concentration). The acrosome reaction and spermatozoa damage was checked using Soybean Trypsin Inhibitor-Alexa conjugate fluorescent staining and phase contrast of light microscope according to Psenicka et al. (2008b, 2008d).

Fertilized eggs were mixed with sperm and water and activated eggs were mixed with hatching water. They were collected in 15, 30, 60, 90, 120, 180, 240 and 450 sec in both cases. Activated and nonactivated spermatozoa and egg samples were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer for 2 days at 4°C.

Samples for TEM were post-fixed in osmium tetroxide for 2 h, at 4°C, washed, dehydrated through an acetone series, and embedded in resin (Polybed 812; Polysciences, Inc.). Series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Microsystems), counterstained with uranyl acetate and lead citrate, and examined in a TEM JEOL 1010 operated at 80 kV.

The fixed samples for SEM were dehydrated in a graded concentration of acetone, and dried using a critical point dryer CPD 2 (Pelco TM). Then, they were coated with gold under vacuum using a SEM Coating Unit E5100 (Polaron Equipment Ltd.), and examined with SEM JSM 7401-F (JEOL Ltd., Tokyo, Japan) or SEM JEOL 6300, equipped with Sony CCD camera.

3. Results

3.1. Spermatozoa activation solution

As the optimal activation solution was found 2.5 mM $CaCl_2$, pH 10, 15 mM tris. This combination activated the most percentage of spermatozoa without high disturbance (Fig. 1). Table 1 shows the pattern for selection of suitable activation solution from means of used

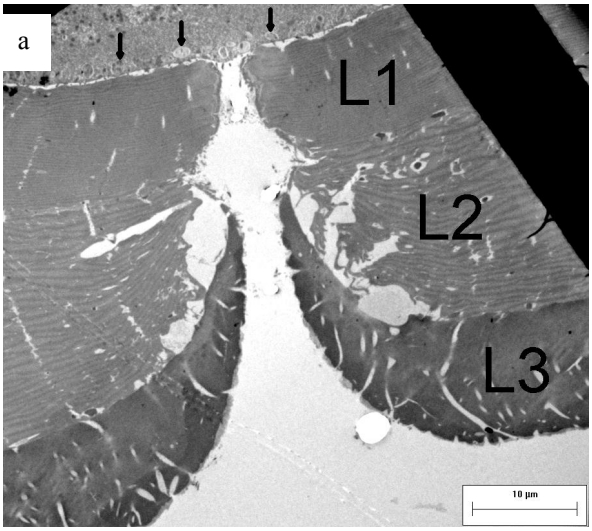
males. Nevertheless the rate of activated spermatozoon acrosomes differed within individuals. The staining using SBTI-Alexa indicated 11,0, 4.8, 5.0, 8.0 and 7.5 % activated acrosomes by water and 71.8, 48.2, 58.3, 76.0 and 69.2 % by selected activation solution, respectively.

Fig. 1. Overlaid picture from face contrast of light microscopy and fluorescence. The signal indicates activated acrosomes by STBI-Alexa and the arrows show acrosomal filament (scale bar = 5 µm).

3.2. Structure of egg coat

The vitelline envelope consisted of three main successive, relatively thick layers from inside to outside: L1 (14.31±1.91 µm), interspaced layer (0.73±0.08), L2 (21.19±3.57 µm) and L3 (12.35±4.37 µm), together with a layer of CGs in unfertilized eggs (Fig. 2a,b,c). The outmost jelly coat layer was composed of less dense, finely flocculent material, which was found on the whole egg and like a stopper over micropyles**,** and completely released by freshwater within one minute (Fig. 2a,c). In addition, the CGs enlarged within time in freshwater (Table 2). The L1 and L2 layers, both containing projections with spaces around them, displayed a filamentous material. The L3 was transformed into a canal, which filled the whole surface of the micropyle from its top to the bottom of the micropyle. TEM revealed that the micropyle consisted of a vestibule and a canal at the level of L1. The level of the interspace layer (transition of the vestibule to the canal) was evidently expanded (Fig. 2a). Our results indicated the presence of two to ten sperm entry sites on each mature egg, covering an area about 50 µm.

Fig. 2 a-c. Transmission electron micrograph (TEM) showing the egg layers (L1, L2, L3) with electron-dense components (EC or white arrows) and cortical granules (black arrows). (a) a longitudinal section of the micropyle of unfertilized egg after 450 sec activation by water. Scale bar: 10 µm. (b) a detail of interspaced layer (IL). Scale bar: 2 µm. (c) a detail of the releasing jelly coat (JC) after 15 sec activation by water. Scale bar: 5 µm.



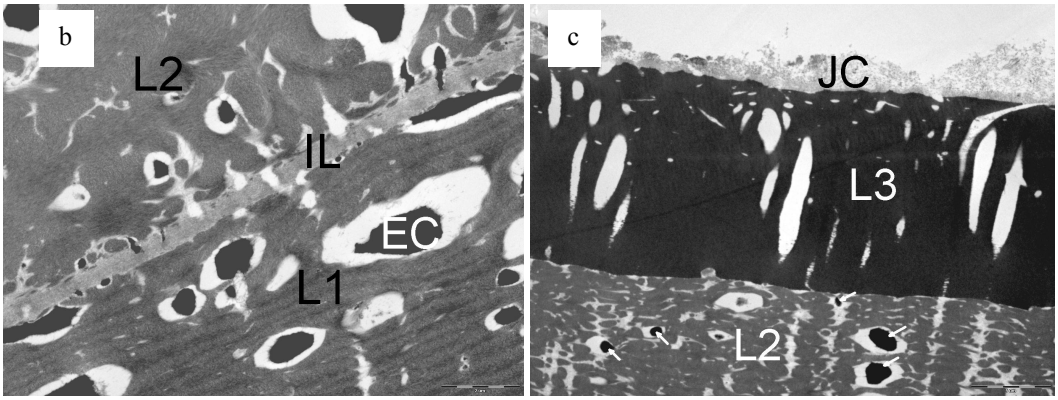
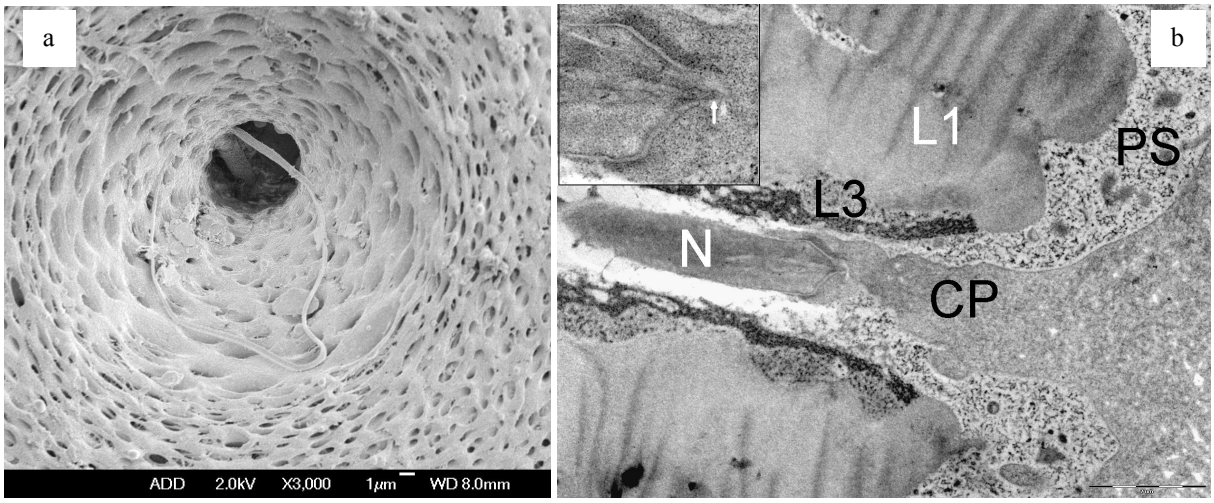


Fig. 3 a-b. (a) Scanning electron micrograph (SEM) showing penetration of spermatozoon into the egg 60 sec after fertilization. Scale bar: 2 µm. (b) Transmission electron micrograph (TEM): longitudinal section showing the fusion of spermatozoon acrosome and its nucleus (N) with egg cytoplasmic projection (CP) and formation of perivitelline space (PS) under egg layers (L1, L3). Scale bar: 2 µm. Inset: Formation of fertilization filament (arrows).



3.3. Fertilization process

The micropyles were filled with spermatozoa about 15 sec post fertilization (Fig. 3a). The spermatozoon, which was almost at the bottom of the micropyle, was merged with a cytoplasmic projection of the egg immersed into the inner micropyle (Fig. 3b). The membrane of the acrosome was decomposed, starting from the apical part. Upon acrosomal reaction the material in the three ENCs and implantation fossa of the sperm head was ejected forward like a fine acrosomal filament 14.22±5.12 µm long on average, which served like a harpoon (Figs. 3b, 4a,b,c).

Fig. 5 shows emptying of implantation fossa after fusion of a spermatozoon with an egg and a release of the flagellum from the nucleus directly under the distal centriole, where the centriolar complex remains with nucleus.

The diameter of the micropylar base (1.95 ± 0.26 µm) was only slightly larger than the diameter of the spermatozoon's head (1.13 ± 0.16 µm). As a result of that no more but one sperm can enter the micropyle. Besides when the spermatozoon was penetrating through the micropyle into the egg the 10 PLPs opened and served as an anchor (Fig. 4a,b,c,d), which is unique feature of the sturgeon spermatozoa. To confirm opening of the PLPs, the distance of PLPs from the nucleus was measured on cross-sections of the PLPs region of activated spermatozoa (0.19±0.12 µm, n=119) and non-activated spermatozoa (0.14±0.04 µm, n=136) with highly significant difference ($p<0.01$). The distance of PLPs of spermatozoa observed from cross sections leading through micropyles was 0.20±0.16 µm, n=58.

Fig. 4a-d. Scanning electron micrograph (SEM) showing acrosome of non-activated (a) and activated spermatozoon (b). Scale bar: 100 nm. (c-d) Transmission electron micrograph (TEM). (c) longitudinal section of activated spermatozoon showing acrosome (A), nucleus (N), posterolateral projections (arrow), fertilization filament (F) and endonuclear canals (EC). Scale bar: 500 nm. (d) Transmission electron micrograph (TEM). Cross section at the level of extended 10 posterolateral projection (arrows) of egg micropyle with spermatozoon; N: nucleus, L: egg layer. Scale bar: 1 µm.

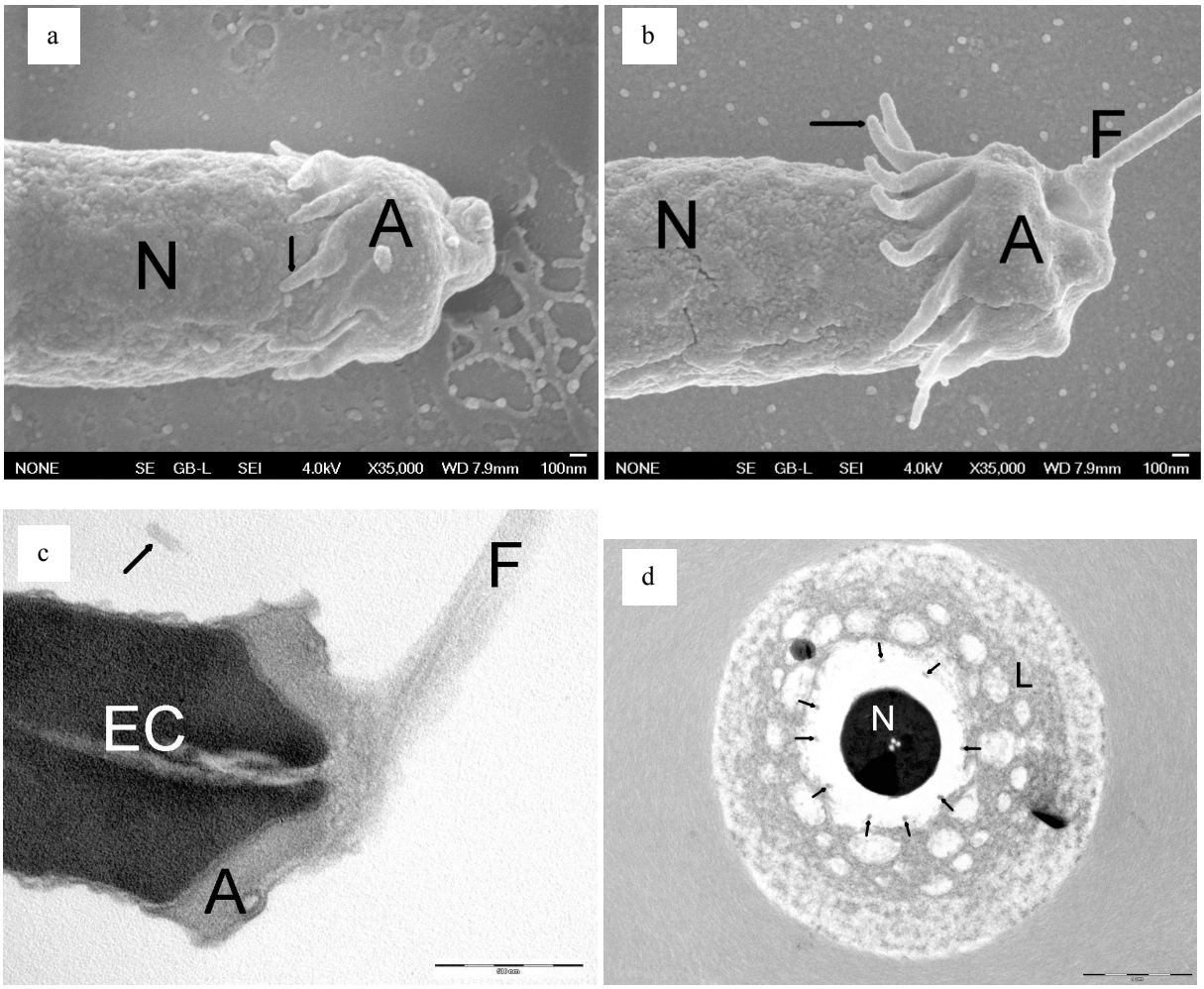
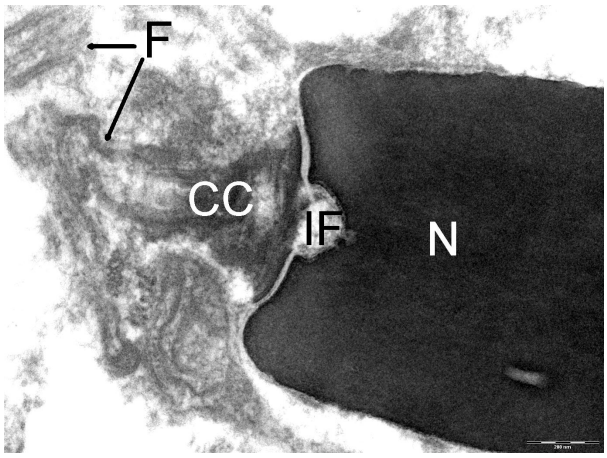


Fig. 5. Transmission electron micrograph (TEM). Longitudinal section of midpiece at the bottom of micropyle after fusion with egg cytoplasm, where the spermatozoon lost flagellum (F). The implantation fossa (IF) was depleted and centriolar complex (CC) remain with nucleus (N). Scale bar: 0.2 µm.



3.4. Block of polyspermy

Immediately after fusion, the entire egg envelope was separated from the oocyte plasma membrane by the enlarging perivitelline space (PS) filled with the material from CGs (Table 3 and Fig. 3b), which agglutinated other spermatozoa (Fig.6).

During formation of PS a fertilization cone was visible at the entry site of sperm in fertilized eggs (Fig. 7). Approximately 60 sec post fertilization the fertilization cone forming from cytoplasm of the egg extended to about 20-30 µm in width, with the ball-like enlarged apex of the cone reaching the micropylar vestibule. Spermatozoa were never able to fuse with the cone membrane. In this study we observed the cone only in fertilized micropyle. In other micropyles only CGs exudates were identified.

Fig. 6. Transmission electron micrograph (TEM) showing longitudinal section of egg layer (l1) and perivitelline space (PS) which agglutinates other spermatozoon with nucleus (N) 120 sec after fertilization. Scale bar: 2 µm.

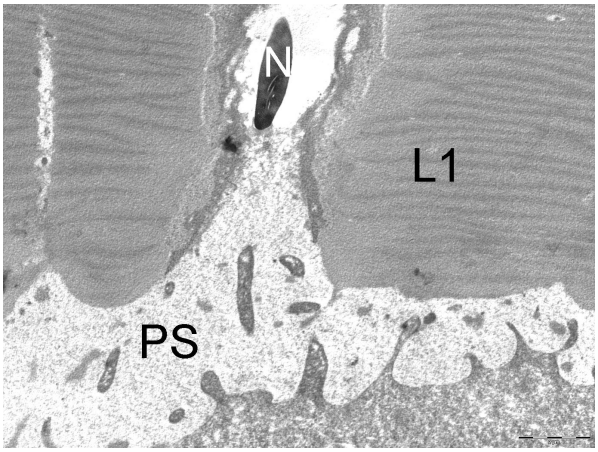
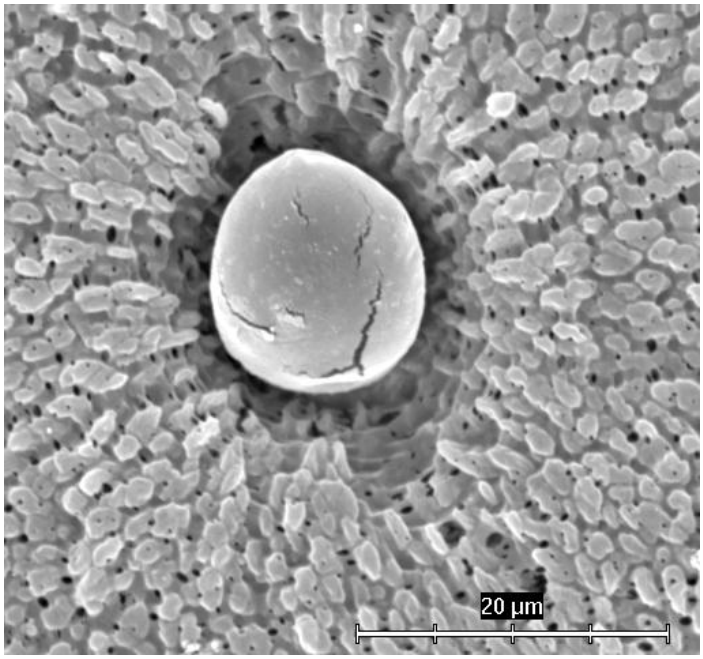


Fig. 7. Scanning electron micrograph (SEM) showing fertilization cone in the micropyle 180 sec after fertilization. Scale bar: 20 µm.



4. Discussion

In general, the typical organelle of the spermatozoon, the acrosome, is considered to be responsible for enabling the spermatozoon to traverse investment coats surrounding an egg. Most spermatozoa undergo an acrosomal reaction in response to an egg component; this acrosomal reaction exposes the contents of the acrosome, which includes enzymes and binding proteins (Dan, 1967; Lopo, 1983; Shapiro and Eddy, 1980). Tunicate, lampreys, hagfish (Eptatretus burgeri and E. stouti) and sturgeons (Cherr and Clark, 1985; Dettlaff et al., 1993; Morisawa, 1999; this study) are reported to possess sperm that form fertilization filaments during the acrosome reaction. According to Psenicka et al. (2008b) and this study, the filament in sturgeon originates from implantation fossa and ENCs. This mechanism differs from all others. Lamprey eggs are covered by a penetration envelope that lacks micropyles (Kille, 1960). Morisawa and Cherr, (2002) described acrosome reaction in hagfish, where the spermatozoa have to penetrate through a U-shape layer filling the bottom of the micropyle (Morisawa, 1999). In sturgeon, the eggs possess an impenetrable envelope, which is perforated by numerous micropyles that could be advantageous in terms of fertilization in fast flowing water but on the other hand there is a risk of polyspermy. Therefore, the presence of acrosomes and long acrosomal processes in sturgeon spermatozoa is inconclusive and does not agree with models of acrosomal evolution (Baccetti, 1979). Based on our findings, we suppose that the filament serves as a quick signal transducer carrying the information for the egg about the presence of sperm in its close proximity and induces the creation of cytoplasmic projection of the egg for fusion with the spermatozoon and block of polyspermy in all other micropyles.

Acrosomes of sturgeon spermatozoa are terminated by PLPs that are radially distributed lobes. Number and size of PLPs vary among species (DiLauro et al., 1998, 1999, 2000, 2001; Psenicka et al., 2007, 2008a; Wei et al., 2007; this study). The distance of PLPs from the nucleus showed differences between activated and non-activated spermatozoa. It seems to be caused by acrosomal filament formation, due to which the outer acrosomal membrane is stretched. Nevertheless, the function of PLPs is not well understood and more studies are needed to accurately determine the role of PLPs structure, their size during fertilization and their role in sperm fertility.

During fusion of the spermatozoon with the egg a fracture of midpiece in transition of distal centriole into the flagellum was observed, which is in the weakest part of flagellum without central microtubules.

The main way how to prevent polyspermy in sturgeon eggs is the creation of PS from CGs immediately after fusion with spermatozoon. The CGs are specialized Golgi-derived secretory granules located just below the plasma membrane of mature unfertilized egg. After the first sperm attaches to the oocyte plasma membrane, the fertilization wave moves through the cortex of the oocyte from the site of sperm attachment to the opposite side of the oocyte. Following this fertilization wave, the content of CGs is released into the PS, which blocks polyspermy (Epel, 1977; this study). In our study when the eggs were activated by water, their jelly coat washed away and CGs swelled but they weren't broken until after the egg had been fertilized. We suppose that so swelled CGs can break more easily and create the PS quicker. That can be another feature of block polyspermy acceleration in addition to the creation of fertilization filament.

Growth of the fertilization cone starts from the top area of the fused sperm head, and eventually reaches the micropylar vestibule. The plasma membrane of supernumerary spermatozoa is unable to fuse with that of the fertilization cone (Kobayashi and Yamamoto, 1981; Kudo, 1980; this study), indicating the importance of the cytoplasmatic process in

polyspermy prevention. Only one fertilization cone per fertilized egg was found in the current study. We assume that the fertilization cone is formed only in the micropyle, where the spermatozoon penetrates the egg and blocks fusion of other spermatozoa with cytoplasmatic projection, which the egg formed after fusion with the fertilized spermatozoon.

5. Conclusions

The main finding of this study was unique function of the acrosome in sturgeon, the opening of fingers-like posterolateral projections during penetration, which can serve like an anchor preventing release from the micropyle of egg.

Despite the multiplicity of micropyles in sturgeon may provide the spermatozoa with an advantage of easier entry finding into the egg, it increases the risk of polyspermy. According to Jaffe and Gould (1985) the polyspermy is lethal for the embryo of most animals. In this study we show the mechanisms by which the gametes defend themselves against the unwanted polyspermy: (a) the swelling of CGs of eggs in water, which can trigger the fertilization wave more easily; (b) the creation of long fertilization filament transmitting a signal to the egg to initiate the perivitelline space; (c) creation of perivitelline space, more important for blocking the rest of micropyles and the fertilization cone against fusion of other spermatozoa with cytoplasmatic projection.

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Chapter 7

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General Discussion

General ultrastructure of spermatozoa within sturgeons

The spermatozoa of sturgeon are composed of an acrosome with posterolateral projections (PPs), a nucleus, a midpiece and a flagellum. Three, sometimes two or four, intertwining endonuclear canals (ECs), bound by membranes, traverse the nucleus longitudinally from the acrosome to an implantation fossa in the sturgeon spermatozoa nucleus. The implantation fossa is on the other side of the nucleus from the acrosome, close to the midpiece. The midpiece consists of a proximal and a distal centriole and three to six mitochondria. The midpiece is separated from the flagellum by cytoplasmic canal. The fibrillar part of the flagellum consists of nine peripheral doublets and a central pair of singlet microtubules. The flagellum is favoured in movement with a fin, which is always parallel to the plane of the central microtubules.

The acrosome of sturgeon spermatozoa

The acrosome is commonly divided into a subacrosome, own acrosome and PPs, which are derived at the posterior part of acrosome. Numbers and sizes of PPs also vary between species (DiLauro et al., 2001; Psenicka et al., 2007; Wei et al., 2007). The acrosome of Siberian *Acipenser baerii* (Psenicka et al., 2007), Chinese sturgeon *A. sinensis* (Wei et al., 2007), sterlet *A. ruthenus* (Psenicka et al., 2009a) and pallid sturgeon *Scaphirhynchus albus* (DiLauro et al., 2001) has 10, 10, 9-10 and 8 PPs, respectively. Unfortunately, there are no data yet for other surgeon species. Therefore, there is high inter-specific variation between the sizes of PPs. The length of PPs in sterlet, lake *A. fulvescens*, Atlantic *A. oxyrhinchus*, shortnose *A. brevirostrum*, Chinese, pallid and Siberian sturgeons has been reported with values of 295, 324, 233, 246, 370, 760 and 940 μm, respectively. DiLauro et al. (2002) divided sturgeon spermatozoa according to shape of their acrosome. Atlantic and shortnose sturgeon have the acrosome in shape of a cap of oak acorn, but a cone-like acrosome was found in lateral section of pallid sturgeon spermatozoa. DiLauro et al. (1998, 1999) and Cherr and Clark (1984) found some granular material in subacrosomal region of spermatozoa in Atlantic, lake and shortnose sturgeon. In addition spermatozoa of white sturgeon *A. transmontanus* contain along with the granular also some filamentous material (Cherr and Clark, 1984).

Ciereszko et al. (1996) found the trypsin-like activity in sturgeon spermatozoa sharing many properties with mammalian acrosin. Acrosin appears to be a widely-distributed conserved protein (Baccetti et al., 1989), which had taken place a billion years ago during the early period of eukaryote evolution (Klemm et al., 1991). For this reason, the presence of acrosin in sturgeon sperm was expected. Immunolabeling method shows that acrosin is present in sterlet spermatozoa and localized in the acrosome and the implantation fossa (Psenicka et al., 2009a). In addition scanning electron microscopy on cryofracture evidences the opening of ECs to the acrosome and implantation fossa as well. That means that the acrosome, ECs, and implantation fossa communicate with each other. Therefore we suppose the ECs and implantation fossa designate as components of acrosome.

Except for the Atlantic sturgeon (DiLauro et al., 1998), which has two EC, all other sturgeon species have three EC in their spermatozoa (Ginsburg, 1977; Cherr and Clark, 1984; DiLauro et al., 2000, 2001; Psenicka et al., 2007; Wei et al., 2007). Psenicka et al. (2008a) also recorded some spermatozoa with two or four EC in Siberian sturgeon. There are differences in the diameters of canals between sturgeon species, with values of 35, 97, 49, 57,

40 and 44 nm in Atlantic, shortnose, lake, pallid, sterlet and Siberian sturgeons, respectively. In addition, the number of mitochondria varies also among sturgeon species.

Comparison of morfometric parameters of sturgeon spermatozoa

Mattei (1991) and Lahnsteiner and Patzner (2008) showed the systematic implications of studies on spermatozoa ultrastructure at family, sub-family and species levels. In *Acipenseriformes*, published data confirm these high variations in morphological parameters (Table 4 in Chapter 2). The spermatozoa of white sturgeon (Cherr and Clark, 1985) were the biggest in most parameters (acrosome, nucleus and midpiece) (lenght of acrosome, nucleus and midpiece 11.82 x 1.25 μm), but the smallest head (5.44 x 0.68 μm) of spermatozoa was found in Chinese sturgeon (Wei et al., 2007).

According to Cosson et al. (1999) the spermatozoa with smaller midpiece tend to swim shorter time than spermatozoa with larger midpiece, but number or amount of mitochondria should be less important for short period of spermatozoa movement. The decrease of spermatozoa velocity as well as that of the beat frequency and wave amplitude of flagella corresponds to the decrease of intracellular ATP concentration. The capacity of mitochondria to supply energy is too slow if related to the consumption by the motility mechanics of a fish spermatozoon during its motility phase. This is consistent with results showing that respiration is at the same level before and after initiation of motility in rainbow trout spermatozoa (Christen et al., 1987). Energy consumed by flagellar motion is generated mainly from the ATP accumulated in the flagellum before the onset of activation. The amount of phosphocreatine needed to cover the ATP degradation never fulfils sufficiently the ATP requirement necessary to balance the need during the motility period. Finally, the amount of mitochondria might not be as important for velocity as the content of ATP accumulated in flagellum before triggering the sperm motility. The energy of midpiece mitochondria is used for spermatozoa movement mainly in case of spermatozoa re-activation (Linhart et al. 2008). The time of rapid movement of spermatozoa can be later improved with some artificial activation solution with high pH 8–9 as recommended for tench by Linhart et al. (2005) or for sperm of sex-reversed male rainbow trout by Kobayashi et al. (2004).

The length of flagellum significantly and content of mitochondria insignificantly correlated with the velocity in tench spermatozoa (Psenicka et al., 2009d). Psenicka et al. (2008a) compared main sperm characteristics in Siberian sturgeon and sterlet. There is also a correlation between the flagellum length and velocity and motility especially at the end of motility.

In sturgeon the fibrillar part of the flagellum consists of nine peripheral doublets and a central pair of singlet microtubules. The fin was always parallel to the plane of the two central microtubules of the flagellum. The fins were always flat, indicating no rotation. The junctions between the peripheral doublets support external and internal dynein arms, which represent the main elements of the propelling machinery. The pair of central microtubules is linked by bridges and it is encased in a central sheath. Nine radial spokes or joins linked the central and peripheral doublets (Psenicka et al., 2007)

The plasma membrane of sturgeon spermatozoa is formed into a lateral fin along most of the flagellum length (e.g. pallid sturgeon, DiLauro et al., 2001). These fin structures are preferentially oriented along the horizontal plane defined by the central microtubules (Billard, 1970) and could help increase the efficiency of wave propagation (Cosson et al., 2000). Silver salmon *Oncorhynchus kisutch* spermatozoa have the membrane in central part of tail spiralized with 12 to 15 coils (Lowman, 1953), but Psenicka et al. (2007) described that this fins were flat in Siberian sturgeon spermatozoa, indicating no rotation and exactly parallel to the plane of the two central microtubules of the flagellum. It meens that the central

microtubules are orientated also in the one plane. The fin is not start in the same way on both sides of the flagellum. The first and second fins started 0.7 and 5.3 μm post midpiece respectively, and continued along the flagellum. The first and second fins ended 3.4 and 5.1 μm from the end of the flagellum respectively. It extended distally up to 705.87±220.04 nm in the middle part of flagellum. It is estimated that the surface area of the flagellum, whose shape is that of a simple cylinder (34 million nm^2) is about 1/4 that of the same cylinder comprising fins (160 million nm^2). This has large implications for water exchange/osmotic regulation at activation. At initiation of movement of fish spermatozoa in fresh water species such as sturgeon, the very first signal perceived by the membrane is osmotic; it is followed by a water flux in either direction, which provokes local membrane distortions due to osmotic constraints. As the sperm flagellar membrane in fishes has these unusual fin-shaped creases (Cosson et al, 2000; Psenicka et al., 2007), these significantly increase the membrane surface area thus contributing to an apparent membrane excess favouring water exchange, but also can be easily distorted, eventually leading to blebs on exposure to extreme osmotic situations (Cosson et al., 2000; Perchec et al., 1996).

Despite the significant correlation between flagella lengths of sterlet and Siberian sturgeon spermatozoa and their velocity, more studies are needed to reveal if there is any correlation between the flagellar length and the size of the flagellar fin and swimming pattern of flagella within motility duration and velocity as well.

Independency or negative dependency observed among morphometric characters of sturgeon spermatozoa generally demonstrates that they are variable primarily in their shape rather than size (if shape is defined as a feature of an object independent of its size and orientation; see e.g. Zelditsch et al., 2004). This applies especially for the midpiece and nucleus, where the lengths were negatively correlated with its widths (Psenicka et al., 2007, 2009a).

Although many authors have mentioned and available data also indicated that the morphological parameters of spermatozoa can be useful in sturgeon systematics, Psenicka et al. (2009b) evidence very high significant differences (up to 30 % changes in sizes, $p<0.01$) between different type of sample preparation in electron microscopy, whence we propose to have a respect to this fact for comparative studies.

Species evaluated in studies represented by Chapter 2 (Siberian sturgeon), Chapter 3 (sterlet) and Chinese sturgeon (Wei et al., 2007), where significant differences were found between them in acrosome length and width, nucleus length and width (anterior, central and posterior), midpiece width (both anterior and posterior) and length of flagellum. There the samples were prepared in the same way and hence these data are comparable. Nevertheless countable characters as number of EC or PP etc. are valuable also without particular description of methodology of ultrastructural studies.

Some deviations in fish spermatozoa

Sufficient number of evaluated individuals is important every time for countable spermatozoa characters as well. Psenicka et al. (2009c) found some structural abnormalities of common carp spermatozoa due to defect of spermiogenesis. Spermatozoa of evaluated males had one, two, but also tree flagella as well as multiple number of centrioles, one bigger or two heads, increase amount of mitochondria, several cytoplasmic canals in midpiece, etc. Also flow cytometry evidence some different contents of DNA. Psenicka et al. (2009d) and Linhart et al. (2006) indicate influence of ploidy level on ultrastructure of tench *Tinca tinca* spermatozoa. At first, increased size of heads of spermatozoon of triploid fish has to be mentioned. That logically follows from increased DNA content in nucleus of individuals with higher ploidy level. Chourrout et al. (1986) even suggested that low fertilization rate in

artificially induced tetraploid males of rainbow trout was caused by the head size of spermatozoa, which would be an obstacle during penetration to the micropyle.

Surprisingly, due to the bigger head, which should be a higher load for spermatozoon, motility parameters of spermatozoa were without significant differences, from which there was presumed a higher potential in terms of sperm movement in study of Psenicka et al. (2009d). Following this idea there was focused on amount of mitochondria, which provide energy for spermatozoa movement and length of flagellum as a tool for movement. Significantly higher amount of mitochondria in spermatozoa of triploid tench was reported. Dong et al. (2005) described this feature in tetraploids of Pacific oysters in comparison with diploid ones as well. It can mean that the amount of mitochondria accurately balanced increase of spermatozoa head of individuals with higher ploidy level. As triploid tench balance lower number of erythrocytes in body with higher content of hemoglobin in erythrocytes (Svobodova et al., 1998), spermatozoa of triploid males can compensate bigger head with higher amount of mitochondria. In addition a bigger size of spermatozoa midpiece of triploid was found. Higher number of mitochondria and bigger size of midpiece was observed also in sturgeon spermatozoa from individuals with higher ploidy level within one species (personal observation).

Psenicka et al. (2009d) suppose that mitochondria content has a function to balance changes in ploidy level, but the main influence on spermatozoa velocity has length of the flagellum as main tool for movement, in which more ATP can be also accumulated.

The unusual difference, which was found in spermatozoa of diploid/triploid tench males, was increasing of axoneme microtubules diameter (Psenicka et al., 2009d). Although this fact needs deeper study with using methodology for microtubules visualization, we suppose that there can be a change in count of protofilaments number, those form the microtubules. Davis and Gull (1983) described differences in numbers of microtubules within several species of nematodes. There, all the examined tissues (nerve, intestinal, pharangeal, and hypodermal cells) contained the same number of microtubules protofilaments within one species.

Flagellar length in teleost species varies from 25 μm in tench (Psenicka e al., 2006) to 94 μm in channel catfish *Ictalurus punctatus* (Jasper et al., 1976). Contrariwise flagellum lenghs within sturgeon species show less differences (33.26 and 42.47 μm in Chinese sturgeon and sterlet) (Wei et al., 2008; Psenicka et al., 2009a) and the size of flagellum fin (568.46±170.08 and 705.87±220.04 μm in sterlet and Siberian sturgeon (Psenicka et al., 2007).

Sturgeon eggs

In sturgeons, the ultrastructure of egg envelope differs among species, which might be considered as divergent larval adaptations associated with different reproductive biology strategies (Vorobyeva and Markov, 1999). Sturgeon eggs contain totally six layers, with total thickness of membranes about 70 μm, three main layers (L1, 2 and 3 from inner), an outermost jelly coat, interspased layer between L1 and L2. One to two rows of CGs are located under the main egg layers. The L1 and L2 layers, both containing screw-like projections with spaces around them, display a filamentous material (Cherr and Clark, 1982, Psenicka et al. 2009e). The L3 contains pores and was transformed into micropyles. The pores are filled with the jelly of jelly coat. TEM revealed that the micropyle consisted of a vestibule and a canal at the level of L1. The level of the interspace layer (transition of the vestibule to the canal) was evidently expanded (Psenicka et al. 2009e). Althoug the thickness of the membranes in the animal pole region is with values about 50 μm lower than in the rest of the egg surface, (Dettlaff and Ginsburg, 1954) most teleost egg envelopes thickness ranges from 2

μm to 50 μm. In sturgeons the number of micropyles varies markedly in different females of a species, and also in the eggs of one female (Podushka, 1993a, b). The number of micropyle has been reported as up to 52 micropyles in the Russian sturgeon (Ginsburg, 1968) and 3 to 16 in the Siberian sturgeon (Debus et al., 2002), covering an area about 50 μm (Psenicka et al. 2009e). Also the size of micropyles differs among species. The outer and inner opening diameter of the micropyle is 15 μm and 1.2 μm in white sturgeon (Cherr and Clark, 1982), and 20 μm and 1.5 μm in beluga *Huso huso* (Vorobyeva and Markov, 1999), respectively. Recently, inter-species differences have been reported regarding acrosome width of sturgeon species (Psenicka et al., 2007; Wei et al., 2007). The diameter of the egg micropyle seems to be related to the diameter of head (acrosome) of the spermatozoa (Psenicka et al., 2009e). This can be considered for further studies. However, the above parameters do not allow for secure species identification (phylogeny aim) due to inter-species differences in size of eggs and number of micropyles.

Activation of sturgeon eggs by water

In agreement to results reported in paddlefish *Polyodon spathula* (Linhart and Kudo, 1997) and white sturgeon (Cherr and Clark, 1985), layers 1 and 2 are composed of fibrils that possess filamentous substructural elements and exhibit no change through activation, fertilization and cleavage. Similar to white sturgeon (Cherr and Clarr, 1985), the jelly coat contains glycosylated proteins, and may block micropyles entrance. In both Siberian (Psenicka et al. 2009a) and white (Cherr and Clark, 1985) sturgeon eggs this amorphous layer is released from the surface and the duct of the L3, and it hydrates within 5 min, when immersed in freshwater. Cherr and Clark (1985) postulated that Ca^{++} and/or Mg^{++} ions are probably required for jelly release and hydration, due to inhibition of hydration in freshwater that lacks Ca^{++} and Mg^{++} ions. Futhermore, hydration could be inhibit or block by inhibitors of trypsin-like enzymes. Our preliminary results infer that sturgeon eggs washed in hatching water, even several minutes, were capable of fertilization and, in addition, the eggs, where the jelly coat was washed, had higher rate of hatching than eggs fertilized in standard way (mixing of eggs, sperm and water together in one time) without any washing (personal observation).

Prevention of polyspermy

Immediately after fusion, when the first spermatozoon penetrates to the oocyte plasma membrane, the entire egg envelope was separated from the oocyte plasma membrane by the enlarging perivitelline space (PS) filled with the material from CGs, which agglutinated other spermatozoa (Cherr and Clark, 1985; Dettlaff et al., 1993). The fertilization wave moves through the cortex of the oocyte from the site of sperm attachment to the opposite side of the oocyte (Psenicka et al. 2009e). Regarding multiplicity of micropyles is the creation of PS the most important mechanism from prevention of polyspermy in sturgeon. The CGs are specialized Golgi-derived secretory granules located just below the plasma membrane of mature unfertilized egg (Epel et al., 1977)., When the eggs were washed by water, their jelly coat, which was found on the whole egg and like a stopper over micropyles**,** was completely release within one minute and CGs swelled but they weren't broken until after the egg had been fertilized. We suppose that so swelled CGs can break more easily and create the PS quicker. That can be another feature of block polyspermy acceleration in addition to the creation of fertilization filament.

Mature eggs of several fish species respond to sperm entry by a formation of fertilization cone at the sperm entry site (Kudo, 1980, 1983; Kobayashi and Yamamoto, 1981;

Kudo and Sato, 1985; Iwamatsu et al., 1991, Linhart and Kudo, 1997). In sturgeon, approximately 60 sec post fertilization the fertilization cone extended to about 20-30 μm in width, with the ball-like enlarged apex of the cone reaching the micropylar vestibule. Spermatozoa are never able to fuse with the cone membrane. The cone is formed only in fertilized micropyle. In other micropyles only CGs exudates are identified. The plasma membrane of supernumerary spermatozoa is unable to fuse with that of the fertilization cone (Kudo, 1980; Kobayashi and Yamamoto, 1981; Psenicka et al. 2009e), indicating the importance of the cytoplasmatic process in polyspermy prevention. It supposes that the fertilization cone above all blocks fusion of other spermatozoa with cytoplasmatic projection, which the egg formed after fusion with the fertilized spermatozoon.

Monitoring of the acrosomal reaction

Psenicka et al. (2008b) summarized methodology, by which the acrosomal process is possible to observe. There electron microscopy, face contrast of light microscopy and fluorescent microscopy are evaluated. Psenicka et al. (2008c,d) discovered usefulness of Soybean Trypsin Inhibitor conjugated with Alexa Fluor® 488 for screening of acrosomal reaction in sturgeon spermatozoa. This method seems to be the most objective for acrosomal integrity evaluation. There was found differences in the integrity of acrosome in fresh sperm within males, between fresh and cryopreserved sperm and different kinds of cryoprotectants. As the best cryoprotectant was found 10% methanol, which has no significant effect on the acrosomal integrity. Contrariwise DMSO (dimethylsulfoxide) caused 100% increase (12%) in acrosomal specific staining determinig damage of the acrosome or acrosomal reaction. While using this staining the suitable activation solution containing 2.5 mM Ca^{2+}, 15 mM tris with pH 10 was selected for sterlet spermatozoa. Sterlet sperm was treated with this selected solution and used for fertilization. There was evidenced a usefulness of acrosome, because the percentage of hatching rapidly decreased and in addition negatively correlated with percentage of disrupted acrosomes within males, in spite of abilities of movement were sustained (personal observation).

Acrosomal process

In general, the typical organelle of the spermatozoon, the acrosome, is considered to be responsible for enabling the spermatozoon to traverse investment coats surrounding an egg. Most spermatozoa undergo an acrosomal reaction in response to an egg component; this acrosomal reaction exposes the contents of the acrosome, which includes enzymes and binding proteins (Dan, 1967; Shapiro and Eddy, 1980; Lopo, 1983). Tunicate, lampreys, hangfish (*Eptatretus burgeri* and *E. stouti*) and sturgeons are reported to possess sperm that form fertilization filaments during the acrosome reaction (Cherr and Clark, 1984; Dettlaff et al., 1993; Morisawa, 1995, 1999a, b, Psenicka et al., 2009e). Lamprey eggs are covered by a penetration envelope that lacks micropyles (Kille, 1960). Morisawa and Cherr (2002) described acrosome reaction in hangfish, where the spermatozoa have to penetrate through a U-shape layer filling the bottom of the micropyle (Morisawa, 1999b). However, sturgeon eggs possess an impenetrable envelope, which is perforated by numerous micropyles. The latter provide spermatozoon direct access to the oolema, except in the JC, which has to be release from the micropyle by water. The multiplicity of micropyles could be advantageous of easier entry finding for spermatozoon in terms of fertilization in fast flowing water but on the other hand there is a risk of polyspermy. Therefore, the presence of acrosomes and long acrosomal processes in sturgeon spermatozoa is inconclusive and does not agree with models

of acrosomal evolution (Baccetti and Afzelius, 1976; Baccetti, 1979) and this mechanism differs from all others. The micropyles of sturgeon eggs are filled with spermatozoa about 15 sec post fertilization. The spermatozoon, which was almost at the bottom of the micropyle, was merged with a cytoplasmic projection of the egg immersed into the inner micropyle. The membrane of the acrosome was decomposed, starting from the apical part. Upon acrosomal reaction the material in the three ECs and implantation fossa of the sperm head was ejected forward through the acrosome like a fine acrosomal filament (14.22 μm long on average), which served like a harpoon (Psenicka et al. 2008b,c,d 2009e). We observed the acrosomal process only at the bottom of the micropyle, but no reaction was observed at the funnel part of the micropyle. With reference to multiplicity of micropyles, it was supposed that the filament serves as a quick signal transducer carrying the information for the egg about the presence of sperm in its close proximity and induces the creation of cytoplasmic projection of the egg for fusion with the spermatozoon and block of polyspermy by PS in all other micropyles (Psenicka et al. 2009e).

Lost of flagellum during fertilization

During the fusion of spermatozoon with the egg a fracture of midpiece in transition of distal centriole into the flagellum was observed, which is in the weakest part of flagellum without central microtubules and the centriolar complex remains with nucleus. It seems that the release of implantation fossa content during formation of fertilization filamanet could couse the loss of flagellum during acrosome reaction. The idea of flagellum loss and the centriole remaining bound to sperm head was supported by the fact that 82 % of spermatozoa showed completely broken or partially damaged flagellum near the midpiece after activation. In addition, no flagellum was found either in sections of fertilized egg using TEM or confocal microscopy. Only a centriole was observed (Psenicka et al. 2009e). Fibrous structure in implantaion fossa was found in Atlantic or shortnose sturgeon spermatozoa (DiLauro a kol., 1998, 1999). It probably connects and keeps the proximal centriole with the ECs. Then the centriole stays with the nucleus after waste of flagellum.

Function of posterolateral projections

Acrosomes of sturgeon spermatozoa are terminated by PPs that are radially distributed lobes, The diameter of the micropylar base in Siberian sturgeon eggs (1.95 ± 0.26 μm) was only slightly larger than the diameter of the spermatozoon's head (1.13 ± 0.16). As a result of that no more but one sperm can enter the micropyle. When the spermatozoon of Siberian sturgeon was penetrating through the micropyle into the egg the 10 PPs opened and served as an anchor, which is unique feature of the sturgeon spermatozoa. To confirm opening of the PPs, the distance of PPs from the nucleus was measured in cross-sections in the PPs region of activated spermatozoa (0.19±0.12 μm) and non-activated spermatozoa (0.14±0.04 μm). The difference was highly significant ($p<0.01$) as well as the distance of PPs of spermatozoa in micropyles (0.20±0.16 μm). The opening seems to be caused by acrosomal filament formation, due to which the outer acrosomal membrane is stretched. Nevertheless, the function of PPs is not well understood and more studies are needed to accurately determine their role in fertilization.

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English Summary

Ultrastructure of spermatozoa and fertilization process in sturgeon

Martin Pšenička

The aim of studies mentioned above was to characterize spermatozoa and fertilization process in sturgeon. Gametes of sturgeon fishes differ from those of teleost fishes mainly by the presence of acrosome in spermatozoon, which creates a long acrosomal filament during penetration into the egg, and multiple micropyles in eggs. The multiplicity of micropyles in sturgeon eggs can be advantage of easier entry finding for spermatozoon, but increases risk of polyspermy. These features don't agree with models of acrosomal evolution and don't occur together in any other examined animals. The present thesis describes ultrastructure of spermatozoa (chapter 2) with focusing on acrosome (chapter 3), suggests methodology for marking and monitoring of the acrosomal reaction and/or integrity of acrosome (chapter 4), evaluates an influence of cryopreservation on acrosome (chapter 5), and usefulness of acrosome for fertilization, describes process of fertilization and blocking of polyspermy in sturgeon (chapter 6).

The sturgeon spermatozoon is differentiated into an elongated head with a nucleus and an acrosome, a midpiece and a flagellum.

The acrosome can be divided into own acrosome, a subacrosome and fingers-like posterolateral projections adjoining to the nucleus. These characters of sturgeon spermatozoa are unique and number, size and shape of posterolateral projections is species specific within sturgeons. Most of sturgeons have the acrosome with right-handed posterolateral projections, except pallid sturgeon. The acrosome of Siberian, Chinese, sterlet and pallid sturgeon have 10, 10, 9-10 and 8 posterolateral projections, respectively.

Three, sometimes two or four, intertwining endonuclear canals, bound by membranes, traverse the nucleus longitudinally from the acrosome to implantation fossa in the sturgeon spermatozoa. Chapter 3 evidences a communication between the acrosome and the implantation fossa by these canals and presence of acrosin in them. That is way it was supposed the endonuclear canals and the implantation fossa designate as components of the acrosome.

The cylindrically shaped midpiece of sturgeon spermatozoa contains a centriolar complex composed of a proximal and distal centriole. It has a function of anchorage the flagellum to the head. Three to six mitochondria were observed in a peripheral section of the midpiece, sometimes surrounding the nucleus. The midpiece is separated from the flagellum by cytoplasmic canal.

The flagellum is favoured in movement with a fin. The fibrillar part of the flagellum consists of nine peripheral doublets and a central pair of singlet microtubules. The fin was always parallel to the plane of the two central microtubules of the flagellum. The fins were always flat, indicating no rotation. The junctions between the peripheral doublets support external and internal dynein arms, which represent the main elements of the propelling machinery. The pair of central microtubules is linked by bridges and it is encased in a central sheath. Nine radial spokes or joins linked the central and peripheral doublets.

Trypsin inhibitor from soybean, Alexa Fluor® 488 conjugate was found as the best tool for marking and monitoring of acrosomal reaction or its integrity. Differences in acrosomal integrity have been already observed in fresh sperm within males, between fresh and cryopreserved spermatozoa and different kinds of used cryoprotectants. Study in chapter

5 labels DMSO with 12 % of the marked acrosomes as worse cryoprotectant then methanol with 6%. This fluorescent dye allowed also finding of an activation solution for the acrosome. The suitable activation solution contained 2.5 mM $CaCl_2$, 15 mM tris, pH 10. These findings allow a deeper study of acrosomal reaction. The last research foreshadows that the acrosome and acrosomal process are necessary for right fertilization, because spermatozoa with inactivated acrosomes were not able to fertilize the eggs. Nevertheless other studies are needed for understanding of this process.

Chapter 6 describes two mechanical functions of the acrosome occuring during penetration. As the first function of acrosome an opening of posterolateral projections was observed, which can be compared with an anchor against release from the micropyle. Distances of posterolateral projections from nucleus in activated spermatozoa activated by found activation solution were significantly different then non-activated spermatozoa ($p<0.01$). The same was proofed also in spermatozoa penetrating through the micropyle. The second found function was a spear–like formation of a long fertilization filament creating probably from endonuclear canals and implantation fossa. That gives a quick signal for formation of cytoplasmic projection, with which the spermatozoon is fused, dissolving of cortical granules and creation of perivitelline space. This mechanism can accelerate the blocking of other micropyles.

The outermost jelly coat can block entry of spermatozoon until the eggs were washed by water and the cortical granules swelled in water but they weren't broken until after the egg had been fertilized. Own fertilized micropyle is blocked by so called fertilization cone filling whole area of this micropyle.

Despite the multiplicity of micropyles in sturgeon may provide the spermatozoa with an advantage of easier entry finding into the egg, this feature increases the risk of polyspermy, which is lethal for the embryo or can affect its ploidy level. These studies explain the mechanisms by which the gametes defend themselves against the unwanted polyspermy: (a) the swelling of cortical granules of eggs in water, which can then trigger the fertilization wave more easily; (b) the creation of long fertilization filament transmitting a signal to the egg to initiate the perivitelline space; (c) the primary creation of perivitelline space, more important for blocking the rest of micropyles, and secondary the creation of fertilization cone against fusion of other spermatozoa with cytoplasmatic projection.

Czech Summary
Souhrn

Ultrastruktura spermií a fertilizačního procesu jeseterů

Martin Pšenička

Cíl prací uvedených výše bylo charakteryzovat spermie a fertilizační proces jeseterů. Gamety jeseterů se liší od gamet kostnatých ryb především přítomností akrozomu u spermií, který vytváří dlouhý fertilizační filament během penetrace do jikry, a množství mikropylí v jikře. Vyšší počet mikropylí u jiker jeseterů může být výhodou snazšího nalezení vstupu pro spermii, ale zvyšuje riziko polyspermie. Tyto vlastnosti nesouhlasí s modelem evoluce akrozomu a spolu se nevykytují u žádných dalších zkoumaných zvířat. Uvedená disertační práce popisuje ultrastrukturu spermie (kapitola 2) se zaměřením na akrozom (kapitola 3), navrhuje metodiku značení a monitorování akrozomové reakce a/nebo integrity akrozomu (chapter 4), zhodnocuje vliv kryoprezervace na akrozom (kapitola 5) a důležitost akrozomu pro fertilizaci, popisuje proces fertilizace a blokace polyspermie u jeseterů (chapter 6).

Spermie jeseterů je diferencovaná na podlouhlou hlavičku s jádrem a akrozomem, středním oddílem a bičíkem.

Akrozom můžeme dělit na vlastní akrozom, subakrozom a prstůmpodobné postakrozomální výběžky přiléhající k jádru. Tyto znaky jeseteřích spermií jsou unikátní a počet, velikost a tvar posterolaterálních výběžků je druhově specifická mezi jesetery. Většina jeseterovitých, vyjma lopatonose, má akrozom s pravotočivými posterolaterálními výběžky. Akrozomy jesetera sibiřského, čínského, malého a lopatonose velkého mají 10, 10, 9-10 a 8 posterolaterálních výběžků.

Tři, někdy dva nebo čtyři, propletené endonukleární kanály, ohraničené membránou, probíhají podélně jádrem od akrozomu až po implantační jamku u spermií jeseterů. Kapitola 3 prokazuje komunikaci mezi akrozomem a implantační jamkou pomocí těchto kanálů a přítomnost akrosinu v těchto částech spermie. Proto navrhujeme endonukleární kanály a implantační jamku označovat jako komponenty akrozomu.

Cylindricky tvarovaný střední oddíl spermie jeseterů obsahoval centriolarní komplex složený z proximální a distální centrioly. Ten má funkci ukotvení bičíku k hlavičce spermie. Tři až šest mitochondrií bylo pozorováno po obvodu středního oddílu, někdy přiléhaly k jádru. Od bičíku byl střední oddíl oddělený cytoplasmatickým kanálem.

Bičík je zvýhodněný v pohybu laterálním lemem. Fibrilární část bičíku se skládá z devíti periferních dublet a centrálního páru jednotlivých mikrotubulů. Laterální lem byl vždy orientován v rovině centrálních mikrotubulů bičíku. Laterální lemy byly vždy v jedné rovině, bez žádných otáček. Spojení mezi periferními dublety zajišťovali externí a interní dyneínová ramínka, která prezentují hlavní prvek hnacího aparátu. Pár centrálních mikrotubulů je spojen můstky a uzavřen v centrální pochvě. Devět radiálních paprsků spojovalo centrální a periferní mikrotubuly.

Trypsin inhibitor from soybean, Alexa Fluor® 488 conjugate byl nalezen jako nejlepší nástroj pro značení a sledování acrozomální reakce nebo jeho integrity. Rozdíly v akrozomální integritě byly již pozorovány u čerstvých spermií mezi mlíčáky, mezi čerstvými a kryoprezervovanými spermiemi a ruzných druhů použitých kryoprotektantů. Studie v kapitole 5 shledává DMSO s 12 % označených akrozomů jako horší kryoprotektant než metanol s 6 %. Tento fluorescent umožňuje také nalezení aktivačního roztoku pro

akrozom. Vhodný aktivační roztok obsahoval 2,5 mM $CaCl_2$, 15 mM tris, pH 10. Tento poznatek umožňuje hlubší studii akrozomální reakce. Poslední výzkum naznačuje, že akrozom a akrozomální proces je nezbytný pro zprávnou fertilizaci, protože spermie s inaktivovaným akrozomem nebyla schopná oplodnit vajíčko. Nicméně další studie jsou potřebné pro pochopení tohoto procesu.

Kapitola 6 popisuje dvě mechanické funkce akrozomu probíhající během penetrace. Jako první funkce akrozomu bylo pozorováno otevírání posterolaterálních výběžků, které mohou být přirovnány ke kotvě bránící vypadnutí z mikropyle. Vzdálenost posterolaterálních výběžků od jádra u aktivovaných spermií pomocí nalezeného aktivačního roztoku byla signifikantně větší než u neaktivovaných ($p<0.01$). To samé bylo prokázáno i u spermií pronikající mikropylárními otvory. Druhou nalezenou funkcí akrozomu bylo kopípodobné formování dlouhého fertilizačního filamentu, vznikajícího pravděpodobně z endonukleárních kanálů a implantační jamky. Ten dává rychlý signál pro formování cytoplasmatického výběžku, se kterým spermie fúzuje, rozpuštění kortikálních granulí a vznik perivitelliního prostoru. Tento mechanismus může zrychlit blokaci dalších mikropylí.

Když je jikra aktivovaná pouze vodou, bez oplození, tak se rozpustí její povrchová rosolovitá vrstva, která jinak může bránit vstupu spermie, a nabobtnají kortikální granule. Ty ovšem až do oplození nepraskají. Vlastní oplozené mikropyle je pak blokováno tzv. fertilizačním kuželem, vyplňující celý prostor tohoto mikropyle.

Přestože vyšší počet mikropylí může poskytnout spermii výhodu snazšího nalezení vstupu do jikry, tak tato vlatnost zvyšuje riziko polyspermie, která je letální pro embryo nebo může ovlivnit jeho plodní úroveň. Tyto studie vysvětlují mechanizmy, kterými se gamety brání proti nechtěné polyspermii: (a) bobtnání kortikálních granulí jikry ve vodě, které potom mohou snadněji spoušťet fertilizačním vlnu; (b) vznik dlouhého fertilizačního filamentu vysílajícího signál do jikry pro iniciaci perivitelinního prostoru; (c) prvořadý vznik perivitelinního prostoru, více důležitého pro blokaci zbylých mikropylárních otvorů, a sekundárně vznik fertilizačního kužele, bránící fúzi dalších spermií s cytoplasmatickým výběžkem.

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List of Publications

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Psenicka, M., Ciereszko, A., Cosson, J., Kaspar, V., Nebesarova, J. and Linhart, O. Function of the Acrosomal Reaction in Sturgeon spermatozoa. The 7th International Symposium "Innovative Marine Life Science for Three *E*s, *E*dibles, *E*nvironment and *E*ducation, in 21st Century", 17-19 Nov 2008, Sapporo, Japan. (poster presentation)

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Alavi, S.M.H., **Psenicka**, **M.,** Policar, T., Rodina, M., Hamackova, J., Linhart, O. Ultrastructure, biochemical characteristics and motility of *Barbus barbus* spermatozoa. 1st International Workshop on Biology if Fish Sperm 29-31 August 2007, the Czech Republic, Vodnany. (oral presentation)

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Wei, Q., Li, P., **Psenicka, M.**, Alavi, S.M.H., Shen, L., Liu, J., Peknicova, J., Linhart O. Ultrastructure and morphology of spermatozoa in Chinese sturgeon (*Acipenser sinensis* Gray 1835) using scanning and transmission electron microscopy. 1st International Workshop on Biology if Fish Sperm 29-31 August 2007, the Czech Republic, Vodnany (poster presentation)

Linhart, O., Alavi, S.M.H., **Psenicka, M.,** Rodina, M., Cosson, J., Mims, S.D., Shelton, W.L. Structure, motility and cryopreservation of sperm of Chondrostean fish species. In: Programme and Abstract: 10th International Symposium on Spermatology, 17-22 September 2006, Madrid, Spain. (oral presentation)

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Training and Supervision Plan during Study

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Research department	Department of Fish Genetics and Breeding
Daily supervisor	
Supervisor	Prof. Dipl.-Ing. Otomar Linhart, DSc.
Period	3th October 2005 until 24th of September 2009

PhD courses	**year**
Fish Reproduction	2006
Biostatistic	2006
Fish Genetics	2006
Ichthyology and Fish Taxonomy	2007
English	2007

Scientific seminars	**year**
Seminar days of RIFCH	2006 2007 2008 2009

International conferences	**Year**
IVth International Workshop on Biology and Culture of the Tench, 20-23 September, Wierzba, Poland (*poster presentation*)	2004
8th International Symposium on Reproductive Physiology of Fish. June 3 – 8, Saint Malo, France (*poster presentation*).	2007
8th Multinational Congress on Microscopy, 17-21 June, Prague, Czech Republic (*poster presentation*)	2007
1st International Workshop on Biology if Fish Sperm, 29-31 August, Vodnany, Czech Republic (*two posters and one oral presentation*)	2007
5th International Workshop on Biology and Culture of the Tench, September 29 – October 3, 2008, Ceresole d'Alba, Italy (*poster presentation*).	2008
International Konference Aqaculture europe 2008, Krakov, Poland, September 15-18. (*oral presentation*)	2008
The 7th International Symposium "Innovative Marine Life Science for Three *E*s, *E*dibles, *E*nvironment and *E*ducation, in 21st Century", 17-19 Nov 2008, Sapporo, Japan. (poster presentation)	2008
Natinal conference	
IX. Ichtologická konference	2006

Foreign stays during PhD study at RIFCH	**Term**	**year**
Prof. Jacky Cosson, Univ. Paris VI, Observatore de Villefranche/mer; Marine Station, Villefranche-sur-mer, France. (Fluorescent microscopy and immunolabeling)	1 month	2006
Prof. Andrzej Ciereszko, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland. (Monitoring of the acrosomal reaction in sturgeon)	2 month	2007
Prof. Arai Katsutoshi, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan. (Biotechnology and Micromanipulation)	1 month	2008

Curriculum Vitae

Surname: Pšenička
First name: Martin
Title: Dipl.-Ing.
Born: 17 January, 1981 in Karlovy Vary
Nationality: Czech
Martial Status: Single

Education: M.Sc. - University of South Bohemia, Agricultural Faculty, Ceske Budejovice, specialization fishery, 2000 - 2005.

Professional experience:
2005 – 2008 – PhD student USB RIFCH, Dep. of Fish Genetics and Breeding
2009 - present PhD student, USB RIFCH, Laboratory of Molecular, Cellular and Quantitative Genetics
PhD courses: Biostatistic, Fish reproduction, Fish genetics, Ichtyology, English language.
Specialization: Reproduction of fish
Knowledge of languages: English – in a word and writing.

Foreign stays during PhD study at RIFCH
2006 Prof. Jacky Cosson, Univ. Paris VI, Observatore de Villefranche/mer; Marine Station, Villefranche-sur-mer, France. (Fluorescent microscopy and immunolabeling)
2007 Prof. Andrzej Ciereszko, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland. (Monitoring of the acrosomal reaction in sturgeon).
2008 Prof. Arai Katsutoshi, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan. (Biotechnology and micromanipulation)

Responsible leader in project of the Grant Agency of USB no. 2/2005/P-VÚRH, Ultrastructure of spermatozoa of Siberian sturgeon (*Acipenser baerii*) and tench (*Tinca tinca*)

Pedagogical experiences: Consultant of one diploma thesis, Seminar days at USB RIFCH (2006, 2007, 2008, 2009), seminars on the topic fish reproduction in Olsztyn, Poland 2007, in Hakkodate, Japan 2008, for the Summer school 2008, courses in terms of Artificial Reproduction in Fish and Biotechnology in Fish, ordering of excursions.

Awards
The prize of dean of the Agriculture faculty for presentation of master thesis in 2005. "Ultrastructure of spermatozoa of Siberian sturgeon *Acipenser baerii* and tench *Tinca tinca*"
The prize of the director of RIFCH for the best publication in 2006. "Psenicka, M., Rodina, M., Nebesarova, J. and Linhart, O. (2006). Ultrastructure of spermatozoa of tench *Tinca tinca* observed by means of scanning and transmission electron microscopy. *Theriogenology* 66, 1355-1363."
The prize of the best presentation in 1st International Workshop on Biology of Fish Sperm, "M. Psenicka, S. M. H. Alavi, M. Rodina, J. Nebesarova, O. Linhart (2007) Studies on ultrastructure of sperm and eggs upon fertilization in Siberian sturgeon (*Acipenser baerii*)"