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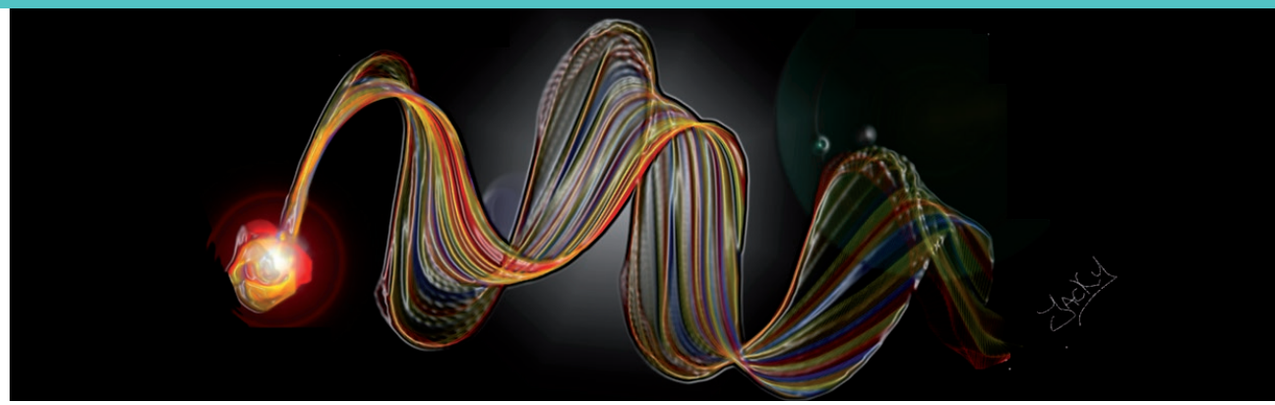
Role of regulatory proteins in fish sperm motility

Význam regulačních proteinů pro pohyblivost rybích spermií



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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

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Review Article

Motility of fish spermatozoa: from external signaling to flagella response

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ABSTRACT

For successful fertilization, spermatozoa must access, bind, and penetrate an egg, processes for which activation of spermatozoa motility is a prerequisite. Fish spermatozoa are stored in seminal plasma where they are immotile during transit through the genital tract of most externally fertilizing teleosts and chondrosteans. Under natural conditions, motility is induced immediately following release of spermatozoa from the male genital tract into the aqueous environment. The nature of an external trigger for the initiation of motility is highly dependent on the aquatic environment (fresh or salt water) and the species' reproductive behavior. Triggering signals include osmotic pressure, ionic and gaseous components of external media and, in some cases, egg-derived substances. Extensive study of environmental factors influencing fish spermatozoa motility has led to the proposal of several mechanisms of activation in freshwater and marine fish. However, the signal transduction pathways initiated by these mechanisms remain clear. This review presents the current knowledge with respect to (1) membrane reception of the activation signal and its transduction through the spermatozoa plasma membrane *via* the external membrane components, ion channels, and aquaporins; (2) cytoplasmic trafficking of the activation signal; (3) final steps of the signaling, including signal transduction to the axonemal machinery, and activation of axonemal dyneins and regulation of their activity; and (4) pathways supplying energy for flagellar motility.

1. Introduction

Sperm quality is a measure of the ability of spermatozoa to successfully fertilize an egg [1]. Spermatozoa motility must be activated to allow them to reach, bind, and penetrate the egg. As a general rule, in fish with external fertilization, spermatozoa are immotile in testis and in seminal fluid [2], and initiation of motility is dependent on the fertilization environment [3–7]. Several factors are known to regulate

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spermatozoa motility. In salmonid fishes, motility is initiated by a decrease in potassium ion (K^+) concentration surrounding the spawned spermatozoa when they are released into fresh water [4]. The main factor in initiation of spermatozoa motility in cyprinid fishes is a decrease in osmolality upon spawning into fresh water [5]. Conversely, hypertonicity of the surrounding medium triggers the motility of spermatozoa of marine teleosts [7]. The motility of spermatozoa in the euryhaline fish, medaka, *Oryzias latipes*, is not so strictly dependent on environmental osmolality, and may be initiated by values ranging from 25 to 686 mOsm/kg [6]. The fore-mentioned examples suggest the existence of a variety of specific signaling pathways for spermatozoa activation. The species-specific differences in sperm sensitivity to environmental osmotic pressure and ion composition, membrane polarization/depolarization processes, involvement of signaling molecules (*e.g.*, cAMP), and phosphorylation of flagella proteins [2,3,8,9] are of special interest. The primary goal of this review is to summarize current knowledge on signaling pathways involved in the process of spermatozoa activation from the reception of the external signal at the level of the plasma membrane to the activation of axonemal dyneins.

2. Activation of fish spermatozoa motility – exogenous signals

2.1. *Duration of spermatozoa motility*

Motility activation of fish spermatozoa is a process lasting fractions of a second, making studies of the biochemical processes underlying the activity of axonemal structures technically difficult [2,8,10]. Estimations of time required for flagellar activity initiation have been described in few fish species. In turbot, *Psetta maxima*, activation of the flagellum after ceasing CO_2 application occurred within 100 ms [11]. In pipefish, *Syngnathus abaster*, whose females deposit eggs into a male organ where fertilization occurs, spermatozoa undergo activation within 80 ms [12]. In most freshwater species, spermatozoa are usually motile for less than 2 min, and, in some cases, are highly active for less than 30 s [13,14]. However, the duration of the sperm motility period can be significantly influenced by the final osmolality, and temperature of the activation media [15], reproduction mode (external or internal fertilization) [3,12,16], and techniques used for preparation of samples for motility observation [10].

Fish species such as the spotted wolf-fish, *Anarhichas minor*, and the 3-spined stickleback, *Gasterosteus aculeatus*, possess spermatozoa that remain motile for several hours after release [17,18]. A prolonged period of spermatozoa motility has also been found in the ocean pout, *Macrozoarces americanus*, and marine sculpin, *Alcichthys alcicornis* [16,19]. Koya et al. [19] found that when the semen of *A. alcicornis* was diluted with a small volume of ovarian fluid, the spermatozoa continued to swim for at least two days, and, with the use of artificial ovarian fluid, motility continued for 7–14 days. The spermatozoa of the ocean pout remained motile in seminal plasma and ovarian fluid for at least 24 h at 4 °C [16]. In addition to ovarian fluid, substances released by the eggs can affect fish spermatozoa motility. Some of these substances are involved in the activation process, while others are sperm-attracting chemicals involved in concentrating spermatozoa in the vicinity of the egg micropyle to increase fertilization success [20].

2.2. *Effect of environmental osmolality on motility activation*

Under natural conditions, motility is induced after the release of spermatozoa from the genital tract into the aqueous environment, where spermatozoa encounter water-soluble components of the external milieu, primarily ions. The environmental osmotic pressure also seems to be consistently involved in activation among species [3,8]. Osmolality of seminal plasma differs significantly between freshwater and saltwater fishes [3]. In freshwater fishes, osmolality ranges from 230 to 346 mOsm/kg (except Acipenseridae at 38–96 mOsm/kg), and for marine fishes, ranges from 249 to 400 mOsm/kg [3]. Thus, fresh water (20–40 mOsm/kg) and sea water (600–1800 mOsm/kg) represent, respectively, a hypotonic and hypertonic environment for a spermatozoon.

The nature of external signal triggering of motility is highly dependent on the fish reproduction environment and the peculiarities of reproductive behavior. Following activation in ambient water, freshwater fish spermatozoa increase cytoplasmic volume in response to hypotonicity. Under hypo-osmotic conditions (45 mOsm/kg), carp, *Cyprinus carpio*, spermatozoa increase in volume several-fold as a result of water influx [21], while rainbow trout, *Oncorhynchus mykiss*, spermatozoa, under similar osmotic conditions (50 mOsm/kg), show less dramatic changes (~30%) [22]. Thus, the tendency of spermatozoa to swell in hypotonic conditions is species-specific. A possible regulatory role of spermatozoa swelling will be discussed later in this review.

In addition to osmolality, other factors may be required for activation of motility in marine fish spermatozoa. For example, activation of herring spermatozoa requires egg-derived substances [23,24]. Two types of sperm-activating factors have been identified in Pacific herring, *Clupea pallasii*, eggs: a water-soluble protein released into the surrounding water [23] and a water-insoluble sperm-motility-initiating factor localized in the vicinity of the micropylar opening of eggs [24]. In contrast, the seminal fluid of Nile tilapia, *Oreochromis niloticus*, contains a sperm motility inhibiting factor [25]. Contact of semen with the surrounding medium leads to activation only when the dilution rate is sufficient to significantly decrease seminal plasma concentration of a high molecular weight glycoprotein that is responsible for immotility.

There are many cases in which isotonic media effectively activate sperm motility in freshwater species including northern pike, *Esox lucius* [26], and Persian sturgeon, *Acipenser persicus* [27]; saltwater species turbot, *Scophthalmus maximus* [28], and haarder, *Mugil soiuy* [29]; and seawater and freshwater acclimated tilapia, *Oreochromis mossambicus* [30].

2.3. *Effect of ionic and gaseous components of the environment on motility activation*

There are extensive data concerning the effects of K^+, Ca^{2+}, Mg^{2+}, and other cations on fish spermatozoa activation [3,7,8],

and it is difficult to distinguish the roles of specific ions. The data dealing with the requirement for extracellular Ca^{2+} and K^+ in the micropyle area for initiation of herring spermatozoa motility suggest that an appropriate balance of monovalent and divalent ions in the medium is essential for activation by spermatozoa-motility-initiating factor in the micropyle area [24].

The general mechanisms of the effects of gaseous components of external media have not been described in detail. Spermatozoa motility in flatfish species halibut, *Verasper variegatus*; flounder, *Verasper moserii*; and turbot, *P. maxima*, has been shown to be arrested by a gentle stream of CO_2, probably *via* the contribution of dissolved CO_2 in equilibrium with HCO_3^- to ion concentration and osmolality [8,11]. Nitric oxide (NO), in turn, was demonstrated to enhance motility of fathead minnow, *Pimephelas promelas*, spermatozoa [31]. The mechanism by which NO affects motility is probably unrelated to osmolality, since the NO concentration used was very low.

Nitric oxide is a biologically active molecule generated by most of an organism's cells that, due to its low molecular weight and lipophilic nature, diffuses quickly through lipid membranes and is implicated in a variety of physiological cellular signaling mechanisms in many tissues. The role of NO in mammalian sperm physiology is well described [32], but little information with respect to its influence on fish sperm is available. Creech et al. [31] demonstrated that low NO concentration in activating media resulted in the enhancement of motility percentage and velocity parameters such as curvilinear velocity, straight-line velocity, and average path velocity of fathead minnow spermatozoa, while higher concentrations of NO decreased all the mentioned parameters. In that study, NO production by spermatozoa was not detected. However, possible involvement of NO in spermatozoa motility regulation *via* NO release from the egg was proposed [31]. In other study [33], it was shown that NO present at high or low concentrations in activating medium did not result in respective inhibition or enhancement of percent motile spermatozoa and velocity in steelhead, *O. mykiss*. Moreover, the latter authors detected NO production in quiescent spermatozoa and suggested the possible regulation of their basic metabolism through NO-derived changes in respiration. Such insufficient and partially conflicting data points to the necessity for further research on the role of NO in fish spermatozoa function.

Based on extensive and multi-faceted studies of environmental factors influencing fish spermatozoa motility, several mechanisms of freshwater and marine fish spermatozoa activation have been proposed [3,7,8,34–36]. A description of the general principles of these mechanisms was summarized by Morisawa [9]. Basically, the environment-dependent activation of ion channels leads to changes in intracellular ion concentration which, in turn, alter membrane polarization and switch on the Ca^{2+} signaling cascade. However, the processes involved in transfer from environmental signal to flagellar activity remain unclear. Information related to these pathways will be reviewed in the following chapters. As information concerning sperm of fish is scarce, data obtained from other animal species will be also considered.

3. Membrane reception of the activation signals and their transduction through the membrane

Under natural conditions, external signals triggering spermatozoa motility act at the level of the plasma membrane, inducing changes, among others, in hyperpolarization/depolarization of membranes, ion channels, and aquaporin activity.

3.1. *Non-ionic participants of signal reception*

The plasma membrane of the spermatozoon head tightly overlays the nucleus, and folding of this membrane at the mid-piece level along the axoneme root results in the superimposition of membrane layers. In fish such as chondrosteans (sturgeon), Salmonidae, and Percidae, the plasma membrane surrounding the axoneme presents paired lateral extensions. Such fin-shaped ribbons (spermatozoa fins) are not observed in Apogonidae, Labridae, and some species of Cottidae, whereas one, two, or no lateral ribbons have been reported in Sparidae, depending on the genus [37]. A recent *in silico* study conducted on sturgeon spermatozoa demonstrated that such fins significantly improved hydrodynamic characteristics of swimming spermatozoa [38]. The presence of these fins may be related to motility control signaling; there is speculation that fins contribute a large increase to the membrane surface area, and this surface may be involved in the water exchange/osmotic regulation of spermatozoa movement [7,39].

Tubbs and Thomas [40] have shown the presence of progestin receptors localized on spermatozoa membranes of Atlantic croaker, *Micropogonias undulatus*. The receptors participated in progesterone-stimulated sperm hyperactive motility. Progestagens are sex steroid hormones important to fish reproduction as in all vertebrates. They regulate oocyte maturation and play an essential role in spermiation [41,42]. Teleost spermatozoa do not possess an acrosome, and the spermatozoa plasma membrane may be a key component of gamete fusion. For example, GM3, a ganglioside localized in the sperm head, was shown to be involved in binding of the rainbow trout spermatozoon to the egg [43]. Some uncharacterized proteins localized in the head region of rainbow trout spermatozoa also play a role in the process of fertilization [44]. Their participation in the regulation of spermatozoa motility parameters is not known.

Lipids are responsible for membrane fluidity, while both proteins and lipids contribute to the overall permeability of membranes to water and ions. Lipid composition of the spermatozoon plasma membrane has been extensively studied, for example, in rainbow trout [45]. Due to the low plasma membrane permeability to water, Bobe and Labbe [46] concluded that rainbow trout spermatozoa do not possess aquaporins, which, if present, would facilitate water penetration. It appears that water transport itself is not a major process in spermatozoa activation in salmonids. The presence of aquaporins was demonstrated in the plasma membrane of head and flagella of spermatozoa of the gilthead sea bream, *Sparus aurata*, and the involvement of aquaporins was reported in a cAMP-mediated phosphorylation of axonemal proteins of

this species [47,48]. Aquaporins may be important participants of the signaling pathway of fish spermatozoa activation, as they are located in the cytoplasmic membrane and are potential sites of external osmotic signal reception. The importance of aquaporins for activation was confirmed by Abascal et al. [49] who reported an inhibitory effect of very low concentrations of $HgCl_2$, an aquaporin inhibitor, on motility of sea bass, *Dicentrarchus labrax*, spermatozoa and by Zilli et al. [48] who described an effect of $HgCl_2$ on both phosphorylation and dephosphorylation involved in activation of gilthead sea bream spermatozoa. Thus, the involvement of water transport in spermatozoa motility signaling pathways may be species-specific and may depend on the spawning environment. This topic, however, needs further investigation.

3.2. *Ion participants in signal reception*

The presence of ion channels has been described in spermatozoa plasma membrane [50], but a more detailed investigation of their activity in motility signaling pathways, especially in fish sperm, is required. Cytosolic pH may be an important component of signaling pathways, since it affects sperm motility [51]. A decrease in internal pH in spermatozoa would directly affect flagellar movement *via* inhibition of dynein activity, as the outer dynein arm ATPase is pH-sensitive [52]. The Na^+/H^+ exchangers (NHE) constitute a family of proteins involved in intracellular pH regulation. Involvement of NHE in spermatozoa activation was reported for common carp [53]. Marian et al. [53] suggested that the regulation of NHE depends on osmolality. Moreover, the Na^+/H^+ exchange may be responsible for participation of sodium ions in rainbow trout spermatozoa motility, while Na^+ channels themselves probably do not play an important role [54].

Results of studies by Krasznai et al. [35] concerning the inhibition of common carp spermatozoa motility by Ca^{2+} channel blockers suggest that Ca^{2+} influx through Ca^{2+} channels and the consequent increase in intracellular Ca^{2+} are major events triggering carp spermatozoa motility. The operation of Ca^{2+} channels may be associated with the activity of K^+ channels. According to Krasznai et al. [35], an opening or closing of K^+ channels in the plasma membrane of the spermatozoon under hypo-osmosis-induced initiation of motility results in a respective local hyperpolarization or depolarization of the plasma membrane. Such transient depolarization may open Ca^{2+} channels, resulting in an influx of Ca^{2+} and activation of the flagellar motility of carp spermatozoa. Moreover, observations of Krasznai et al. [55] indicate that osmo-regulated mechanosensitive ion channels of carp spermatozoa are also involved in the activation mechanism. The involvement of K^+ and Ca^{2+} channels in triggering motility initiation has also been shown in rainbow trout [54].

Some research has emphasized a significant role of the Na^+/Ca^{2+} exchanger in spermatozoa motility of several fish species [55,56]. It was shown that bepridil, a blocker of the Na^+/Ca^{2+} exchanger, prevented common carp spermatozoa motility [55]. An increase in $[Ca^{2+}]_i$ and decrease in $[Na^+]_i$ was found after motility of Pacific herring spermatozoa was initiated by contact with a glycoprotein sperm-motility-initiating factor in the egg chorion. Vines et al. [56] suggested that the opening of voltage-sensitive Ca^{2+} channels and reverse Na^+/Ca^{2+} exchange result in elevation of $[Ca^{2+}]_i$ and thus initiate sperm motility in herring.

The precise regulatory mechanisms of ion channel activity and the channels' participation in the hyperpolarization of the spermatozoon membrane, associated with the activation of spermatozoa motility [3,35], are still not well understood.

4. From membrane to axoneme: the cytoplasmic trafficking of signals

Internal space of a spermatozoon is limited, but a thin layer of cytoplasm links areas of the cell and contains mediators allowing the cell to decode the signals received and to deliver them to the appropriate sites. For example, an increase in intracellular ion concentration may lead to the activation of adenylyl cyclase (AC), which, in turn, regulates the initiation of motility by a cAMP-dependent mechanism [48]. In mammals, tyrosine phosphorylation of several proteins is up-regulated by reactive oxygen species (ROS). Reactive oxygen species may stimulate tyrosine phosphorylation *via* suppression of tyrosine phosphatase activity or increase in intracellular cAMP availability [57]. Involvement of ROS-dependent signal cascades in the regulation of fish spermatozoa motility is poorly understood. It was recently demonstrated that ROS-dependent mechanisms could contribute to the regulation of flagellar motility in tilapia *via* non-axonemal superoxide dismutase phosphorylation [58]. Other examples of possible participation of reactive oxygen and nitrogen species (NO) in spermatozoa motility regulation were described in fathead minnow [31], steelhead [33], and Asian catfish, *Heteropneustes fossilis* [59]. However, further studies are required to understand the precise roles of reactive oxygen and nitrogen species in cytoplasmic signal transduction. Nevertheless, due to the brevity of the period of spermatozoa activation in fish, to establish the sequence of the signal transduction pathway/s from signal reception to axoneme *via* cytoplasmic trafficking is a challenge.

5. Final steps in signaling: signal reception by the axoneme

5.1. *cAMP signaling in spermatozoa motility regulation and its spatial organization*

Regulation of spermatozoa motility is linked to the AC/cAMP signaling pathway in mammals, fishes, and sea urchins [60–62]. The contribution of cAMP-dependent protein kinase A (PKA) as a regulator of motility is well known in some animals [63,64]. In mammals, two types of adenylyl cyclases have been characterized: a transmembrane AC (tmAC) and a soluble AC [65]. Soluble AC is responsible for cAMP synthesis, whereas the role of tmACs in sperm is not well understood. Cyclic AMP is also important in sea urchin spermatozoa signaling. Spermatozoa of the purple sea urchin, *Strongylocentrotus purpuratus*, possess both types of AC. Soluble AC is present in the spermatozoa head, and several tmACs are differentially distributed throughout the cell [66]. Moreover, studies of White et al. [67] strongly indicate that protein kinase C

is also a central signaling mediator associated with the maintenance of motility of sea urchin spermatozoa.

The link between the increase in cAMP concentration and motility initiation at the axoneme level has been primarily investigated in Salmonidae. It involves a complex series of phosphorylation and dephosphorylation events such as cAMP-dependent phosphorylation of the 15 kDa movement-initiating phosphoprotein [68], PKA [69], and a 22 kDa dynein light chain [70]. However, the cause-and-effect relationship between protein phosphorylation and initiation of spermatozoa motility requires elucidation for our understanding of basic mechanisms of motility signaling.

Special attention should be paid to the spatial organization of the components of cAMP signaling. Spatial regulation of the cAMP-signaling pathway is achieved through the association of PKA with A-kinase-anchoring proteins (AKAPs), which keeps enzymes in proximity to their effectors or substrates [71]. The functions of some AKAPs, providing compartmentalization of cAMP signaling in different cell types, were reviewed by Logue and Scott [72]. Many components of the cAMP signaling pathway are present in the flagellum and are closely associated with the axonemal structure. Two axonemal AKAPs have been identified in the *Chlamydomonas* flagellar axoneme [73], which may indicate that the axoneme itself constitutes a scaffold arrangement for the spatial organization of cAMP signaling. Delineating of such cAMP, or other second messenger, microdomains is important if we are to understand the organization of cellular signaling.

5.2. *Dynein machinery as the ultimate goal of regulation*

Dynein constitutes one of the major families of molecular motors that produce directed movement along axonemal microtubules. They are organized as a macromolecular complex containing large, intermediate, and small protein subunits. The intermediate- and light-chains (IC and LC) of dynein regulate its motor activity. The heavy chain (HC) contains the motor machinery transforming chemical energy collected from ATP hydrolysis into directed mechanical force. Schematic depictions of regulation of the sophisticated dynein machinery that take into account the extreme complexity of this system and show the structural, physical, and chemical aspects of its regulation have been created [50,74–79]. They improve our general understanding of spermatozoa structure and involvement of signaling cascades in flagellar response to external stimuli and provide a background for further exploration of axoneme activity.

One of the possible control mechanisms is based on the redox state of dynein ATPase. The α and β HCs of the *Chlamydomonas* dynein are both closely associated with thioredoxin-related proteins, LC5 and LC3 [80]. A thioredoxin-like segment has also been identified in outer arm dynein IC1 protein of sea urchin spermatozoa [81]. According to Harrison et al. [75], identification of these redox-active motifs in the cilia/flagella of such diverse organisms suggests that their role may be of general significance for dynein biological function. The data of Morita et al. [58] with respect to the involvement of redox and phosphorylation pathways in the regulation of osmotic tolerance and motility of euryhaline tilapia spermatozoa could be considered additional support for the importance of redox-based signaling in spermatozoa physiology. In spite of a large amount of data indicating high redox activity in spermatozoa, the enzymes responsible for regulating their redox status, the importance of specific ROS, and the molecular mechanisms by which these reactive species exert their biological activity are still not fully characterized.

Among components of the axoneme involved in the regulatory process responsible for maintaining a regular beating wave, the dynein regulatory complex (DRC) [82] has drawn special attention. DRC is involved in the coordination of dynein arm activity. Flexible nexin molecules linking adjacent doublets are also proposed to be a component of the regulatory mechanism [83], as they act as long elastic proteins which restrain sliding of axonemal microtubule doublets. The recent studies of Heuser et al. [84] provide new insights into the protein structure of the nexin link and its potential functions, concluding that the DRC is closely associated with the nexin link and suggesting the term "nexin-DRC" (NDRC). However, the precise mechanism of NDRC activity is still not fully described and this field requires further study. The role of the central pair is also not elucidated [78], but it is known that mutations resulting in partial or total deletion of the central pair structure can lead to a lack of flagellar motility. Total removal of the outer dynein arms produces a 50% reduction in beat frequency, but has little effect on the form of axonemal waves [85].

Thus, the numerous components of an axoneme most likely possess overlapping or complementary roles in both the mechanics and regulation of beating, and knowledge of the structures and interactions of all components are important for understanding the overall mechanism of axonemal movement.

6. Bioenergetics of flagellar motility

A prerequisite of spermatozoa motility is that the hydrolysis of ATP be catalyzed by dynein ATPase, which liberates chemical energy and is coupled to the mechanical sliding of adjacent microtubules. Energy-supplying pathways for spermatozoa may differ according to fish species and may include glycolysis, phospholipid catabolism and triglyceride metabolism, the Krebs cycle, and oxidative phosphorylation [86–88]. The latter provides energy insufficient to compensate for ATP utilization by dynein ATPases, which is probably one of the chief reasons for short duration of spermatozoa motility in fish [89].

Characterizing the energetics of fish spermatozoa motility must take into account both the energy necessary for survival in the genital tract, where substrates for energy-supplying pathways are possibly delivered by tissues, and the energy required for motility when sperm is liberated into the surrounding medium, the latter phase being devoid of energy molecules [86]. Numerous types of organic molecules can be metabolized for energy generation. Spermatozoa of fish with internal fertilization rely on both endogenous and exogenous substrates, while spermatozoa of fish with external fertilization must rely entirely on endogenous energy readily available and transportable in the axonemal compartment by the

appropriate shuttle [86,90,91]. Thus, strategies for generation and use of energy primarily depend on the mode of fertilization.

In fish with external fertilization, ejaculated spermatozoa depend entirely on energy previously stored or produced by the mitochondria. However, the cellular site of energy production, the mid-piece, which contains mitochondria, is not the same as the site of energy consumption, the axonemal dynein ATPases. Energy must be transferred from one site to another, but the diffusion of ATP may be severely restricted by the narrow space available within the flagellum, which is mostly occupied by the axoneme, and by the low diffusion rate of ATP [90]. Alternative mechanisms of energy transfer are essential. This may include shuttling of phosphocreatine (PCr) and creatine with the participation of creatine kinase (CK) isoenzymes, especially distinctly localized CK isoenzymes in spermatozoa [92], one being located in mitochondria and another in the flagellum. This phosphocreatine shuttle was described in detail by Tombes et al. [91] and was recently re-evaluated by Takao and Kamimura [90].

Spermatozoa of salmonids and cyprinids possess PCr and CK, which catalyzes the formation of ATP from PCr and ADP [87,93]. Therefore, spermatozoa regenerate ATP from PCr and motility-generated ADP *via* CK. They can also regenerate ATP by the action of adenylate kinase, which catalyzes the formation of ATP from ADP [93]. The elucidation of possible fish species-specific peculiarities of phosphocreatine shuttle and adenylate kinase system participation in energy supply of axoneme is of great importance and interest.

Since the energy necessary for spermatozoa motility is provided by ATP hydrolysis catalyzed by dynein ATPases, the initiation of fish spermatozoa motility should be immediately followed by a decrease in ATP level. This is well documented. Significant decrease in ATP content within the first 10 s of motility was observed in spermatozoa of Eurasian perch, *Perca fluviatilis* [94]. A sharp decrease in ATP level in the first seconds of motility with stable concentration to the end of the motility period was observed in Siberian sturgeon, *Acipenser baerii*, spermatozoa [95]. Decrease of ATP level in carp spermatozoa occurs within the motility period and plateaus after motility ceases at approximately 2 min post activation [89], later than in other fish species. However, motility initiation of carp spermatozoon is associated with an increase in cell volume [21,96]. Therefore, an even more rapid decrease of ATP concentration may take place at motility initiation of carp spermatozoa because of the combined effects of ATP consumption and volume increase. The dynamics of ATP content during the motility period reflects species-specific differences in bioenergetic pathways in spermatozoa. Elucidation of these differences is a subject for future investigation.

7. Conclusions

In fish spermatozoa, motility initiation is well described biochemically but not phenomenologically. It is a brief event, with the described events lasting milliseconds, difficult to capture by microscopic imagery, and may represent a challenge to the observer. Spermatozoa activation is a complex and highly organized process. In general, the reception of external activating stimuli is followed by signaling conducted by second messengers (cAMP, cGMP, Ca^{2+}, ROS, NO) (Fig. 1), and, finally, axonemal motion through activation of axonemal dyneins by protein phosphorylation or dephosphorylation signals [7,50,76]. A major common feature among species is osmolality signaling. Salmonids are thought to be ion (K^+) specific regarding the activation signal, but this K^+ control can be by-passed by a short pre-exposure to high osmolality conditions [97], leading to the conclusion that the activation mechanism remains osmotic. The site of specific osmotic sensors on the spermatozoa membrane remains unknown. Specific rafts in the membrane could constitute crucial areas on the membrane surface [98].

Reception of external signals takes place at the level of the spermatozoon plasma membrane, with many molecules and molecular complexes participating in the process (Fig. 1). Specificities of flagellar membrane composition and lipid sorting have been mentioned [99], as has protein sorting, which differentiates the composition of the flagellar membrane from that of the head membrane. This could be considered an indication that a possible pathway of signal reception may be the membrane composition itself, a characteristic little studied in fish. Ion channels in fish spermatozoa are known to be involved in environmental signal reception [35,55]. However, the processes of signal transduction from the environment to the flagella at the level of ion channel activity are still not clear, and the topic requires further comprehensive interspecific studies. In addition to ion channels, aquaporins and membrane receptors are known to participate in signal reception. Special interest has been paid to the aquaporin family involvement in osmotic reactions [7,100]. Aquaporins highly accelerate water exchange, which becomes close to free diffusion. The time delay for aquaporin response is in the range of 10 ms in lipid vesicles under osmolarity level differences experienced by fish spermatozoa at activation [101]. The question remains of whether this response time is sufficient to fit into the time window leading to full flagellum activation. The finding of associations between ion channel and aquaporin activity and signaling by second messengers such as cAMP, cGMP or Ca^{2+} is promising.

It is well accepted that ROS are signaling molecules that, as with other second messengers, transduce messages from the extracellular milieu to generate a specific cellular response. Reactive oxygen species play defined functions, through redox modifications of a great diversity of molecules participating in most signaling pathways described to date [102]. Involvement of ROS in signaling pathways in spermatozoa seems to be a possibility, since spermatozoa possess a limited amount of cytoplasm, which does not allow diffusion of large signaling molecules. Although it seems intuitive that the high reactivity of some ROS means that the site of their production is critical to their biological effects, there are examples of ROS, such as hydrogen peroxide, that exhibit low reactivity appearing to mediate intercellular communication. Although it is assumed that hydrogen peroxide diffuses freely across biological membranes, specific aquaporins have been found to facilitate hydrogen peroxide diffusion across cell membranes [103]. The role of ROS in sperm physiology has been extensively studied in mammals, especially in connection with their participation

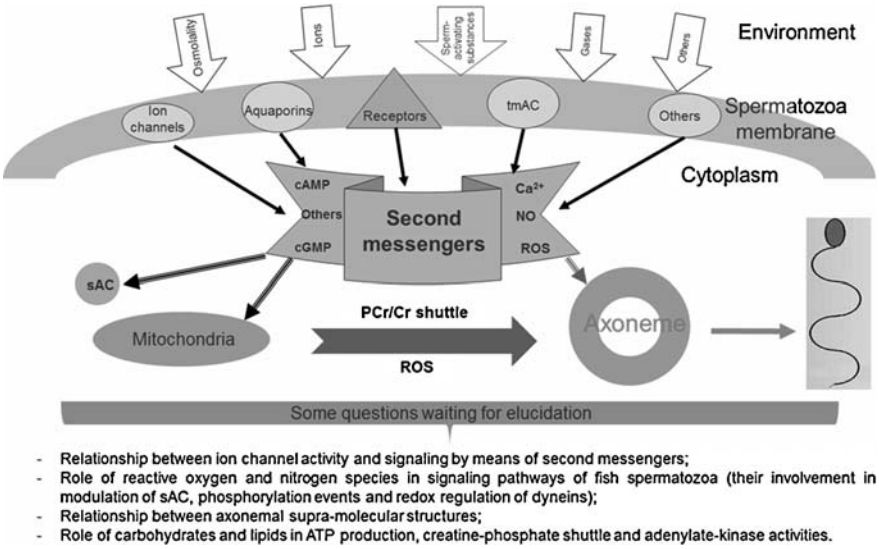

Fig. 1 – Spermatozoa motility activation from external signals through membrane and cytoplasmic transit to axoneme. Participants of the process and questions for elucidation are presented. *Abbreviations:* sAC: soluble adenylyl cyclase; tmAC: transmembrane adenylyl cyclase; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; NO: nitric oxide; PCr/Cr: phosphocreatine/creatine; ROS: reactive oxygen species.

in capacitation [104,105]. Less data exist on the role of ROS in fish sperm physiology. The primary focus of researchers has been the negative effects of ROS and activity of non-enzymatic and enzymatic antioxidant systems [106–109]. The vast majority of available data indicates that the activation of free radical oxidation in circumstances such as environmental contamination, and cryopreservation leads to spermatozoa motility deterioration. In contrast to mammalian spermatology, positive effects of ROS for fish spermatozoa are yet to be elucidated. A similar situation is seen with respect to the role of NO in fish spermatozoa physiology. It is known that NO-synthase activity is present in different tissues in some fish species [110–112], but data about the presence of NOS and the effects of NO on fish spermatozoa motility are scarce.

Regarding the energy control of fish sperm motility, the role of ADP and PCr during the motility period as effectors on dynein should be considered. Accumulation of ADP during this period, as a competitive inhibitor of ATP-ase activity of dyneins, along with the lack of ATP, largely contributes to the arrest of motility. The regulatory effect of changes in macroergic phosphate content during fish spermatozoa motility period is of interest for elucidation. The final steps of activation signaling are the most intriguing aspect of flagella activity studies. While some distinct processes of flagellar micro-machinery activity have been described [113–115], the detailed description of flagellar beating requires use of modern techniques for better understanding of the relationships among axonemal super-molecular structures. The study of existence and involvement of spatially organized microdomains, providing transduction of activation signals to the axoneme, is of interest and currently in initial stages of investigation.

Why are so many potential actors involved in the transduction of the signal from membrane to axoneme? A simple unifying model could be a change in internal ionic concentration. Either dynein motors are directly sensitive to this ionic concentration [11], or the dynein regulatory complex constitutes the regulator in charge of collecting information from all actors. Also the potential roles of central structures and calcium interaction with the dynein regulatory complex, well investigated in *Chlamydomonas* [113], remain to be explored in detail in fish sperm axonemes. Another important point to consider in the response of axonemes of fish sperm is the longitudinal heterogeneity of distribution of dynein subspecies along the axonemal length (shown for *Chlamydomonas* by Yagi et al. [116]). Spermatozoa motility signaling is a complex and highly orchestrated process that has not been extensively studied in fish. The elucidation of its essentials aspects will expand the overall understanding of fish spermatozoa functioning and subsequent improvements in assisted reproductive technologies.

Conflict of interest

We declare no actual or potential conflict of interest regarding the submitting manuscript. Viktoriya Dzyuba and Jacky Cosson.

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1.2. The main objects chosen for the investigation in the present study

The general overview of the current knowledge for involvement of different enzymatic activities in fish spermatozoon motility includes the reception of the signal(s) for the processes of motility activation at the level of spermatozoon membrane, the transduction through this membrane, cytoplasmic trafficking of the activation signal, transduction to the axoneme machinery, activation of axonemal dyneins and regulation of their activity, and the energy supply for flagellar motility. While each step involves a complicated network of regulation, this current work focused on a study of the enzymatic activities associated with regulation *via* energy supply during sperm motility and regulation of sperm maturation *via* antioxidant and proteolytic enzymes. A short description of the reasoning behind the objectives of the present study is provided here.

1.2.1. Energetic aspects of spermatozoon motility regulation

It is commonly accepted that the source of energy for flagella activity is the hydrolysis of ATP (Cosson, 2013). The hydrolysis of ATP is catalyzed by dynein ATPases. Dyneins are molecular motors, which are organized as a large macromolecular complex consisting of small, intermediate, and large protein subunits. The large subunits (so-called heavy chains) transform chemical energy of ATP into directed mechanical force needed for the sliding of adjacent microtubules operating *via* instant phosphorylation and dephosphorylation of the components of the axonemes. Fish spermatozoa have a wide variety of pathways for energy supply, allowing numerous types of substrates to be metabolized for ATP generation (Lahnsteiner et al., 1993; Ingermann, 2008). These pathways are glycolysis, phospholipid catabolism and triglyceride metabolism, the Krebs cycle, and oxidative phosphorylation (Billard and Cosson, 1990; Ingermann, 2008; Lahnsteiner et al., 2010a). The energy that is supplied by the latter is not sufficient to compensate for ATP utilization by dynein ATPases. This is probably one of the main reasons for the short duration of spermatozoan motility in fish (Perchec et al., 1995). However, the level of total intracellular ATP is proposed to be the one of determinants of spermatozoa fertilizing ability (Zilli et al., 2004). The metabolic pathways for ATP generation and its distribution along the length of the flagellum may be species-specific and have not been fully explored. Existence of different metabolic strategies for storage and generation of ATP was suggested by Ingermann (2008).

ATP produced in the mid-piece of spermatozoon needs to be transferred to the places of its consumption – dyneins which are distributed along entire length of the flagellum. However, such a transport along the flagellum cannot be realized *via* diffusion because of substantially limited space internal and low diffusion rate of ATP. It was shown by Kaldis et al. (1997) that diffusion flux rates of ATP, ADP and inorganic phosphate in the primitive sperm of the marine lugworm *Arenicola marina* are very low. Therefore some alternative mechanisms of energy transfer may be of concern. For example, one possibility might be shuttling of phosphocreatine (PCr) and creatine (Cr), for which diffusion flux rates have been shown to be some orders of magnitude higher than for ATP, ADP and inorganic phosphate (Kaldis et al., 1997). The shuttling of PCr and Cr is carried out by creatine kinase (CK) isoenzymes distinctly localized in spermatozoon (Kaldis et al., 1997) and catalyzing the formation (regeneration) of ATP from PCr and ADP (Kamp et al., 1996; Saudrais et al., 1998; Woolsey and Ingermann, 2003; Ingermann et al., 2011).

The PCr shuttling was described in detail for sea urchin *Strongylocentrotus purpuratus* spermatozoa by Tombes et al. (1987); experimental results and their theoretical analyses give strong support for the participation of PCr shuttle in high energy phosphate transport. In

their earlier study, the authors compared the effect of fluorodinitrobenzene (FDNB, inhibitor of CK) on the motility of live and demembranated spermatozoa and showed that only live spermatozoa, where CK would be important in providing ATP to the flagellum, exhibited an inhibition of flagellar motility after treatment with low concentrations of FDNB (Tombes and Shapiro, 1985). Fluorodinitrobenzene treatment resulted not only in the attenuation of sliding velocity in the distal regions of the flagellum, that can be related to the supply of ATP along the flagellum, but also in the fact that FDNB-inhibited spermatozoa generate bending patterns with shorter wavelengths. And, finally, as a result of quantitative analysis of energy transport in spermatozoon flagellum Tombes et al. (1987) showed substantial gradients of PCr and creatine, and came to the conclusion that PCr diffusion must clearly be the one major route for high energy phosphate transport along the sea urchin spermatozoon flagellum. In this respect, the wave attenuation appearing when ATP delivery is weak along the sea urchin flagellum is reminiscent of the same wave attenuation (damping) phenomenon described in fish spermatozoon flagella during the progression of the motility period (Cosson et al., 1997).

ATP diffusion coefficient was recently re-evaluated by Takao and Kamimura (2008); they used fluorescence recovery after a photobleaching technique for the determination of diffusion coefficients of fluorescein derivatives of similar molecular masses in sea urchin flagella, in contrast to determination in aqueous solution (Brokaw, 1966) or in muscle fibers (Tombes et al., 1987). The rate of ATP diffusion was found to be about three times lower than that reported in the study of Tombes et al. (1987). Applying the same model as Tombes et al. (1987) and the lower value of ATP diffusion coefficient assumed in their simulation of intraflagellar ATP diffusion, Takao and Kamimura (2008) concluded that the rate of ATP diffusion inside spermatozoa is high enough to support the continuous motility of sea urchin sperm flagella in case of the creatine shuttle system functioning.

The hydrolysis of ATP by dyneins leads to generation of ADP, which is known to be an inhibitor of dyneins, and at the same time it is the source for ATP regeneration by adenylate kinase (AK) reaction. So, AK and CK could be considered as regulators of motility *via* ADP/ATP regeneration mechanisms, acting in close proximity to flagellar motors – dyneins. The relative contribution of these two systems (AK and CK) of ATP regeneration and function of the creatine-phosphocreatine shuttle in spermatozoa have been thoroughly investigated only in rainbow trout by Saudrais et al. (1998); they confirmed the presence of PCr shuttle and AK in trout spermatozoa and found that AK was less effective in ATP regeneration than CK. The similar results for the higher effectiveness of CK (compared to AK) in ATP production was also found by Woolsey and Ingermann (2003). Other studies describe the separate activity of AK and CK in spermatozoa of different fish species (Lahnsteiner et al., 1998; Gronczewska et al., 2003; Grzyb and Skorkowski, 2006; Lahnsteiner and Mansour, 2012); two of these (Gronczewska et al., 2003; Grzyb and Skorkowski, 2006) did not study the relationship between these activities and flagellum motility.

While the main metabolic pathways for ATP generation are well studied for different fish species, the question remains whether the mechanisms of ATP regeneration are similar among fishes, or if there are species differences in the pathway of energy supply for spermatozoon movement. This question of species difference prompted the investigation of the pathways in the spermatozoa of carp and sterlet, which are in different subclasses of actinopterygians and they have spermatozoa of differing structure, mode of motility activation and significantly different motility duration.

1.2.2. Role of proteolysis regulators in sturgeon sperm maturation

Although different approaches have been suggested for evaluating sperm quality, motility is the most common criterion used for this purpose, since high motility is a requisite for fertilization and it correlates strongly with fertilization success (Rurangwa et al., 2004). Maturation is the acquisition by completely formed (morphologically) spermatozoa of the ability to initiate motility and therefore to be capable of fertilization (Schulz et al., 2010). This process is well investigated in many animal species, especially in humans and domestic mammals (de Lamirande et al., 1997; Marengo, 2008; Sostaric et al., 2008) because of its importance in assisted fertility technologies. In mammals, spermatozoa acquire the potential for motility as they leave the testis and pass along the epididymis. It is generally recognized that calcium ions, reactive oxygen species, cyclic AMP, different protein kinases and protein phosphatases (for serine/threonine and tyrosine residues of proteins), and the extracellular signal-regulated protein kinase in regulation of human sperm capacitation are involved (de Lamirande et al., 1997; O'Flaherty et al., 2005).

In some insects, such as tobacco hornworm *Manduca sexta* and water strider *Aquarius remigis*, the addition of trypsin is sufficient to initiate the motility of immotile sperm taken from the seminal vesicle (Friedlander et al., 2001; Miyata et al., 2012).

However, the process of spermatozoan maturation has been described for only a few teleosts [rainbow trout, chum salmon and Japanese eel (Morisawa and Morisawa, 1986; Miura and Miura, 2001)] and, generally for applications in aquaculture, has not been considered to be a major limiting factor. In rainbow trout and chum salmon, it was demonstrated that spermatozoa from the sperm duct were motile when diluted in a potassium-free medium, while spermatozoa from the testis were immotile, or less motile in the same medium (Morisawa and Morisawa, 1986, 1988). In salmonids, sperm maturation has been induced by incubation of testicular spermatozoa in artificial seminal plasma containing bicarbonate at pH 8.2, probably through the elevation of intrasperm cyclic AMP levels (Morisawa and Morisawa, 1988). Similar results have been reported for Japanese eel spermatozoa by Miura et al. (1995) and Ohta et al. (1997). From these results it is clear that spermatozoa acquire the potential for motility activation through exposure to some factors in the sperm duct.

Recently we described the sperm maturation process in sturgeons, establishing its localization and main components (Dzyuba et al., 2014). The excretory system in sturgeon is different from that in teleosts; the sperm and urinary ducts are not completely separated. Sterlet testicular sperm and sperm collected from Wolffian ducts had significant differences. While sperm that is collected from the Wolffian ducts is commonly used in artificial propagation, testicular spermatozoa were not able to be activated in an activating medium. However, they can acquire this capacity after *in vitro* incubation in urine or seminal fluid from Wolffian duct; this reaction is temperature- and time-dependent. We also found that removal of high molecular weight substances from seminal fluid found in the Wolffian ducts or the addition of soybean trypsin inhibitor prevented maturation of testicular sperm which had been incubated in seminal fluid from Wolffian duct (Dzyuba et al., 2014). Based on these findings, we propose that the presence of high molecular weight substances is a prerequisite for sperm maturation and suggest that proteolytic activity regulates sturgeon sperm maturation.

Seminal plasma proteolytic enzymes are known to be involved in regulation of spermatozoa motility in mammals, fish and nematodes (de Lamirande and Gagnon, 1986; Cosson and Gagnon, 1988; Inaba and Morisawa, 1991; Smith and Stanfield, 2012). Regional distribution of proteases and protease inhibitors in the testicular and epididymal fluids of domestic mammals allowed Métayer et al. (2002) to suggest their role in sperm maturation process. But there are no available data about the possible involvement of proteases in fish sperm

maturation, especially taking into account the fact that this process for sturgeons has just been discovered. At the same time, as sturgeon spermatozoa possess acrosin-like activity and anti-trypsin activity of sturgeon seminal fluid may have a role in controlling spermatozoon acrosin-like activity (Ciereszko et al., 1996), the balance between proteolytic and anti-proteolytic activities of seminal fluid may affect spermatozoon-related proteases functioning at spermatozoa maturation.

Thereby, the comprehensive investigation of differences between proteases and their inhibitors present in seminal fluid from sturgeon testicular sperm and sperm collected from Wolffian ducts and peculiarities of their functioning will greatly facilitate our understanding of their role in the process of sturgeon spermatozoa maturation.

1.2.3. Role of prooxidant-antioxidant balance in fish spermatozoa functioning

Reactive oxygen and nitrogen species (ROS and RNS) are collective terms often used to include not only oxygen and nitrogen radicals ($O_2^{\bullet-}$, $OH^{\bullet}$, $NO^{\bullet}$) but also some non-radical derivatives of oxygen and nitrogen (H_2O_2, O_3, HOCl, HNO_2, $ONOO^-$ and so on) (Halliwell and Gutteridge, 1999). Owing to their short half-life, high reactivity and to the fact that they are normally generated by the cells during key metabolic processes, ROS and RNS are excellent candidates to play the role of second messengers. It is now well accepted that ROS are signaling molecules that, as in case of other second messengers, transduce messages from the extracellular milieu to generate a specific cellular response. ROS have defined functions through redox modifications of a great diversity of molecules, participating in almost every signaling pathway described up to date (Covarrubias et al., 2008).

The respective roles of ROS and RNS in sperm physiology have been thoroughly studied in mammals. Reactive oxygen and nitrogen species, such as the superoxide anion ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$), are known to be modulators of different physiological processes and signal transduction cascades (O'Flaherty et al., 2006b; de Lamirande and O'Flaherty, 2008). Their essential role as modulators of capacitation is recognized in humans (Herrero et al., 1999, 2003; O'Flaherty et al., 2006a), bovines (O'Flaherty et al., 2003), and mice (Herrero et al., 2003) spermatozoa. Reactive oxygen and nitrogen species produced by spermatozoa (mainly $O_2^{\bullet-}$ and $NO^{\bullet}$) from the beginning of capacitation (O'Flaherty et al., 2003; de Lamirande and Lamothe, 2009) control the increase in cAMP (Aitken et al., 1998) and most of the known phosphorylation events, as well as tyrosine nitration of proteins (de Lamirande et al., 1997, 2009; Aitken et al., 1998; Herrero et al., 2001; O'Flaherty et al., 2006a, 2006b; de Lamirande and Lamothe, 2009).

The role of redox regulated pathways in mammalian sperm cell biology was reviewed in depth by Baker and Aitken (2004). They conclusively confirmed that in spite of extensive biochemical and experimental data indicating that mammalian spermatozoa are intensely redox active cells and that this activity controls many different aspects of sperm function (nuclear condensation, mitochondrial stabilization, motility activation, hyperactivation, capacitation, acrosomal exocytosis, sperm-oocyte fusion and so on), we have still not characterized the enzymes responsible for regulating the redox status of sperm cells, the relative importance of specific ROS, or the molecular mechanisms by which these reactive metabolites exert their biological activity (Baker and Aitken, 2004).

As for fish sperm, the data on the role of ROS in their physiology are much weaker. The data of Morita et al. (2011) about the involvement of redox and phosphorylation pathways in the regulation of osmotic tolerance and motility of the euryhaline tilapia (*Oreochromis mossambicus*) could be considered as support of the importance of redox-based signaling for fish spermatozoa physiology.

In contrast to biological signaling activity, excessive production of ROS and RNS and/or their incomplete elimination can result in the development of oxidative stress, which is one of the reasons that spermatozoan function deteriorates in various animal species, including mammals and fish (Sikka et al., 1995; Agarwal and Saleh, 2002; Aitken et al., 2003; Kasimanickam et al., 2007; Turner and Lysiak, 2008; Shiva et al., 2011; Hagedorn et al., 2012). In order to prevent the development of oxidative stress, spermatozoa are equipped with non-enzymatic and enzymatic antioxidants (Rhemrev et al., 2000; Chabory et al., 2010). But because of the limited cytoplasmic volume and, therefore, the low amount of endogenous antioxidants, sperm cells rely chiefly on the antioxidant capacity of seminal fluid to protect spermatozoa from oxidative damage (Potts et al., 2000; Baumber and Ball, 2005; Ciereszko, 2008; Tavilani et al., 2008; Lahnsteiner et al., 2010b).

Most researchers have focused their studies on the negative effects of ROS (intensity of free radical oxidation of biomolecules and level of its products) and activity of non-enzymatic and enzymatic antioxidant systems (Dietrich et al., 2005; Zhou et al., 2006; Chen et al., 2010; Lahnsteiner et al., 2010b, 2011; Li et al., 2010, 2011; Shaliutina et al., 2013). The vast majority of the available data indicate that the activation of free radical oxidation in different circumstances (for example, environmental contaminations, cryopreservation and so on) leads to sperm motility deterioration. In our opinion, the data concerning peroxidation processes intensity and antioxidant system stability at *in vitro* fish sperm storage are somewhat contradictory. In brown trout, thiobarbituric acid-reactive substance content in spermatozoa and seminal fluid was shown to increase after 48 h of storage (Lahnsteiner et al., 2010b), while the level of all studied non-enzymatic antioxidants and activity of antioxidant enzymes did not change. In the spermatozoa of both Siberian and Russian sturgeons, significant increase in thiobarbituric acid-reactive substance content has also been shown after 3 and 6 days *in vitro* storage (Shaliutina et al., 2013), but an increase in superoxide dismutase activity prefaced this increase in both species.

Data on the intensity of peroxidation processes and the antioxidant system capacity of sturgeon seminal fluid at maturation, *in vivo* and *in vitro* semen storage are lacking. Scientific benefits from study of these processes are related to understanding of possible signaling *via* free radicals activity occurring during sperm maturation.

As fish spermatozoa undergo the drastic changes of environment required for motility activation, peroxidation processes could accompany these changes, but their role is still not clear. An indirect indication of the involvement of an increase in membrane fluidity in marine and freshwater fish sperm motility activation reported by Krasznai et al. (2003) and possible changes in carp sperm membrane properties occurring in response to hypotonic shock (Dzyuba et al., 2013) allow prediction of the regulation of sperm motility by membrane fluidity properties. As membrane fluidity is well known to depend on lipid saturation level and this level is undoubtedly influenced by peroxidation processes (Dobretsov et al., 1977), the peroxidation processes occurring during sperm motility phase are of high interest.

1.3. The goals of the thesis

1. Investigation of the presence of the creatine-phosphocreatine shuttle in sterlet sperm and evaluation of the relative contribution of creatine kinase and adenylate kinase in ATP regeneration in the motile phase of demembranated spermatozoa of sterlet and carp, taxonomically distant fish species in which spermatozoa differ in structure, mode of motility activation, and motility duration.

2. Study of the involvement of seminal fluid proteases and anti-proteolytic activity in sterlet spermatozoa maturation.

3. Evaluation of the changes in thiobarbituric acid-reactive substance content, superoxide dismutase and catalase activity, and uric acid concentration in seminal fluid of sterlet *Acipenser ruthenus* sperm collected from testes and from the Wolffian ducts at various times after induction of spermiation (*in vivo* storage) and after short-term *in vitro* storage. Elucidation of the oxidative stress involvement in response of fish spermatozoa to hypotonic treatment.

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CHAPTER 2

ENZYME ACTIVITY IN ENERGY SUPPLY OF SPERMATOZOON MOTILITY IN TWO TAXONOMICALLY DISTANT FISH SPECIES (STERLET *ACIPENSER RUTHENUS*, ACIPENSERIFORMES AND COMMON CARP *CYPRINUS CARPIO*, CYPRINIFORMES)

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ENZYME ACTIVITY IN ENERGY SUPPLY OF SPERMATOZOON MOTILITY IN TWO TAXONOMICALLY DISTANT FISH SPECIES (STERLET *ACIPENSER RUTHENUS*, ACIPENSERIFORMES AND COMMON CARP *CYPRINUS CARPIO*, CYPRINIFORMES)

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ABSTRACT

As spermatozoon motility duration differs significantly among fish species, the mechanism of ATP generation/regeneration and its distribution along the flagellum may be species-dependent. The current study compared the role of creatine kinase with that of adenylate kinase in ATP regeneration during motility of demembranated spermatozoa of taxonomically distant fish species, sterlet and common carp, allowing investigation for the presence of the creatine-phosphocreatine shuttle in sterlet spermatozoa. The flagellar beat frequency of demembranated spermatozoa was measured in reactivating media in presence or absence of ATP, ADP, phosphocreatine, and creatine kinase and adenylate kinase inhibitors. Following demembranation, adenylate kinase, creatine kinase, and total ATPase activity was measured in spermatozoon extracts. Beat frequency of demembranated spermatozoa was found to be positively correlated with ATP levels in reactivating medium and to reach a plateau at 0.8 mM and 0.6 mM ATP for carp and sterlet, respectively. It was shown for the first time that sterlet axonemal dynein ATPases have a higher affinity for ATP than do those of carp. Supplementation of reactivating medium with ADP and phosphocreatine without ATP resulted in beat frequencies comparable to that measured with 0.3–0.5 mM ATP for both studied species. The presence of the phosphocreatine/creatine kinase phosphagen system and its essential role in ATP regeneration were first confirmed for sturgeon spermatozoa. The inhibition of creatine kinase exerted a high impact on spermatozoon energy supply in both species, while the inhibition of adenylate kinase was more pronounced in sterlet than in carp. This was confirmed by the quantification of enzyme activity in spermatozoon extracts. We concluded that spermatozoa of these taxonomically distant species utilise similar systems to supply energy for flagella motility, but with different efficacy.

Keywords: *adenylate kinase, beat frequency, creatine kinase, demembranated spermatozoa, fish sperm*

Introduction

Spermatozoon motility must be activated to initiate the processes that form the basis of successful fertilization: spermatozoa must reach, bind to, and penetrate the egg. In external fertilization, spermatozoa cannot obtain energy from the substrate of the milieu into which they are shed and therefore must possess considerable endogenous energy, mostly to sustain motility. Glycolysis, phospholipid catabolism and triglyceride metabolism, the Krebs cycle, and

oxidative phosphorylation are the main energy-supply pathways for fish spermatozoa (Billard and Cosson, 1990; Ingermann, 2008; Lahnsteiner et al., 2010). While it is generally accepted that the source of energy for flagella activity is the hydrolysis of ATP (Cosson, 2013), the metabolic pathways involved in its generation/regeneration and distribution along the length of the flagellum may be species-specific and are not fully understood. Possible existence of differing metabolic strategies for storage and generation of ATP was suggested by Ingermann (2008), based on differences in spermatozoon motility duration among fish species (Cosson, 2010).

As a result of ATP hydrolysis during spermatozoon movement, ADP is generated and may be used as a source of ATP regeneration *via* adenylate kinase (AK) or, in the presence of phosphocreatine (PCr), by the action of creatine kinase (CK). The contribution of AK and CK to ATP regeneration was studied in demembranated rainbow trout spermatozoa by Saudrais et al. (1998) who showed the presence of a creatine-phosphocreatine (Cr/PCr) shuttle and found that AK was less effective in ATP regeneration than CK.

The present study was carried out to investigate the presence of the Cr/PCr shuttle in sturgeon sperm and to evaluate the relative contribution of CK and AK in ATP regeneration in the motile phase of demembranated spermatozoa of sterlet and carp, taxonomically distant fish species in which spermatozoa differ in structure, mode of motility activation, and motility duration.

Materials and methods

Ethics

All experiments were performed in accordance with National and Institutional guidelines on animal experimentation and care, and were approved by the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany based on the EU-harmonized Animal Welfare Act of the Czech Republic.

Fish experimental models

Experiments were conducted using common carp *Cyprinus carpio* and sterlet *Acipenser ruthenus*. The species were selected based on their differing motility duration, 30–40 s in carp (Billard et al., 1995; Perchec Poupard et al., 1997) and approximately 4 min in sterlet (Lahnsteiner et al., 2004), as well as their modes of spermatozoon motility activation, osmotic in carp (Perchec Poupard et al., 1997) and ionic in sterlet (Alavi and Cosson, 2006). Spermatozoa of carp and sterlet also show differences in structure, including the presence of an acrosome in sterlet, which is absent in carp. Carp and sterlet spermatozoa differ in head shape (basically spherical in carp, elongated in sterlet) and size (estimated average head length 5.14 μm in sterlet, including mid-piece and acrosome, and 2.45 μm in carp), as well as in flagellum length (30.7 μm in carp and 42.3 μm in sterlet) (Psenicka et al., 2009a, 2009b).

Fish rearing conditions

The experimental fish groups were reared at the hatchery of South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic.

Five mature common carp males (2.5–3 kg) were maintained in 4000 L aquaculture tanks at a temperature of 18 °C for 14 days prior to hormone injection.

Six mature sterlet males (0.6–1.0 kg) were transferred from aquaculture ponds (water temperature 8–10 °C) into a 0.8 m^3 closed water recirculation system. Within 24 h, water temperature was increased to 15 °C, and fish were held four days before beginning experimentation.

Sperm collection

Spermiation in carp and sterlet was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% NaCl solution at 1 and 4 mg kg^{-1} body weight, respectively. Carp sperm was collected into 10-mL plastic syringes 24 h after injection using abdominal massage. Sterlet sperm was collected at the urogenital sinus by aspiration using a 4-mm plastic catheter connected to a 20-mL syringe 24 h after hormone injection. Sperm samples showing 80–100% motility after dilution with water from the recirculation system were used for experiments. Spermatozoon motility parameters were assessed using standard video-microscopy techniques. Sperm was stored at 4 °C before and during experiments. In all samples, spermatozoon concentration was determined using a Burker cell hemocytometer (Meopta, Czech Republic) and Olympus BX 50 phase contrast microscope (200× magnification; Olympus, Japan).

Spermatozoon demembranation and reactivation

For carp, two µl of sperm was mixed at room temperature with 198 µl demembranating medium (DM) [0.15 M KCH_3COO, 20 mM Tris, pH 8.2, 0.5 mM ethylenediaminetetraacetic acid, 0.1 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.04% Triton X-100]. After 40 s, 6–9 µl of this mixture was transferred into 191–194 µl reactivating medium (RM) to obtain a spermatozoon concentration of $0.6x10^7$ cells/mL. For sterlet, five µl of sperm was mixed at room temperature with 45 µl DM (20 mM NaCl, 20 mM Tris, pH 8.2, 0.5 mM ethylenediaminetetraacetic acid, 1 mM DTT, 0.04% Triton X-100). After 60 s, 11–50 µl of this mixture was transferred into 150–189 µl RM to obtain a spermatozoon concentration of $0.6x10^7$ cells/mL. Beat frequencies (BF) were measured immediately after the RM addition. Composition of DM/RM and procedures for demembranation/reactivation were selected and adapted based on experimentation and previous studies (Billard et al., 1993; Perchec et al., 1996; Perchec Poupard et al., 1997; Saudrais et al., 1998; Linhart et al., 2002, 2003).

The RM for carp was composed of 0.15 M KCH_3COO, 20 mM Tris, pH 8.2, 0.5 mM EGTA, 1 mM DTT, 0.5% pluronic acid, and 1 mM $MgCl_2$; and for sterlet of 20 mM NaCl, 20 mM Tris, pH 8.2, 2 mM EGTA, 1 mM DTT, 1 mM $MgCl_2$, 200 µM cyclic adenosine monophosphate, and 0.5% pluronic acid. Depending on experimental conditions, the RM may also have contained ATP (vanadate free) or ADP in varying concentrations; 15 mM PCr with or without 20 µM fluorodinitrobenzene (FDNB, inhibitor of creatine kinase), or 20 µM P^1,P^5-di(adenosine-5′) pentaphosphate (diApp, inhibitor of adenylate kinase) (Saudrais et al., 1998). All reagents were purchased from Sigma-Aldrich Co.

Beat frequency assessment

Flagellar BF was measured at room temperature (18–20 °C), using stroboscopic illumination (ExposureScope®, Czech Republic) and dark field microscopy with reference to the calibrated frequency of the flash illuminator, both in individual spermatozoa or the spermatozoon population (Billard and Cosson, 1992; Cosson et al., 1985).

Determination of enzyme activity in energy supply of spermatozoon motility

Adenylate kinase, CK, and ATPase activity was measured in spermatozoon extracts after demembranation/reactivation. Sperm samples were mixed at room temperature with corresponding DM and, after 40 or 60 s for carp and sterlet, respectively, specified amounts were transferred to the RM without ATP. The ratios of the sperm/DM/RM volumes were selected to produce experimental conditions similar to those used for BF analysis. The mixtures were centrifuged for 30 min at 16 000 x *g* at 4°C, and the resulting supernatants were held at –80 °C for use in evaluation of AK, CK, and ATPase activity.

Adenylate kinase, CK, and ATPase activity was measured using the Bioluminescence Cytotoxicity Assay Kit (BioVision, Catalog Number K312-500), Creatine Kinase Activity Colorimetric Assay Kit (BioVision, Catalog Number K777-100), and EnzChek® Phosphate Assay Kit (Molecular Probes, Catalog Number E-6646) following the manufacturers recommendations.

Calculations and statistical analysis

Statistical analysis was conducted under the assumption that flagellar BF could be considered a descriptor of total enzyme activity leading to axoneme beating, allowing application of the Michaelis-Menten equation for the description of trend lines.

Beat frequency kinetic characteristics were measured under varying concentrations of ATP with and without 0.1 mM ADP, a presumed competitive inhibitor of dynein-ATPase, in RM. Beat frequencies exhibited by five spermatozoa in each sperm sample and at each ATP concentration were determined and incorporated into analysis by the Michaelis-Menten model according to the formula $BF = \frac{BF_{max} \times [ATP]}{K_m + [ATP]}$, where BF was the measured beat frequency at a given ATP concentration [ATP], and BF_{max} and K_m were calculated maximum BF and the Michaelis constant, respectively. Plotting of kinetic curves and calculations of mean and standard error for BF_{max} and K_m were conducted using GraphPad Prism version 6 for Windows (La Jolla, CA, USA, www.graphpad.com). To analyze the inhibition of dynein ATPase by ADP, Lineweaver-Burk plots (double reciprocal plot of BF vs. ATP concentration) with description of trend lines equation with corresponding R^2 and *P* were generated using Statistica v. 12 (Statsoft Inc, Tulsa, OK, USA). Significance of differences in K_m and BF_{max} was checked by the t-test in the *difference test calculator* in Statistica v. 12 (Statsoft Inc, Tulsa, OK, USA). Statistical significance was accepted as $P < 0.05$.

Time-dependent changes in BF were described by trend lines obtained from the BF data of individual spermatozoa. At least 50 data points were incorporated into the analysis to plot each line. Trend lines were plotted by applying one phase exponential association of BF vs. post-reactivation time $BF = BF_{max} \times (1 - e^{-kt})$, where BF is beat frequency measured at time t, and BF_{max} and k are calculated parameters, using GraphPad Prism version 6 for Windows (La Jolla, CA, USA, www.graphpad.com). Significance of differences in k and BF_{max} were checked by t-test in the *difference test calculator* in Statistica v. 12 (Statsoft Inc., Tulsa, OK, USA). Statistical significance was accepted as $P < 0.05$. For multiple comparisons, Bonferroni correction for *P* was applied.

Due to a low number of CK, AK, and ATPase activity quantifications, a nonparametric Mann–Whitney U-test was used for between-species comparisons. Data were presented as mean ± SE. Statistical significance was accepted as $P < 0.05$.

Results and discussion

Beat frequency relative to ATP concentration

Beat frequency of demembranated carp and sterlet spermatozoa showed positive correlation with ATP level in the RM (Fig. 1). At 0.05 mM ATP, carp spermatozoa showed slight motility, but it was possible to estimate their BF (27 ± 2 Hz) only with ATP in the RM at 0.1 mM or higher. When ATP concentration was increased, carp spermatozoon BF increased, reaching a plateau value of 70 Hz with 0.8–1.2 mM ATP. In sterlet, flagella BFs were 32 ± 3 Hz with 0.05 mM ATP, 38 ± 3 Hz with 0.1 mM ATP, and reached a plateau (63–67 Hz) at 0.6 mM ATP (Fig. 1).

Beat frequencies of demembranated carp and sterlet spermatozoa with 1 mM ATP in the RM were higher than previously reported values in native spermatozoa. Native carp spermatozoa have been shown to exhibit flagella BF ranging from 30–50 (Perchec Poupard et al., 1998) to 50–60 Hz (Perchec et al., 1995). Boryshpolets et al. (2013) obtained a mean flagella BF of 52 ± 2 Hz in sterlet using high-speed video microscopy. Further, as a result of active movement, native spermatozoa lost energy, and flagella BF essentially decreased. In carp spermatozoa, ATP level during dilution in activating medium has been shown to significantly decrease within two minutes and BF to decrease from 50–60 Hz to 7–10 Hz during the same time period (Perchec et al., 1995). The present study found demembranation of spermatozoa to result in dramatically increased flagella BF and motility duration in both fish species. Maximum BF with 1.0 mM ATP was approximately 21% higher than that previously reported in native carp and sterlet spermatozoa (Perchec et al., 1995; Boryshpolets et al., 2013). With 1.0 mM ATP, demembranated carp and sterlet spermatozoa were motile for more than 10 min (data not shown). With demembranation, the axonemal mechanism comes into direct contact with the external environment, and the spermatozoon is supplied with a higher and more consistent concentration of ATP than is available within the native cell. ATP content in native carp spermatozoa was shown to be in the range of 8–9 nmol per 10^8 spermatozoa (Perchec Poupard et al., 1998). The presence of ATP at 1.0 mM in RM may result in significant increase in motility duration and BF in demembranated spermatozoa.

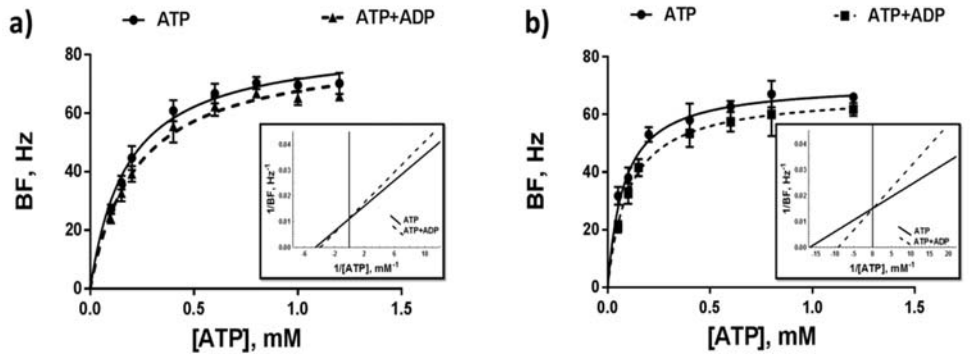

Figure 1. *Relationship of beat frequency in demembranated spermatozoa to concentration of ATP. (a) Carp. (b) Sterlet. (ATP) Concentration of ATP in reactivating medium was 0.05–1.2 mM. (ATP + ADP) ATP reactivating concentrations were the same as for ATP group with addition of 0.1 mM ADP. Values are mean ± SE; trend lines plotted from Michaelis-Menten model. See Table 1 for calculated parameters of trend lines. Inset: reciprocal plot of beat frequency vs. ATP concentration (Lineweaver-Burk plot). See Table 2 for calculated parameters for trend lines.*

Table 1. *Calculated Michaelis-Menten parameters for demembranated sterlet and carp spermatozoa reactivated in ATP-containing media.*

Species	Parameter	Substrate[1]	
		ATP	**ATP +ADP**
Carp	BF_{max}, Hz	85 ± 1	83 ± 1
	K_m, mM	0.180 ± 0.008*	0.219 ± 0.008
	R^2 for trend line	0.94	0.95
Sterlet	BF_{max}, Hz	71 ± 1	68 ± 1
	K_m, mM	0.079 ± 0.004*	0.104 ± 0.005
	R^2 for trend line	0.89	0.92

[1] Substrate: ATP – 0.05–1.2 mM ATP in reactivating medium; ATP + ADP – ATP concentrations same as ATP group with addition of 0.1 mM ADP.
* – within rows indicates significant difference ($P < 0.05$). For the Michaelis constant (K_m) and maximum beat frequency (BF_{max}), data are presented as mean ± SE.

The obtained data are in accordance with results of Saudrais et al. (1998), who found that native trout spermatozoa, which showed initial flagella BF of 60–70 Hz, decreased flagella BF to 20 Hz at 20 s and ceased swimming at 35–40 s. In contrast, demembranated trout spermatozoa in RM with 1.0 mM ATP exhibited BFs of 60–100 Hz and consistent movement for more than 15 min.

Some assumptions can be made with respect to the internal ATP concentration of native fish spermatozoa. In trout spermatozoa, assuming a volume of $16x10^{-9}$ µL per spermatozoon (Christen et al., 1987), and assuming that the cell volume is constant during the motility period, the initial ATP concentration (before swimming) would be 1–5 mM. The calculated final ADP concentration is about 2 mM in arrested (after swimming) spermatozoa, while the ATP concentration drops to 0.5–1 mM or less at the cessation of motility. As the K_m of the dynein ATPase for ATP was found to be 80–180 µM, such relatively high final ATP concentration cannot fully explain the cessation of motility as dynein activity arrest due to a lack of substrate (Cosson, 2013).

Table 2. *Lineweaver-Burk plot parameters for sterlet and carp demembranated spermatozoa reactivated in ATP-containing media.*

Species	Parameter	Substrate[1]	
		ATP	**ATP + ADP**
Carp	Equation	$\frac{1}{BF} = 0.0110 + \frac{0.0025}{[ATP]}$	$\frac{1}{BF} = 0.0115 + \frac{0.0030}{[ATP]}$
	R^2 for trend line	0.966	0.967
	P	< 0.001	< 0.001
Sterlet	Equation	$\frac{1}{BF} = 0.152 + \frac{0.0009}{[ATP]}$	$\frac{1}{BF} = 0.0155 + \frac{0.0016}{[ATP]}$
	R^2 for trend line	0.854	0.955
	P	< 0.001	< 0.001

[1] Substrate: ATP – 0.05–1.2 mM ATP in reactivating medium; ATP + ADP – ATP concentrations same as ATP group with addition of 0.1 mM ADP.

It is known that increased ADP concentration in proximity to the axoneme exerts an inhibitory effect on flagella beating (Okuno and Brokaw, 1979). To check the possible inhibitory effect of ADP on dynein ATPase, 0.1 mM ADP was added to the RM. The dependency of BF on ATP concentration with or without ADP was in accordance with Michaelis-Menten kinetics (Table 1).

The addition of ADP resulted in a lower BF at every studied ATP concentration, reaching a plateau at 65–67 Hz in carp and 58–60 Hz in sterlet. For both species, calculated BF_{max} did not significantly change with addition of ADP (Table 1).

Apparent K_m for ATP was 180 ± 8 μM for carp and 79 ± 4 μM for sterlet. A reciprocal plot of BF vs. ATP concentration revealed that apparent K_m for ATP was 180 μM for carp and 79 μM for sterlet. This suggests that sterlet axonemal dynein ATPases have a higher degree of affinity to ATP than do those of carp. Further support for this may be that BF of demembranated sterlet spermatozoa reached a plateau at ATP concentration of 0.6 mM as opposed to 0.8 mM in carp. The apparent K_m for ATP was increased upon addition of ADP, in contrast to BF_{max}, which remained unchanged, evidence for competitive inhibition of dynein ATPase by ADP. It should be noted that the observed increase in K_m for ATP with addition of ADP was higher in sterlet, at 32%, than observed in carp (22%). Competitive inhibition of dynein ATPase by ADP is also apparent in the Lineweaver-Burk plot (Fig. 1, Table 2), which indicated that ADP is a stronger competitive inhibitor of sterlet axonemal dynein ATPases than of those in carp.

The value of K_m for ATP in carp is similar to the K_m of 200 μM estimated in trout by Saudrais et al. (1998) under conditions similar to those of the present study. Gatti et al. (1989) showed that trout 19S sucrose gradient-purified outer arm dynein exhibited an apparent K_m for ATP of 40 ± 16 μM. Saudrais et al. (1998) explained this difference citing results of Warner and McIlvain (1986) showing that the rebinding of the soluble forms of the 13S and 21S dyneins of *Tetrahymena* cilia to extracted doublet microtubules activates ATPase and decreases the affinity of the two enzymes for ATP, producing a K_m shift from 20–40 μM to more than 100 μM. Such increase in K_m values at rebinding of dyneins to the microtubule lattice could be the reason for the high K_m found in the present study, as we used demembranated spermatozoa with undamaged axonemes. Nevertheless, the striking difference in K_m of ATP in sterlet and carp spermatozoa, and, correspondingly, in their axonemal dynein ATPase affinity to ATP, remains to be clarified.

The relationship between ATP and ADP is not straightforward: recent results of Lindemann et al. (2014) show that changes in the balance of ATP, Mg^{2+}, and ADP are critical to establishment of the beat cycle of mammalian spermatozoa. Whether the same situation exists in fish spermatozoa remains to be elucidated. The relationship between ATP and ADP was explored in sea urchin spermatozoa by Yoshimura et al. (2007), who reported that, at neutral pH, ATP concentration of 1.0 mM inhibits motility, and this inhibition can be counteracted by ADP. The authors hypothesized that ATP-induced inhibition and ADP-induced activation are probably acting through phosphorylation/dephosphorylation of dynein outer arm linked protein(s). This suggests that information about internal pH of fish spermatozoa during the motility period would be of value.

The present results and previous research indicate that demembranation/reactivation of fish spermatozoa results in a general pattern of increase in flagella BF and motility duration. The question remains whether the mechanism is similar among fishes, or if there are species differences in the pathways supplying energy for spermatozoon movement, prompting the study ADP or PCr instead of ATP as a motility substrate in spermatozoa of carp and sterlet, representatives of different subclasses of actinopterygians with spermatozoa of differing structure and mode of motility activation.

Beat frequency time-dependency on energy substrate availability

When RM was supplemented with 0.5 mM ADP only, no axonemal movement was observed immediately after mixing in either studied fish species. After 1.5 min, slow beating of flagella was initiated. Thereafter, the BF increased as a function of time, reaching maximum values of 30 Hz in carp at 15 min and 28 Hz in sterlet at 17 min (Fig. 2a). Calculation of one phase exponential association of BF vs. time post-reactivation showed high R^2 for trend lines, which may be an indication of appropriateness of the selected method for the description of time dependency of flagella beating (Table 3). The calculated BF_{max} was higher in sterlet, while the k coefficient had a higher value in carp (Table 3). As the k coefficient reflects the steepness of the trend lines, its significantly higher value in carp suggested higher AK activity in this species.

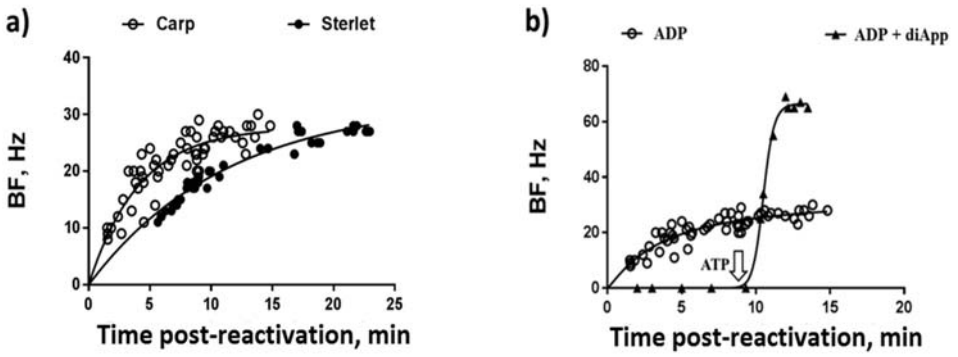

Figure 2. *Beat frequency of demembranated spermatozoa in reactivating medium containing ADP. (a) Carp and sterlet spermatozoa. (b) Carp spermatozoa without and with 20 μM P^1,P^5-di(adenosine-5′) pentaphosphate (diApp, inhibitor of adenylate kinase). With diApp, 1 mM ATP was added 9 min post-reactivation (arrow). See Table 3 for calculated parameters.*

Table 3. *One phase exponential association parameters for time-dependency of beat frequency of demembranated carp and sterlet spermatozoa activated by ADP.*

Parameter	Carp	Sterlet
BF_{max}, Hz	28 ± 1	32 ± 1*
k	0.244 ± 0.024	0.089 ± 0.004*
R^2 for trend line	0.795	0.933

* – within row indicates significant difference ($P < 0.05$). For maximum beat frequency (BF_{max}) and k coefficient, data are presented as mean ± SE.

When diApp, a specific inhibitor of AK, was present in RM devoid of ATP, no movement of demembranated spermatozoa was observed (Fig. 2b). Addition of 1.0 mM ATP under these conditions resulted in immediate motility with high BF in carp (Fig. 2b). When ATP was added 9 min post-reactivation, flagella became motile, reaching maximum BF values of 69 Hz after 3 min. Similar results were obtained for demembranated sterlet spermatozoa (data not shown).

ADP involvement in spermatozoon energy supply may have been due to the presence of AK in the studied samples. Adenylate kinase is known to produce either ADP or ATP and AMP depending on the respective concentrations of these three nucleotides. In spermatozoon

flagella, in which ATP is utilized rapidly, AK predominantly produces ATP and AMP. Adenylate kinase activity has been found in flagella of evolutionarily diverse organisms, from unicellular protozoa to mammals (Pullen et al., 2004; Cao et al., 2006). As in the present study, the inhibition of motility by diApp, a specific inhibitor of AK, when ADP was used as energy substrate, was reported for bovine spermatozoon flagella (Schoff et al., 1989). Adenylate kinase activity has been detected in spermatozoa of burbot *Lota lota*, brown trout *Salmo trutta,* and rainbow trout *Salmo gairdnerii* (Saudrais et al., 1998; Lahnsteiner and Mansour, 2012).

As was discussed by Ingermann (2008), spermatozoa of salmonids and cyprinids possess PCr and CK, which catalyzes the formation of ATP from PCr and ADP. Therefore, spermatozoa regenerate ATP from PCr and motility-generated ADP *via* CK and, additionally, from ADP by the action of AK. According to Saudrais et al. (1998), the contribution of AK to energy supply is lower than that of CK. Data on the effectiveness of the Cr/PCr shuttle in energy supply of sturgeon spermatozoa are limited. Its activity is probably low, leading reduced transfer of energy from the mid-piece (containing mitochondria) to the distal part of the flagellum. This may be the source of the decrease of wave amplitude in the distal part of the flagellum observed in Siberian sturgeon spermatozoa (Billard et al., 1999).

To evaluate the contribution of CK and, consequently, the existence of a Cr/PCr shuttle in carp and sterlet spermatozoon motility, RM containing ADP was supplemented with 15 mM PCr (Saudrais et al., 1998). This addition was associated with increased motility and flagella BF. Flagellar beating appeared in the initial two minutes post-reactivation, reaching maximum values of 60 Hz at 13 min in carp and 50 Hz at 11 min in sterlet (Fig. 3). The combination of ADP and PCr supplied carp flagella axonemes with energy sufficient for BFs approximately 86% of plateau values observed with 0.8–1.2 mM ATP. The level was 75% that with ATP in sterlet axonemes.

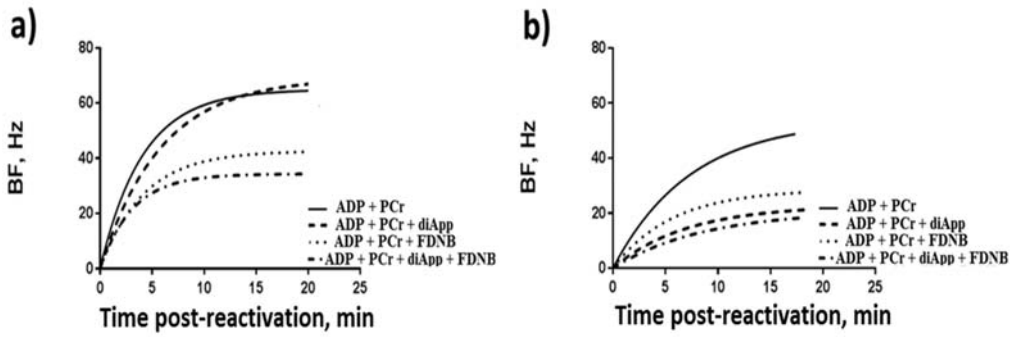

Figure 3. *Time vs. beat frequency in demembranated spermatozoa reactivated in medium of differing composition. (a) Carp. (b) Sterlet. (ADP + PCr) – with 0.5 mM ADP and 15 mM phosphocreatine (PCr); (ADP + PCr + FDNB) – with 0.5 mM ADP + 15 mM PCr combined with 20 μM fluorodinitrobenzene (FDNB); (ADP + PCr + diApp) – with 0.5 mM ADP + 15 mM PCr combined with 20 μM P^1,P^5-di(adenosine-5′) pentaphosphate (diApp); and (ADP + PCr + diApp + FDNB) – with 0.5 mM ADP + 15 mM PCr combined with 20 μM P^1,P^5-di(adenosine-5′) pentaphosphate and 20 μM fluorodinitrobenzene. Trend lines are plotted from 50–60 data points. See Table 4 for calculated parameters.*

For evaluation of the relative contribution of AK and CK to the energy supply of carp and sterlet spermatozoa, flagella BF was measured in RM containing 0.5 mM ADP and 15 mM PCr as well as with either or both diApp (AK inhibitor), FDNB (CK inhibitor). In carp spermatozoa,

maximum BF was 60 Hz (ADP + PCr), 63 Hz (ADP + PCr + diApp), 42 Hz (ADP + PCr + FDNB), and 36 Hz (ADP + PCr + diApp + FDNB). This suggests that the primary contributor to energy supply was CK, as its inhibition alone or together with AK resulted in a more pronounced decrease in BF, and AK inhibition alone had no significant effect. The outcome with sterlet spermatozoa was slightly different. Sterlet BF was sensitive to the presence of inhibitors of both AK and CK, showing BF in the following order: 50 Hz (ADP + PCr), 41 Hz (ADP + PCr + FDNB), 31 Hz (ADP + PCr + diApp), and 17 Hz (ADP + PCr + diApp + FDNB) (Fig. 3; Table 4).

Table 4. *One phase exponential association parameters for time-dependency of beat frequency of demembranated carp and sterlet spermatozoa activated by ADP + PCr.*

Species	Parameter	ADP + PCr	ADP + PCr + diApp	ADP + PCr + FDNB	ADP + PCr + diApp + FDNB
Carp	BF_{max}, Hz	65 ± 2[a]	69 ± 4[a]	43 ± 2[b]	34 ± 2[b]
	k	0.25 ± 0.01	0.17 ± 0.03	0.24 ± 0.02	0.32 ± 0.05
	R^2 for trend line	0.917	0.868	0.874	0.620
Sterlet	BF_{max}, Hz	54 ± 3[a]	23 ± 5[b]	29 ± 3[b]	21 ± 2[b]
	k	0.14 ± 0.02	0.14 ± 0.08	0.18 ± 0.04	0.12 ± 0.03
	R^2 for trend line	0.8564	0.5207	0.6845	0.8566

Values with different letters within rows are significantly different ($P < 0.05$). For maximum beat frequency (BF_{max}) and k coefficient, data are presented as mean ± SE.

Inhibition of CK produced the greatest impact on energy supply in carp spermatozoa, while, for sterlet spermatozoa, AK appeared mostly crucial for the maintenance of flagella movement.

Ingermann (2008) stated that ATP regeneration systems AK and CK can delay, but do not prevent, the dramatic decline in ATP level during motility. Carp and sterlet spermatozoa, as is the case in the majority of externally fertilizing fishes, are considered to rely on pre-motility accumulated endogenous energy and nutrient stores. Billard et al. (1999) found that ATP content in Siberian sturgeon spermatozoa was lower than reported for teleosts. The difference in initial levels of ATP in carp and sterlet spermatozoa is most likely the primary reason for the observation that similar ADP and PCr content resulted in differing efficacy of motility restoration. On the other hand, it has been suggested that sturgeon spermatozoa do not depend entirely on pre-accumulated ATP for movement, but possess the capacity to regenerate energy molecules during swimming (Billard et al., 1999). Our data implied that AK and CK are not sufficient to fully supply energy needed by sterlet spermatozoa, and one could assume the existence of other ATP regeneration system(s), for example, arginine kinase.

Along with CK, arginine kinase is a representative of the phosphagen systems. Phosphagen systems carry out the processes of trans-phosphorylation of ADP to ATP and function in the transport of high-energy phosphate from the place of its formation (in spermatozoa typically mitochondria located in the mid-piece) to the place of its consumption (dynein ATPases distributed along the flagellum). The role of these systems is critical in cells displaying high and variable rates of energy turnover, such as primitive-type spermatozoa (Ellington, 2001). Using a facilitated diffusion model for spatial ATP buffering, Ellington and Kinsey (1998) compared the efficacy of the major phosphagen systems for cellular transport of energy and found them to differ only slightly in capacity for energy transport.

The model describing the PCr shuttle originates from Tombes and coauthors (Tombes and Shapiro, 1985; Tombes et al., 1987) on sea urchin spermatozoa. Basically, in the ATP/PCr shuttle, ATP produced in the mitochondrion is transformed by mitochondrial CK into PCr, which

diffuses along the flagellum more efficiently than does ATP. The PCr is re-transformed locally into ATP by flagellar CK, which transfers inorganic phosphate from PCr to ADP generated by dynein-ATPase activity (Cosson, 2013). Tombes et al. (1987) provided a quantitative analysis of flagellar bending reduction in the distal portion of the flagellum when CK was inhibited and revealed that demembranated spermatozoa in which CK is inhibited show normal axonemal bending upon reactivation with ATP. Extension and validation of the model were more recently published by Takao and Kamimura (2008) who calculated the length limit of native spermatozoon flagella: in a flagellum longer than 60 μm, concentration of ATP at the tip was estimated to be much lower than 100 μM, potentially too low to maintain beating at adequate frequency. This could explain the rigidity of the distal part of the fish spermatozoon at the end of the motility period (Cosson, 2010, 2013). The maximum feasible flagellar length in the model of Takao and Kamimura (2008) is 100 μm. It should be noted that activity of a flagellum beating at high frequency could contribute to increasing the intra-flagellar diffusion rate by a shaking effect of molecules such as ATP (Takao and Kamimura, 2008).

The PCr/CK system is present in the vertebrates, lower chordates, and lower and higher invertebrate groups, while an arginine phosphate/arginine kinase system is widely distributed throughout invertebrates and present in lower chordates, but absent in vertebrates. As the PCr/CK system evolved from an arginine phosphate/arginine kinase system-like ancestor (Ellington, 2001), the possibility of an arginine phosphate/arginine kinase system, or another phosphagen system, in sterlet spermatozoa cannot be excluded, especially taking into account the ancient origin of the sturgeon compared to teleosts and the observed lower efficacy of ADP and PCr in restoring the BF of demembranated sterlet spermatozoa to a maximum level (Fig. 3, Table 4).

Enzyme activity involved in energy supply of spermatozoon motility

Among the studied soluble enzymes, CK activity showed the highest absolute values in both carp and sterlet (Table 5). Sterlet spermatozoon extracts displayed approximately three-fold (224 vs. 80 U/10^9 spermatozoa) the CK activity and two-fold (1.03 vs. 0.48 U/10^9 spermatozoa) the ATPase activity observed in carp spermatozoa. Conversely, sterlet spermatozoon extracts showed significantly lower AK activity, at approximately one quarter of the level observed in carp spermatozoa. The low baseline activity of CK in carp and AK in sterlet could be the source of the most pronounced effects of their inhibition on energy supply for flagella movement in the respective species. At the same time, it can be assumed that longer average spermatozoon motility period in sterlet (4 min) compared to carp (40 s) could be partially explained by the higher CK activity.

Table 5. *Enzyme activity in energy supply in demembranated carp and sterlet spermatozoon soluble extracts.*

Activity	Carp (n = 5)	Sterlet (n = 6)
Adenylate kinase, mU/10^9 spz	6.37 ± 0.69	1.57 ± 0.11*
Creatine kinase, U/10^9 spz	80.0 ± 5.4	223.7 ± 22.4*
ATPase, U/10^9 spz	0.48 ± 0.02	1.03 ± 0.07*

* – within rows indicates significant difference ($P < 0.05$). The data are presented as mean ± SE.

Conclusions

The results of this study indicated that spermatozoa of two taxonomically distant fish species, common carp and sterlet, which exhibit characteristic differences in their structure, mode of motility activation, and motility duration, possess similar systems for energy supply of flagella motility. It was shown for the first time that the PCr/CK phosphagen system is present in sturgeon spermatozoa and plays an essential role in regeneration of ATP. The efficacy of the studied ATP regeneration systems differs in the investigated fish species. Adenylate kinase and CK provide demembranated carp spermatozoa with sufficient energy to allow flagellar beat frequency at essentially the same level as motile native spermatozoa. In sterlet, functioning of the AK and CK systems was shown to be potentially less effective in energy maintenance for motility of demembranated spermatozoa, although observed beat frequencies are similar to those found in native spermatozoa.

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CHAPTER 3

STURGEON SPERM MATURATION: INVOLVEMENT OF PROTEOLYSIS REGULATORS

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

3.2. Dzyuba, B., Boryshpolets, S., Cosson, J., Dzyuba, V., Fedorov, P., Saito, T., Psenicka, M., Linhart, O., Rodina, M., 2014. Motility and fertilization ability of sterlet *Acipenser ruthenus* testicular sperm after cryopreservation. Cryobiology 69 (2): 339–341.

3.3. Dzyuba, V., Słowińska, M., Dzyuba, B., Cosson, J., Ciereszko, A., Štěrba, J., Rodina, M., Linhart, O., 2015. Proteolytic and anti-proteolytic activity during sterlet spermatozoon maturation. (manuscript)

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Short Communication

In vitro sperm maturation in sterlet, *Acipenser ruthenus*

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ABSTRACT

The aim of the study was to examine sperm maturation in sturgeon and to establish the localization of the maturation. We demonstrated that sperm maturation occurs in sturgeon outside the testes *via* dilution of sperm by urine. The process involves the participation of high molecular weight (>10 kDa) substances and calcium ions.

1. Introduction

Sperm maturation is a process resulting in the acquisition of the potential for motility and fertilization by morphologically developed spermatozoa. It is considered to be the final physiological stage of spermiogenesis, and is well known in taxonomically distant groups, such as mammals [1] or insects [2]. In fish, mature spermatozoa initiate full motility immediately after their release into an appropriate environment, while immature spermatozoa are not able to initiate efficient motility under the same conditions. However, such a process has been found only in a restricted number of teleost species [3,4] and it has not yet been described in the sturgeon.

In the sturgeon, the efferent ducts coming from the testis directly contact the kidneys, and testicular sperm present in Wolffian ducts is probably diluted by urine. It seems that such arrangement leads to low semen osmolality, low content of protein in seminal fluid and low sperm concentration – the characteristics specific for sturgeons [5]. However, dilution of testicular sperm by urine remains speculative and needs to be proved experimentally. The physiological importance of this process in sperm maturation cannot be excluded. The aims of the present study were to examine sperm maturation in the sturgeon and to identify its maturation site. For this purpose, we investigated sperm concentration and motility as well as seminal plasma ion (K^+, Na^+, Ca^{2+}) concentrations in sperm collected from the testis and Wolffian ducts. Further, we

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examined whether sperm maturation in the sturgeon is under control of: (1) external enzymatic activity, as it was found in mammals [1], or (2) the ions as it was shown for other fish species [4].

2. Materials and methods

All experiments were performed according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. During the natural spawning season (April–May), six sterlet (*Acipenser ruthenus*) males (3–4 year-old, 0.6–1.0 kg body weight, bw) were transferred from fish-farming ponds (water temperature 8–10 °C) into the 0.8 m^3 closed water recirculation system, located at the hatchery of the South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic. Thereafter, the water temperature was increased to 15 °C within 24 h, and before the beginning of the experiment, fish were held four days without feeding. Urine was collected from the urogenital sinus by aspiration using a plastic catheter (4 mm diameter) connected to a 10 mL syringe, and was stored at 4 °C for 24–30 h. Spermiation was stimulated by one intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution (4 mg/kg bw). Spermatozoa were collected from: (1) urogenital (Wolffian) ducts by the procedure described for urine collection, 24 h after stimulation of spermiation (Wolffian duct sperm, used in fisheries for artificial sturgeon propagation), and (2) testis (testicular sperm). Immediately after Wolffian duct sperm collection, the fish were euthanized by striking the cranium followed by exsanguination. After euthanasia, the digestive tract was removed, and testicular sperm was collected after incision of the efferent ducts [6].

Seminal fluid was obtained as a result of sperm centrifugation at 4 °C: (1) 300 × *g* for 10 min, and then (2) the resulting supernatant, 5000 × *g* for 15 min. Supernatants obtained after the second centrifugation were used in the study. Seminal fluid osmolality was measured using a Vapor Pressure Osmometer 5520 (Wescor, Logan, UT, USA) and was expressed in mOsm/kg. Concentrations of sodium (Na^+), and potassium (K^+) ions were measured by potentiometry using Ion Selective Electrodes (ISE, Bayer HealthCare, Tarrytown, NY, USA). Calcium (Ca^{2+}) ion concentration was measured by absorption photometry applying o-cresolphthalein complexone method [7]. The protein concentration was determined by absorption photometry with Bradford reagent.

Tris–HCl buffer (10 mM, pH 8.0) containing 0.25% pluronic (a substance that prevents spermatozoa from sticking to slides) was used as activating medium (AM). To trigger sperm motility, Wolffian duct sperm and testicular sperm were diluted in AM with dilution rates 1:100, and 1:1000, respectively. Dilution rates were selected according to requirements of the motility assessment procedure, because of differences in sperm concentration in Wolffian duct and testicular sperm samples. Sperm suspensions were thoroughly mixed for 2 s. Sperm motility was recorded for 1–2 min post-activation using video microscopy combined with stroboscopic illumination (ExposureScope®, Czech Republic). Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL) and percent of motile cells (motility rate) by micro-image analyzer (Olympus Micro Image 4.0.1. for Windows, Japan) on five successive overlapping video frames. Overlapping tracks of sperm heads permitted the calculation of VCL (defined as a total point-to-point distance traveled by the spermatozoon over the 0.16 s – the time period between the first and fifth frames) and motility rate. Motility parameters for Wolffian duct sperm were evaluated within a 1 h-period after collection. The response of the testicular sperm to dilution with AM was tested immediately after collection and at the end of experiment (2 h later).

Sperm concentration was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) and Olympus BX 50 phase contrast microscope (200× magnification; Olympus, Japan). To estimate the sperm dilution rate during testicular sperm passage through the kidney, the concentration ratio of testicular sperm/Wolffian duct sperm was calculated for each fish. To investigate sperm maturation, testicular sperm was incubated (dilution rate 1:200) in three solutions: (1) seminal fluid from Wolffian duct sperm, (2) urine, and (3) artificial seminal fluid (ASF: 18 mM NaCl, 3 mM KCl, and 0.2 mM $CaCl_2$). To investigate the influence of Ca^{2+} on sperm maturation, ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA, Ca^{2+} chelator; final concentration 2 mM) and verapamil (calcium channel blocker; final concentration 100 μM) were added to seminal fluids from Wolffian duct sperm. To investigate the pH involvement in sperm maturation, Tris was added to ASF (final concentration 10 mM), and pH of the resulting solution was adjusted to 6.5 or 8.5 by adding 100 mM HCl. To test the importance of trypsin-like activity for motility of Wolffian duct sperm, trypsin inhibitor from soybean (SBTI) was added to seminal fluid or urine (final concentration 20 μg/mL), and sperm motility was checked after 10 min of incubation. To investigate whether high molecular weight substances are involved in sperm maturation, seminal fluid from Wolffian duct sperm was centrifuged 20 min at 1000 × *g* in Vivaspin Concentrators (Sartorius Stedim Biotech GmbH, Germany) with 10 kDa cutoff. The low molecular weight fraction of seminal fluids from Wolffian duct sperm was used as testicular sperm incubating medium.

Data distribution and homogeneity of dispersion were tested by the Shapiro–Wilks test and Levene's test, respectively. Normally distributed data were analyzed by one-way ANOVA followed by Fisher LSD test. Due to a low number of observations ($n = 6$), a nonparametric Kruskal–Wallis ANOVA followed by the Mann–Whitney *U*-test was used for comparison of motility rate, motility duration and ion concentration in seminal fluid. Data were presented as mean ± SD. Statistical significance was accepted at $p < 0.05$. All analyses and graph plotting were conducted using Statistica V 9.1 computer program (Statsoft Inc, Tulsa, OK, USA).

3. Results and discussion

Concentration of testicular sperm ($28 \pm 9 \times 10^9$/mL) was significantly higher than that of Wolffian duct sperm ($0.5 \pm 0.4 \times 10^9$/mL). Osmolality and cation (Na^+, K^+ and Ca^{2+}) content were significantly lower in Wolffian duct sperm than

Table 1 – Chemical characteristics of urine and seminal fluids of sterlet.

Fluid	Examined parameters					
	$[Na^+]$ (mM)	$[K^+]$ (mM)	$[Ca^{2+}]$ (mM)	Total protein (mg/mL)	Osmolality (mOsm/kg)	pH
Urine	15 ± 2^a	2 ± 1^a	0.22 ± 0.09^a	0.07 ± 0.04^a	32 ± 8^a	8.0 ± 0.3^a
SFTS	106 ± 7^b	15 ± 2^b	0.57 ± 0.19^b	3.82 ± 0.40^b	250 ± 6^b	6.4 ± 0.3^b
SFWS	16 ± 2^a	3 ± 1^a	0.19 ± 0.06^a	0.09 ± 0.04^a	34 ± 7^a	8.1 ± 0.4^a

SFTS: seminal fluid of testicular sperm; SFWS: seminal fluid of Wolffian duct sperm.
Different letters designate significant differences among the fluids ($p < 0.05$, $n = 6$).

in testicular sperm (Table 1). Since testicular sperm and Wolffian duct sperm were collected before and after sperm passage through the kidney, respectively [6], the differences between both sperm types may result from dilution of testicular sperm. The testicular sperm/Wolffian duct sperm concentration ratio was used as an approximate estimation of testicular sperm dilution. In the current study, the dilution rate ranged from 26 to 262. It is possible that urine is the diluting factor since ion concentrations are similar in urine and seminal fluid of Wolffian duct sperm (Table 1). This supposition is also supported by previous reports, showing similarities in ionic composition of seminal fluid and urine in sterlet [8,9]. Extracellular K^+ inhibits sturgeon flagellar activity in a concentration dependent manner [10,11], and this inhibition can be overcome by Ca^{2+} [12]. Thus, high K^+ and low Ca^{2+} concentrations in seminal fluids from Wolffian duct sperm, conditions which may result from testicular sperm dilution with hypotonic urine [8,12], seem to be the factors that prevent sperm motility activation. We have observed significant differences in testicular sperm and Wolffian duct sperm responses to dilution with activating medium. The activation medium activated the Wolffian duct sperm motility, but was not able to initiate testicular sperm motility immediately after collection and at the end of the experiment (2 h later). The Wolffian duct sperm was characterized by high motility rate and VCL values (Table 2) typical for sterlet [13]. In our opinion, the difference in the ability to trigger motility may be an indicator of sperm maturity.

It is of interest that after pre-incubation of testicular sperm in urine or seminal fluids from Wolffian duct sperm, motility was initiated in AM (Table 2). This is consistent with the notion that sperm maturation occurs in the sturgeon. It also suggests that under *in vivo* conditions the maturation takes place within 10 min after the testicular sperm passage through the kidney and results from dilution of testicular sperm with urine. The maximum level of motility rate was reached after 10 min of pre-incubation, while maximal VCL required 25 min of pre-incubation (Table 2). As sperm velocity is determined by flagellar energy-dependent activity [12], the differences in VCL observed for various testicular sperm incubation times may arise from differences in intracellular ATP concentration. It is possible that intracellular ATP level gradually increases during sperm maturation, but this hypothesis requires additional research.

Removal of high molecular weight substances from the seminal fluids from Wolffian duct sperm resulted in a lack of testicular sperm motility after 10 min of incubation ($n = 6$). It appears that the presence of high molecular weight substances in seminal fluids from Wolffian duct sperm is a prerequisite for spermatozoa maturation. It has been reported that sturgeon spermatozoa have a trypsin-like activity and that seminal fluids from Wolffian duct sperm has very low anti-trypsin activity [14]. In the current study, the inhibition of trypsin-like activity with SBTI blocked spermatozoa maturation. Additionally, pre-incubation of Wolffian duct sperm with SBTI did not change the motility rate and duration ($p > 0.05$, $n = 6$, data not shown). Thus, the results suggest that a proteolytic activity may be involved in sturgeon spermatozoa maturation, as it has been described in mammals [15] and insects [2].

Incubation of testicular sperm with ASF (designed to mimic the ionic composition of seminal fluids from Wolffian duct sperm) at pH 6.5 (seminal fluid from testicular sperm) or 8.5 (seminal fluid from Wolffian duct sperm) led to the maturation of spermatozoa. The spermatozoa acquired the ability to activate motility after 120 min of incubation (Table 2), but motility rate and VCL were significantly lower than those of testicular sperm incubated in seminal fluids from Wolffian duct sperm or urine. Longer periods of incubation (up to 5 h at both pH values, data not shown) in ASF did not increase sperm

Table 2 – Motility parameters of sterlet testicular spermatozoa and Wolffian duct spermatozoa in the *in vitro* study.

Sperm type	Pre-incubation time	Treatment during pre-incubation	Motility (%)	VCL (μm/s)	Motility duration (s)
WS	–	Control, no treatment	90 ± 6^b	185 ± 26^c	132 ± 9^b
TS	10 min	Urine	87 ± 8^b	141 ± 24^b	133 ± 12^b
TS	10 min	SFWS	87 ± 6^b	151 ± 36^b	129 ± 9^b
TS	25 min	SFWS	85 ± 5^b	177 ± 22^c	139 ± 11^b
TS	120 min	SFWS	86 ± 5^b	177 ± 17^c	139 ± 11^b
TS	120 min	ASF(pH 6.5)	19 ± 7^a	124 ± 30^a	39 ± 4^a
TS	120 min	ASF (pH 8.5)	36 ± 16^a	143 ± 22^{ab}	44 ± 8^a

WS: Wolffian duct sperm, motility was evaluated within 1 h after collection, no treatment was applied; TS: testicular sperm; SFWS: seminal fluid of Wolffian duct sperm; ASF: artificial seminal fluid. Different letters depict significant differences within a column ($p < 0.05$).

motility. Therefore, in contrast to salmonids, extracellular pH itself does not initiate sperm maturation in sturgeons. Removal of calcium ions from incubating media or blocking of sperm Ca^{2+} channels by amiloride was demonstrated to completely inhibit sperm maturation ($n = 6$, data not shown), suggesting that Ca^{2+} channels play a significant role in sperm maturation of the sturgeon. Further studies are required to determine the relationship between Ca^{2+} uptake and proteolytic activity during sperm maturation in sturgeons.

In summary, in the current study, we demonstrated for the first time that spermatozoa maturation occurs in sturgeons. Sperm maturation in this species is dependent on high molecular weight substances present in seminal fluids from Wolffian duct sperm and involves activation of Ca^{2+} channels. Furthermore, we showed that the sperm maturation takes place outside the testes, and that proteolytic activity of seminal fluids and sperm Ca^{2+} channels are involved in the process.

Conflict of interest

None declared.

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Brief Communication

Motility and fertilization ability of sterlet *Acipenser ruthenus* testicular sperm after cryopreservation ☆

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ABSTRACT

Sturgeon spermatozoa are immotile in the testis and acquire the potential for motility after contact with urine in Wolffian duct. The present study tested if *in vitro* incubation of testicular sperm in seminal fluid from Wolffian duct sperm leads to the acquisition of sperm fertilization ability. Sterlet sperm was taken from the testes, matured *in vitro* and cryopreserved. The fertility and motility of cryopreserved semen were tested. Matured testicular sperm showed freeze–thaw survival rates similar to Wolffian duct sperm, which is commonly used in sturgeon artificial propagation. Matured testicular sperm and Wolffian duct sperm post-thaw motility rate and curvilinear velocity were not significantly different, while duration of matured testicular sperm motility was significantly shorter than that of Wolffian duct sperm. Development rates of embryos obtained with post-thaw matured testicular sperm and Wolffian duct sperm were not significantly different. *In vitro* maturation of sterlet testicular sperm can potentially be useful in sperm cryobanking.

Use of testicular sperm is required in artificial reproduction when collection of ejaculated sperm leads to obtaining of sperm of low quantity or in case of the death of the donor. Testicular sperm is commonly used in culture of some fish species, such as catfish [7]. Postmortem collected sperm is a source of viable genetic material for fish sperm cryobanking [9]. However, two problems are linked with the use of testicular sperm: (1) The collection of non-fully mature sperm because of the maturation process along the genital ducts, (2) The aging of sperm in the case of the death of the breeder. Sturgeons represent a group of highly exploited fish species that are considered threatened or endangered [8]. Expansion of cryobiological methods for preservation of sturgeon testicular sperm is needed and potentially useful for application in aquaculture and species conservation.

In sturgeon, testicular spermatozoa do not become motile upon transfer to, and dispersion in, fresh water. That is associated with specific anatomy of excretory pathways – the efferent ducts coming from the testes directly contact the kidneys, and testicular sperm is diluted by urine in Wolffian ducts. Recently we have demonstrated that a maturation step is necessary for sturgeon spermatozoa to acquire the potential for activation, and this step takes place exactly in Wolffian duct (from which mature sperm is collected by catheterization for practical purposes) [4]. Testicular sturgeon spermatozoa maturation can be simulated *in vitro* by pre-incubation in a urine-containing solution such as seminal fluid obtained from Wolffian duct sperm or urine itself. However, it is not clear whether spermatozoa matured *in vitro* possess fertilizing ability and could retain the ability after cryopreservation. Motility itself is not sufficient for sturgeon spermatozoa fertilizing ability because of the presence of an acrosome and dependence on acrosome reaction [10]. Investigation of these questions is important for understanding the processes involved in sturgeon sperm maturation and could be applied to expand existing methods of sturgeon artificial propagation.

The goal of this study was to investigate the cryoresistance and post-thaw fertilizing ability of sturgeon testicular spermatozoa subjected to a maturation phase before and after cryopreservation.

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Sterlet *Acipenser ruthenus* was selected as a model sturgeon species as procedures of *in vitro* gamete manipulation and fertilization are widely applied in artificial propagation of this species.

All experiments were performed according to the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice. Experiments on sperm were conducted in experimental facilities of Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic.

During the natural spawning season (April – May), 6 sterlet males (3–4 years old, 0.6–1.0 kg body weight, BW) were transferred from fish-farming ponds (water temperature 8–10 °C) to a 0.8 m^3 closed water recirculation system. The water temperature in closed water recirculation system was increased to 15 °C over the course of 24 h. Prior to initiation of experiments, fish were held 4 days without feeding.

Spermiation was stimulated by a single intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution (4 mg/kg BW) [3]. Twenty four hours post-stimulation, spermatozoa were collected from the urogenital ducts by aspiration using a 4 mm plastic catheter connected to a 10 ml syringe (Wolffian duct sperm, commonly used in fisheries for artificial sturgeon propagation). Immediately after Wolffian duct sperm collection, the fish was euthanized by a blow to the skull and exsanguination. The digestive tract was removed, and testicular sperm was collected via incision of the efferent ducts [1].

Seminal fluid was obtained from Wolffian duct sperm centrifugation at 4 °C 300*g* for 10 min. The supernatant was collected and centrifuged at 5000*g* for 15 min. Supernatants obtained from the second centrifugation were used in the experiments.

For maturation, testicular sperm was incubated for 25 min in the seminal fluid at a dilution rate of 1 vol of testicular sperm to 50 vol of seminal fluid [4]. Wolffian duct sperm and matured testicular sperm were frozen using conventional freezing procedures: prior to freezing the samples were diluted 1:1 in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0 [5] containing 10% methanol (methanol concentration after sperm dilution is 5%). Diluted sperm was placed in 0.5 ml straws (CRYO-VET, France) and suspended 3 cm above liquid nitrogen in a Styrofoam box for 10 min and then plunged into the liquid nitrogen. Sperm solution was thawed for 6 s in a 40 °C water bath. Thawed sperm was used immediately for motility or fertilization assays.

To investigate whether premature spermatozoa survive cryopreservation, testicular sperm was frozen after 1 min incubation in seminal fluid from Wolffian duct sperm, a period insufficient for sperm maturation. Samples were frozen and thawed as described above. Motility of these samples was evaluated immediately after thawing and after 25 min post-thaw storage. Fertilizing ability of spermatozoa immediately after thawing was also evaluated.

For triggering motility, sperm samples were diluted at 1:100 in an activation medium consisting of 10 mM Tris–HCl buffer, pH 8.0, containing 0.25% Pluronic® F-127 (SIGMA-ALDRICH, catalogue number P2443) to prevent spermatozoa from adhering to microscope slides. Sperm suspensions were thoroughly mixed for 2 s. Motility was recorded for 1–2 min post-activation using video microscopy combined with stroboscopic illumination (ExposureScope®, Czech Republic). Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL) and percent of motile cells (motility rate) by micro-image analyzer (Olympus Micro Image 4.0.1. for Windows, Japan) on five successive overlapping video frames. After overlapping of frames, tracks of spermatozoa heads became visible, permitting calculation of VCL (defined as total point-to-point distance traveled by the spermatozoon in 0.16 s, the time period between the first and fifth frames) and motility rate [3]. Sperm motility duration was measured as the time from activation to cessation of motility in approximately 95% of spermatozoa. Spermatozoa concentration was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on Olympus BX 50 phase contrast microscope (Olympus, Japan).

For evaluation of fertilization and embryo development, sterlet females were injected with carp pituitary powder dissolved in 0.9% (w/v) NaCl solution 36 h (0.5 mg kg^{-1}) and 24 h (4.5 mg kg^{-1}) before stripping [2]. Eggs from three females pooled in equal parts (2 g, approx 140 eggs) were inseminated in a Petri dish with sperm immediately after thawing. Based on the spermatozoa concentration of the sample, the volume of sperm was adjusted to obtain a 10^5 spermatozoa/egg ratio. Gametes were activated with 8 ml hatchery water, and, after 1 min, ova were transferred to an incubator with aerated, dechlorinated, and UV-sterilized tap water at 16 °C. Living (transparent, with visible embryo inside) and dead (opaque, whitish) eggs were counted in each Petri dish during incubation and dead eggs were removed. Live embryos were counted after the second cleavage division at 4 h post-fertilization to calculate fertilization rate, and at the eyed stage at 72 h post-fertilization to calculate development rate. Fertilization and development rates were expressed as the proportion of live embryos at corresponding post-fertilization times of the initial number of eggs incubated according to recommendations for sturgeon fishery practices according to Dettlaff et al. [2].

Table 1
Motility parameters of Wolffian duct and testicular spermatozoa after maturation before and after freeze/thaw.

Sperm sample	Motility (%)	VCL (μm s^{-1})	Motility duration (s)
Wolffian duct sperm			
Fresh	92 ± 8[a]	187 ± 25	125 ± 38[a]
Frozen/thawed	57 ± 11[b]	185 ± 37	117 ± 15[a]
Matured testicular sperm			
Fresh	80 ± 9[a]	187 ± 21	54 ± 13[b]
Frozen/thawed	48 ± 16[b]	195 ± 31	48 ± 8[b]
No matured testicular sperm			
Fresh	0*	0	0
Frozen/thawed	0	0	0
Frozen/thawed after 25 min post-thaw storage	0	0	0

Values with different letters are significantly different ($P < 0.05$, Mann–Whitney *U*-test with Bonferroni correction). No significant differences were found in VCL (ANOVA, $P = 0.37$).

*Samples of no matured testicular sperm, showing no motility, were not included into statistical analysis to avoid artificial decrease of sensitivity of statistical method applied.

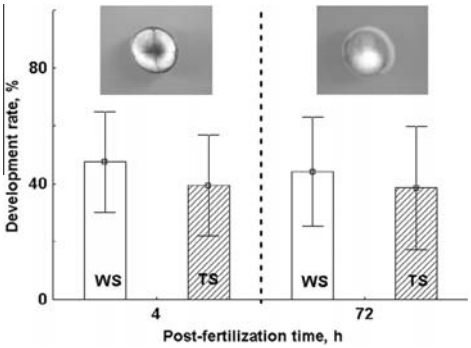

Fig. 1. Development rate of sterlet embryos obtained by frozen–thawed Wolffian duct sperm (WS) or testicular sperm after *in vitro* maturation (TS). The photos of developing embryo at 4 and 72 h post-fertilization are shown above the columns. No significant difference among groups was found ($P = 0.48$, Kruskal–Wallis test).

Data distribution and homogeneity of dispersion were tested by the Shapiro–Wilks test and Levene's test, respectively. Normally distributed data were analyzed by ANOVA followed by Fisher's LSD test. Due to a low number of observations ($n = 6$), a nonparametric Kruskal–Wallis test followed by the Mann–Whitney *U*-test with Bonferroni correction was used for comparison of motility rate, motility duration, and fertilization and development rates. Data are presented as mean ± SD. Statistical significance was accepted at $P < 0.05$. All analyses and plotting were conducted using Statistica v 9.1 (Statsoft Inc, Tulsa, OK, USA).

We confirmed that testicular sturgeon spermatozoa acquire the potential for activation only after pre-incubation in seminal fluid from Wolffian duct sperm. In fresh sperm, the motility rate and VCL of matured testicular sperm in the initial period of motility were not significantly different from that in Wolffian duct sperm (Table 1). These results are in accordance with those of our previous study [3]. However, motility duration of matured testicular spermatozoa was significantly shorter than observed for Wolffian duct spermatozoa. This finding could not be explained from the limited existing data. As sperm motility is an ATP consuming process [6], we propose that future studies include investigation of bioenergetic pathways affected by sturgeon sperm maturation.

Testicular sperm frozen after 1 min pre-incubation in Wolffian duct seminal fluid did not become motile either immediately after thawing or after a post-thaw incubation period. A fertilization test conducted with those sperm samples resulted in no normally developing embryos.

Matured sterlet testicular spermatozoa showed freeze–thaw survival similar to Wolffian duct sperm. Their motility rate and VCL were not significantly different while motility duration was significantly lower (Table 1). However, the shorter motile period did not appear to be a limiting factor for post-thaw fertilizing ability, as development rates of embryos obtained with testicular and Wolffian duct sperm were not significantly different ($P = 0.48$, Kruskal–Wallis test, Fig. 1). The absence of significant differences between embryo development rates at 4 and 72 h post-fertilization together with previously published results of similarity of hatching rate and development rate at 72 h post-fertilization [3] indicate high fertilizing ability. Thus, we observed that with *in vitro* maturation, sterlet testicular sperm acquires fertilizing ability, which, after the freeze/thaw process, is not different from that of Wolffian duct sperm. These results constitute a solid base for development of methods allowing the use of testicular sperm in cases such as accidental death of valuable broodstock being at spermiating stage or failure to obtain Wolffian duct sperm required for artificial sturgeon propagation in manner similar to practice with other fish species [7].

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PROTEOLYTIC AND ANTI-PROTEOLYTIC ACTIVITY DURING STERLET SPERMATOZOON MATURATION

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ABSTRACT

While the role of proteolysis regulators in mammalian sperm maturation is recognized, there are no available data for their role in fish spermatozoon maturation. Immotile sterlet testicular spermatozoa (TS) are capable of activation after pre-incubation in seminal fluid from Wolffian duct sperm (WS). Addition of trypsin inhibitor to the pre-incubation medium disrupts spermatozoon maturation. The present study evaluated the involvement of seminal fluid (SF) proteases and anti-proteolytic activity in the sterlet spermatozoon maturation process. Casein and gelatin zymography and quantification of amidase and anti-proteolytic activity were conducted in sturgeon seminal fluid from Wolffian duct sperm (SFWS) and seminal fluid from testicular sperm (SFTS), along with spermatozoon extracts from WS, TS, and TS after in vitro maturation (TSM). We did not find significant species differences in proteolytic profiles of SFWS or between SFWS and SFTS protease profiles. Zymography revealed differences in spermatozoon extracts: WS extracts were characterized by the presence of a broad proteolytic band ranging from 48 to 41 kDa, while TS extracts did not show such activity until after in vitro maturation. The differences in amidase activity coincided with these results. It may not be the levels of proteolytic and anti-proteolytic activity per se, but the alterations in their interactions triggering a cascade of signalling events, that is crucial to the maturation process.

Keywords: *amidase activity, anti-proteolytic activity, casein and gelatin zymography, Siberian sturgeon sperm, spermatozoon maturation, sterlet sperm*

1. Introduction

The process of spermatozoon maturation, the acquisition of capability for motility and fertilization by morphologically complete spermatozoa (Schulz et al., 2010), has been thoroughly studied in many mammalian species (de Lamirande et al., 1997; Marengo, 2008; Sostaric et al., 2008). In mammals, spermiogenesis is completed in two sequential steps: maturation, which occurs during the post-testicular sperm transit through the epididymis, and capacitation, occurring in the female genital tract. During these processes, spermatozoa

undergo complex and well-orchestrated biochemical and structural modifications resulting in changes of motility parameters and, as a consequence, in fertilization ability. The participation of calcium ions, reactive oxygen species, cyclic AMP, protein kinases and phosphatases, and proteolysis regulators is well described for mammalian spermatozoon maturation (de Lamirande et al., 1997; Gatti et al., 2004; O'Flaherty et al., 2005; de Lamirande and O'Flaherty, 2008; Caballero et al., 2011; LaFlamme and Wolfner, 2013; Dacheux and Dacheux, 2014).

The process of spermatozoon maturation has been described for a limited number of teleost fishes, including rainbow trout *Oncorhynchus mykiss*, chum salmon *Oncorhynchus keta*, and Japanese eel *Anguilla japonica*. It has been demonstrated that spermatozoa acquire the potential for motility *via* the environment of the sperm duct, such as its pH and bicarbonate ion concentration (Morisawa and Morisawa, 1988; Ohta et al., 1997). The spermatozoon maturation process has recently been described in sturgeon (Dzyuba et al., 2014a), and high molecular weight substances in, and proteolytic activity of, seminal fluid were proposed as regulatory factors.

Seminal plasma proteolytic enzymes are known to be involved in regulation of spermatozoon motility in diverse groups of animals, including mammals, fish, and nematodes (de Lamirande and Gagnon, 1986; Cosson and Gagnon, 1988; Inaba at al., 1998; Smith and Stanfield, 2012). Variation in distribution of similar types of proteases and their inhibitors in the epididymal fluid of ram, boar, and stallion (Métayer et al., 2002) suggests a role for proteolysis regulators in spermatozoon maturation.

The ability of the proteolytic enzyme trypsin to initiate *in vitro* motility of immotile spermatozoa from the seminal vesicle was observed in tobacco hornworm *Manduca sexta* (Friedlander et al., 2001) and water strider *Aquarius remigis* (Miyata et al., 2012). Based on these findings, Friedlander et al. (2001) suggested that trypsin-induced structural changes in spermatozoa mimicked the changes occurring *in vivo* during normal maturation of *M. sexta* spermatozoa. Miyata et al. (2012) proposed a model in which trypsin-like serine protease cleaves a protease-activated receptor-2-like protein, activating it to transmit the motility activation signal through initiation of a signalling cascade that inactivates an endogenous phosphatase and activates a mitogen-activated protein kinase pathway, resulting in stimulation of flagellar motility.

There are currently no available data on the involvement of proteases and their inhibitors in the fish spermatozoon maturation process, and this process is only recently reported for sturgeon. Based on our previous results concerning the disruption of sterlet testicular spermatozoa *in vitro* maturation by the addition of trypsin inhibitor into pre-incubation medium (Dzyuba et al., 2014a) as well as reported data of trypsin-like activity in sturgeon spermatozoa and low anti-trypsin activity in fluids of Wolffian duct sperm (Ciereszko et al., 1994), we investigated the possible involvement of proteases and their inhibitors in sturgeon spermatozoa maturation.

The comprehensive investigation of differences between proteases and their inhibitors present in seminal fluid from sturgeon testicular sperm and those in sperm collected from Wolffian ducts and specifics of their function will help to clarify the role of proteolysis regulation in the process of sturgeon spermatozoon maturation.

2. Materials and methods

Experiments were conducted according to the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany based on the EU-harmonized Animal Welfare Act of the Czech Republic.

2.1. Fish rearing conditions

Experiments were conducted using sterlet *Acipenser ruthenus,* Siberian sturgeon *Acipenser baerii*, and beluga *Huso huso* sperm. These fishes were reared at the hatchery of South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic and Fischzucht Rhonforelle GmbH & Co. KG, Gersfeld, Germany.

During the natural spawning season, mature sterlet males (3–4 year-old, 0.6–1.0 kg), Siberian sturgeon (5–6 year-old, 3–5 kg), and beluga (15–20 year-old, 40–50 kg) were transferred from aquaculture ponds (water temperature 8–10 °C) into a closed water recirculation system. Over the course of 24 h, water temperature was increased to 15 °C, and fish were held four days without feeding before beginning experimentation.

2.2. Sperm collection and seminal fluid and spermatozoon processing

Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% NaCl solution at 4 mg kg^{-1} body weight. Wolffian duct sperm (WS) was collected by aspiration from the urogenital (Wolffian) ducts using a 4 mm plastic catheter connected to a 20 mL syringe, 24 h after stimulation of spermiation. Immediately after WS collection, fish were euthanized by a blow to the cranium, followed by exsanguination. The digestive tract was removed, and testicular sperm (TS) was collected *via* incision of the efferent ducts (Dzyuba et al., 2014b).

Sperm samples were centrifuged at 300 g at 4 °C for 10 min followed by 15 min at 5,000 g. Supernatants were collected and centrifuged at 10,000 g at 4 °C for 15 min. Supernatants from WS obtained after the third centrifugation were designated SFWS and divided into two aliquots. One aliquot of each SFWS was used for TS *in vitro* maturation described below, and the second was frozen at -80 °C until analysis. Supernatants from TS were subjected to additional steps of centrifugation at 10,000 g and 16,000 g, both for 15 min. Resultant supernatants from TS were designated SFTS and were frozen at -80 °C until use. Pellets left after centrifugation of WS and TS were frozen at -80 °C and later used for obtaining of spermatozoon extracts.

For maturation, sterlet testicular sperm was incubated for 25 min in SFWS from sterlet, Siberian sturgeon, and beluga at a dilution rate of 1:50 v/v testicular sperm and SFWS (Dzyuba et al., 2014a). These samples were designated TS after *in vitro* maturation (TSM) and centrifuged as described above for WS. Pellets left after centrifugation of TSM were frozen at -80 °C and later used for obtaining of spermatozoon extracts.

In addition, SFWS was concentrated using a Vivaspin 500 concentrator with 10,000 MWCO PES membranes (Sartorius Stedim Biotech GmbH 37070 Goettingen, Germany) at 1,000 g at 4 °C. Concentrated SFWS was frozen at -80 °C until use for SDS-PAGE and determination of amidase and anti-proteolytic activity.

Spermatozoon extracts from the sperm pellets of WS, TS, and TSM were obtained through re-suspension in 4 M urea, sonication, and centrifugation according to Richardson et al. (1988). Supernatants were used for further experimentation.

The protein concentration in all samples was determined by spectrophotometry using Bradford reagent (Sigma-Aldrich) following the manufacturer's instructions.

2.3. Spermatozoon motility analysis

Spermatozoon motility was assessed in both Wolffian duct sperm and testicular sperm pre-incubated for 25 min in seminal fluid from WS at a dilution rate of 1:50, TS:SFWS (Dzyuba et al., 2014a). Spermatozoon motility was activated in medium containing 10 mM Tris-HCl, pH 8.0, and 0.25% pluronic F-127 (Sigma-Aldrich) to prevent spermatozoa from adhering to slides, and motility parameters were assessed. Spermatozoon motility was recorded for 1–2 min post-activation using video microscopy combined with stroboscopic illumination (ExposureScope®, Czech Republic). For estimation of percent motile spermatozoa (motility rate) and motility duration, video recordings were analyzed by micro-image analyzer (Olympus MicroImage 4.0.1. for Windows, Japan) on five successive overlapping video frames.

2.4. Zymography of gelatinolytic and caseinolytic activity

Samples of spermatozoon extract, SFWS, and SFTS were subjected to electrophoresis in gelatin- or casein-containing (0.1%) polyacrylamide (10% acrylamide) gels in the presence of SDS under non-reducing conditions (Siegel and Polakoski, 1985). SDS-PAGE electrophoresis was conducted in the Mini-PROTEAN Tetra system (BioRad) at 200 V and 40 mA (for two gels). After electrophoresis, the gels were washed twice at room temperature with 2.5% Triton X-100 for 20 min each washing and were incubated in 50 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl and 0.02% Triton X-100 with or without 5 mM $CaCl_2$ for 24 h at 37 °C. After incubation, the gels were stained with 0.025% Coomassie Brilliant Blue for 48 h. The stained gels were stored in 2% acetic acid. Areas of proteolysis appeared as clear zones against a blue background. Molecular mass was estimated using pre-stained SDS-PAGE standards, broad range (Bio-Rad, US): myosin – 202.4 kDa; β-galactosidase – 114.8 kDa; bovine serum albumin – 73.1 kDa; ovalbumin – 47.9 kDa; carbonic anhydrase – 34.1 kDa; soybean trypsin inhibitor – 27.0 kDa; lysozyme – 17.0 kDa; and aprotinin – 6.0 kDa. The molecular mass of the proteolytic bands were estimated with the GelQuant programme, v. 2.7.0 (DNR Bio-Imaging Systems Ltd., Israel).

2.5. Amidase and anti-proteolytic activity

Seminal fluid and spermatozoon extract amidase activity was measured according to Kennedy et al. (1989) with modifications by Ciereszko et al. (1996, 1998). The amidase activity was assayed in 50 mM Tris-HCl, 0.1% bovine serum albumin, and 20 mM $CaCl_2$, pH 8.2. The reaction mixture contained 1 mM substrate (N_α-benzoyl-D,L-arginine 4-nitroanilide hydrochloride, BAPNA). Seminal fluid or spermatozoon extract was added to the reaction mixture and incubated at 25 °C for 5 min. The reaction was initiated by BAPNA, and the increase in absorbance at 405 nm was measured. One unit (U) of amidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol BAPNA min^{-1} at 25 °C, using the molar extinction coefficient of 9950 M^{-1} cm^{-1} (Thurston et al., 1993).

Seminal fluid and spermatozoon extract anti-proteolytic activity was evaluated by inhibition of cod trypsin (Zymetech, Iceland) amidase activity as previously except for the addition of cod trypsin (36 U L^{-1}) in the reaction mixture. Addition of SF and spermatozoon extract inhibited trypsin activity. Non-inhibited trypsin activity was also measured. Inhibitory activity was expressed as units L^{-1}. One inhibitory unit (U) corresponds to the apparent amount of inhibitor required to block one unit of trypsin activity (Mommens et al., 2008).

2.6. Statistical analysis

A nonparametric Kruskal–Wallis test followed by the Mann–Whitney U-test with Bonferroni correction was used for comparison of motility rate, motility duration, and amidase and anti-proteolytic activity, because of the low number of observations (n = 6–8). Results were presented as mean ± SD. Statistical significance was accepted at $P < 0.05$. All analyses were conducted using Statistica v. 9.1 (Statsoft Inc, Tulsa, OK, USA).

3. Results and discussion

Sterlet testicular spermatozoa show motility only with preliminary incubation for 10–25 min in SFWS or urine (Dzyuba et al., 2014a). Removal of high molecular weight substances from SFWS and addition of soybean trypsin inhibitor has been shown to result in a lack of testicular spermatozoon motility after pre-incubation (Dzyuba et al., 2014a). Hence the presence of high molecular weight substances, including those with proteolytic activity, in the spermatozoon environment is a prerequisite for their maturation.

Pre-incubation of sterlet TS in SF from sterlet, Siberian sturgeon, and beluga WS revealed that the maturation effect of SF from Wolffian duct sperm was not species-specific, as motility rate and motility duration after 25 min pre-incubation did not significantly differ among the seminal fluids examined (Table 1).

In TS before pre-incubation, motility was not detected. No significant difference was detected among species (Kruskal–Wallis test, $P > 0.05$).

Table 1. *Motility of sterlet Wolffian duct spermatozoa (WS) and testicular spermatozoa (TS) after 25 min pre-incubation in seminal fluid from sterlet, Siberian sturgeon, and beluga Wolffian duct sperm (mean ± SD).*

Sperm type	Pre-incubation medium	Motility (%)	Motility duration (s)
WS	no treatment	82 ± 15	141 ± 37
TS	Sterlet SFWS	74 ± 5	146 ± 12
TS	Siberian sturgeon SFWS	70 ± 7	136 ± 26
TS	Beluga SFWS	74 ± 9	151 ± 11

As inhibition of trypsin activity during pre-incubation in SFWS inhibits testicular sperm motility (Dzyuba et al., 2014a), we studied proteolytic and anti-proteolytic activity of SF from sterlet and Siberian sturgeon WS.

In sterlet, caseinolytic activity of SFWS was characterized by the consistent presence of bands of molecular weight 188 kDa (often double) and 80 kDa (Fig. 1a). Several samples of SFWS contained caseinolytic bands of 269 and 63 kDa. The addition of 5 mM $CaCl_2$ to the gel incubation medium did not result in increase of the observed band intensity or in additional proteolytic bands. Gelatin zymography revealed the presence of the same proteases found with casein zymography (269 kDa in some samples, 188 kDa, and 80 kDa) and allowed visualization of an additional protease of molecular weight 145 kDa (Fig. 1b). The presence of $CaCl_2$ in the development buffer resulted in an increase in intensity of all bands and revealed an additional gelatinolytic band of 63 kDa (double in several samples, with molecular weight of 62 and 65 kDa).

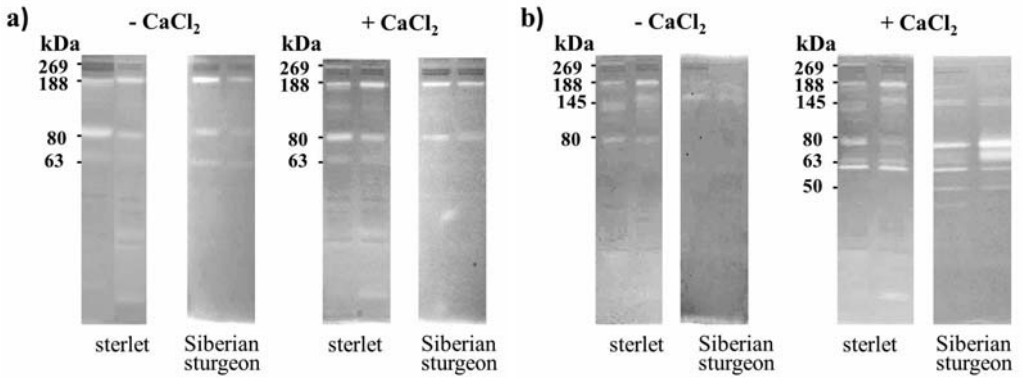

Figure 1. *Caseinolytic (a) and gelatinolytic (b) activity of seminal fluid from Wolffian duct sperm of sterlet and Siberian sturgeon. ± $CaCl_2$ = absence or presence of $CaCl_2$ in gel development media.*

As in sterlet, Siberian sturgeon SF showed several bands of caseinolytic activity (269 in some samples, 188, 80, and 63 kDa) that were not stimulated by addition of calcium ions (Fig. 1a). In contrast to sterlet, the gelatinolytic profile of Siberian sturgeon SF was weak when $CaCl_2$ was absent with only faint bands of 188 and 145 kDa, while, with $CaCl_2$, multiple bands, of varying intensity ranging from 80 to 50 kDa, were revealed (Fig. 1b) in addition to those bands faintly detectable in the absence of calcium ions.

We found no differences in caseinolytic activity in sterlet and Siberian sturgeon seminal fluids, while gelatinolytic activity of proteases in Siberian sturgeon SF in absence of $CaCl_2$ was lower than that in sterlet SF. Nevertheless, the effect of $CaCl_2$ addition to gel incubation medium, intended to reveal presence of metalloproteinases, was similar for both tested species. Zymography revealed no effect of calcium ions on caseinolytic activity, but there was an obvious impact of Ca^{2+} on gelatinolytic activity, which was manifested by intensification of the bands observed in absence of Ca^{2+} and appearance of new bands. A similar effect was found in perch SF by Krol et al. (2011). The more pronounced effect of calcium ions on gelatinolytic activity of Siberian sturgeon SF suggests that the majority of proteases with gelatinolytic activity in SF of this species are metalloproteinases.

Levels of amidase and anti-proteolytic activity showed species differences (Table 2). Seminal fluid of Siberian sturgeon sperm was found to exhibit approximately two and four times the amidase and anti-proteolytic activity of sterlet, respectively.

Table 2. *Amidase and anti-proteolytic activity of seminal fluid from sterlet and Siberian sturgeon Wolffian duct sperm.*

Activity	Sterlet	Siberian sturgeon
Amidase activity, U L^{-1}	0.47 ± 0.27	1.93 ± 1.16
Anti-proteolytic activity, U L^{-1}	8.67 ± 3.13	18.30 ± 5.51*

* indicates significant within-row difference (Mann–Whitney U-test, $P < 0.05$).

To our knowledge, this is among the first studies in which gelatin and casein zymography was used to detect proteolytic activity in sturgeon seminal fluid. Alavi et al. (2014) failed to detect gelatinolytic activity in SF of sterlet. Zymography is commonly used for the investigation of proteolytic activity in SF of teleost species (Kowalski et al., 2003a, 2003b; Mommens et al., 2008; Krol et al., 2011). The gap in the data on proteolytic activity in sturgeon

SF may be rooted in the history of trypsin-like activity detection in sturgeon spermatozoa (Ciereszko et al., 1994, 1996). This activity, resembling that of acrosin of mammalian sperm, was ascribed to sturgeon spermatozoa, primarily based on the presence of an acrosome, an organelle that is not present in teleost fish spermatozoa (Jamieson, 2009). This led to the assumption that sturgeon spermatozoa express proteolytic activity, while their seminal fluid is mainly characterized by anti-proteolytic activity that would protect spermatozoa against uncontrolled proteolysis.

Current literature lacks data on total amidase activity of sturgeon SF, possibly supporting this speculation. The level of amidase activity determined in the present study in sterlet and Siberian sturgeon SF was comparable to that reported in seminal plasma of European perch *Perca fluviatilis* L. (Krol et al., 2011), and was significantly lower than values observed in burbot *Lota lota* seminal plasma (Lahnsteiner et al., 1997). The striking difference in the reported level of proteolytic activity in sterlet and perch SF compared to burbot SF could reflect species differences or be a consequence of differing assessment methods.

The levels of anti-proteolytic activity in SF of the studied species observed in the present study are consistent with findings by Piros et al. (2002) of a significantly higher level of this activity in Siberian sturgeon SF compared to sterlet.

Despite the observed species differences in gelatinolytic profiles in absence of $CaCl_2$ and anti-proteolytic activity of sterlet and Siberian sturgeon SF, the motility rate after TS pre-incubation was similar.

Results of pre-incubation in SF on TS maturation varied, regardless of SF species. In some instances, TS needed more prolonged incubation to acquire the potential for motility. Seminal fluid from WS that triggered at least 70% TS motility after 10 min of pre-incubation was designated SF with strong maturing activity, and SFWS in which only 15–20% of TS became motile after 10 min of pre-incubation was referred to as SF with weak maturing activity. Several samples exhibiting strong and weak maturing activity were selected from Siberian sturgeon SFWS for comparison of their proteolytic and anti-proteolytic activity.

There was no significant difference in caseinolytic profiles (± $CaCl_2$) between the SF with strong and weak activity (Fig. 2), and high variability among samples was observed. The only difference was slightly higher intensity of the detected bands in samples with weak maturing activity. Samples exhibiting no caseinolytic activity were found in both groups. Comparison of gelatinolytic activity in the two groups in absence of $CaCl_2$ (Fig. 3a) did not reveal obvious differences, with the exception of a single sample showing weak maturing activity that expressed no caseinolytic activity (Fig. 2). In that sample, two faint bands (61 and 56 kDa) of gelatinolytic activity were observed (Fig. 3a). Addition of $CaCl_2$ to the gel incubation medium resulted in slight intensification of bands present in the absence of calcium ions and in the appearance of a set of bands, differing in intensity, ranging from 50 to 80 kDa, even in samples that did not previously display proteolytic activity. Nonetheless, no significant differences were observed between SF exhibiting strong and weak maturation effects (Fig. 3b).

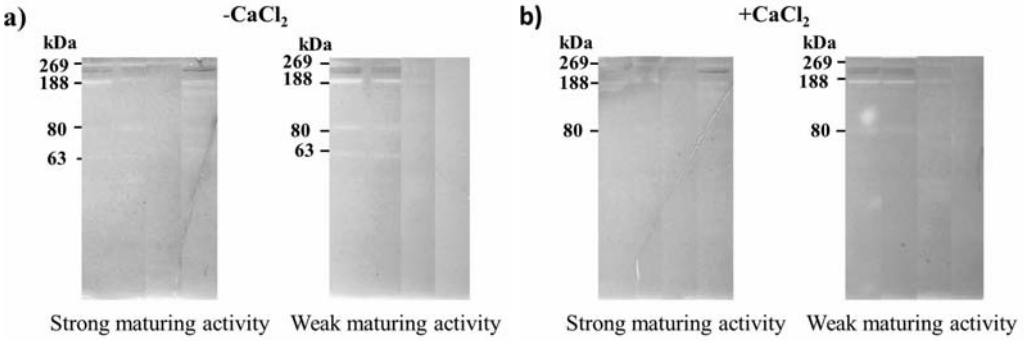

Figure 2. *Caseinolytic activity of seminal fluid from Wolffian duct sperm of Siberian sturgeon possessing strong and weak maturing activity in absence (a) or presence (b) of $CaCl_2$ in gel development media.*

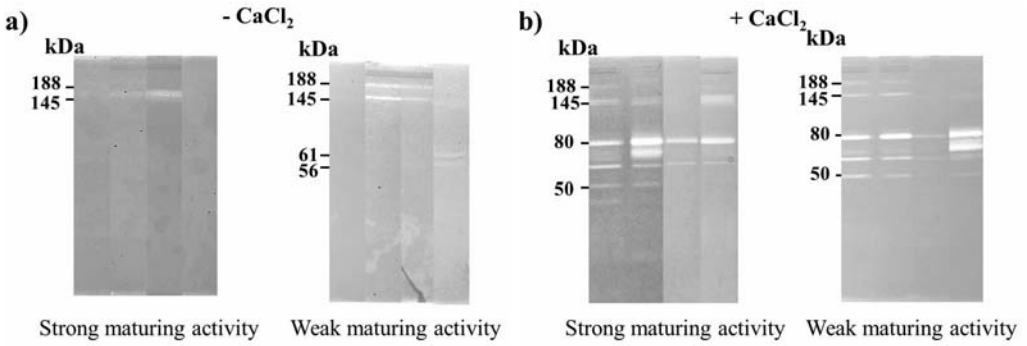

Figure 3. *Gelatinolytic activity of seminal fluid from Wolffian duct sperm of Siberian sturgeon possessing strong and weak maturing activity in absence (a) or presence (b) of $CaCl_2$ in gel development media.*

No significant differences in the level of amidase and anti-proteolytic activity were found between samples showing strong and weak maturing activity (Table 3). No significant within-row difference was detected (Mann–Whitney U-test, $P > 0.05$).

Table 3. *Amidase and anti-proteolytic activity of seminal fluid from Siberian sturgeon Wolffian duct sperm showing differing abilities to induce maturation of sterlet testicular spermatozoa.*

Activity	Strong maturing activity	Weak maturing activity
Amidase activity U L^{-1}	1.26 ± 0.56	2.60 ± 1.28
Anti-proteolytic activity U L^{-1}	19.99 ± 6.21	16.61 ± 4.96

Since no relationship between proteolytic and anti-proteolytic activity of studied SFWS (independent of species and maturation effect) and effectiveness of *in vitro* maturation of testicular spermatozoa was found, differences in seminal fluids from TS and WS that could potentially influence the maturation process of spermatozoon entering kidneys and Wolffian ducts were investigated. Casein and gelatin zymography and evaluation of amidase and anti-proteolytic activity of SFTS were conducted.

Caseinolytic activity of sterlet SFTS was consistently represented by two bands of molecular weight 188 kDa (often double with a second of 167 kDa) and 80 kDa (Fig. 4a). These bands were also characteristic of SFWS (Fig. 1). The caseinolytic band of 269 kDa observed in several samples of seminal fluid from WS was not present in SFTS. In addition to 188 and 80 kDa bands, SFTS also expressed faint bands of 63 kDa (not present in all SFWS samples). As in SFWS, no effect of calcium ions was observed.

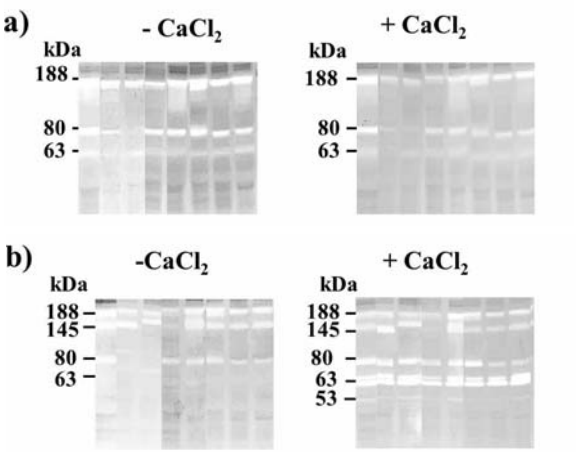

Figure 4. *Caseinolytic (a) and gelatinolytic (b) activity of seminal fluid from sterlet testicular sperm in the absence or presence of $CaCl_2$ in gel development media.*

Gelatinolytic activity of SFTS was represented by 188, 145, 80, and 63 kDa bands when calcium ions were absent (Fig. 4b). In the presence of $CaCl_2$, the intensity of these bands increased, and additional bands with molecular weight 66, 62 (instead of 63 kDa), and 53 kDa were revealed.

Comparison of SFWS and SFTS proteolytic profiles suggested that proteolytic enzymes in the seminal fluid of sturgeon sperm are characterized by substrate specificity, as the presence of a 145 kDa band was observed only with gelatin zymography. There were no differences in protease patterns of TS and WS seminal fluids indicating that the primary molecular species of proteases in SFWS originate in the testis. The only exception was protease of molecular weight 269 kDa, which was present in some samples of SFWS and was not detected in SFTS.

Amidase and anti-proteolytic activity in seminal fluids from sterlet TS was observed to be 19.86 ± 8.87 and 21.41 ± 8.23 U L^{-1}, respectively, which was significantly higher (Mann–Whitney U-test, $P < 0.05$) than in SFWS (Table 2). During spermatozoon transit through kidneys and Wolffian ducts, there was a significant decrease in activity. The loss of potential for motility upon trypsin inhibition during testicular spermatozoa incubation in SFWS (Dzyuba et al., 2014a), suggests that decrease in anti-proteolytic activity in the medium surrounding spermatozoa during transit through kidneys and Wolffian ducts is essential for maturation.

Considering the absence of significant differences in proteolytic and anti-proteolytic activity in samples showing strong and weak maturation effects, we presume that it is not the level of activity *per se*, but changes in their interactions, which trigger alteration of the spermatozoon membrane surface crucial to the maturation process.

To assess this presumption, proteolytic activity of WS, TS, and testicular spermatozoa after *in vitro* maturation (TSM) was assessed. Caseinolytic profiles of WS and TS extracts were dramatically different. Wolffian duct spermatozoon extracts were characterized by the presence of proteolytic bands of 57 and 33 kDa and a broad proteolytic area of molecular

weight ranging from 41 to 48 kDa, while TS extracts revealed only a proteolytic band of 33 kDa (Fig. 5a). An extract of TSM showed the same activity as that of WS extract. Gelatin zymography did not reveal proteolytic activity in all samples analyzed in the absence of $CaCl_2$ in gel incubation medium (Fig. 5b). Addition of $CaCl_2$ to gel incubation medium revealed four bands of gelatinolytic activity with molecular weight ranging from 63 to 77 kDa in TS extracts. These bands were of extremely low intensity in WS extracts and became less intense in TSM. The metalloproteinases identified in TS extracts may be attached to the spermatozoon surface, but their origin is unknown. Along with these bands, a faint broad area of gelatin digestion was detected in the range of 41–46 kDa in WS and TSM. Low gelatinolytic activity in this region of the gel limited evaluation of molecular weight for individual proteases.

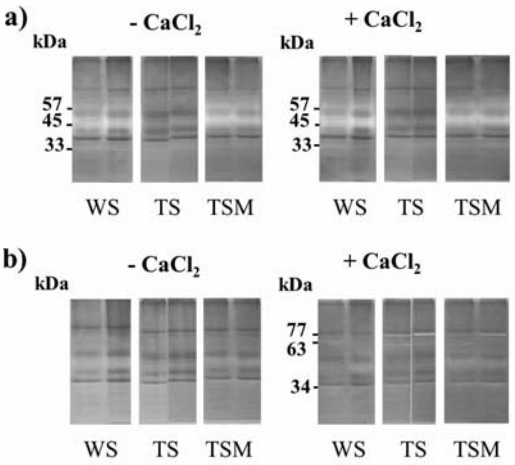

Figure 5. *Caseinolytic (a) and gelatinolytic (b) activity of extracts of sterlet Wolffian duct sperm (WS), testicular sperm (TS), and testicular sperm after in vitro maturation (TSM) in the presence or absence of $CaCl_2$ in gel development media.*

Amidase activity in TS extracts was found to be lower than in WS extracts (P = 0.07), and was significantly increased in TSM (Table 4). The alterations in amidase activity coincided with the characteristics of the proteolytic pattern, especially that with caseinolytic activity. The spermatozoon extracts, regardless to their origin, did not reveal anti-proteolytic activity.

Table 4. *Amidase and anti-proteolytic activity of sterlet Wollfian duct spermatozoa (WS), testicular spermatozoa (TS), and testicular spermatozoa (TSM) after in vitro maturation.*

Activity	WS	TS	TSM
Amidase activity, U g^{-1} protein	20.84 ± 14.74	7.26 ± 3.80	55.87 ± 16.05*
Anti-proteolytic activity, U g^{-1} protein	ND	ND	ND

* indicates significant difference compared to TS (Mann–Whitney U-test, $P < 0.05$); ND – not detected.

Generalizing all obtained data, the following sequence of events can be postulated: A hypothetical protease 'A' located on the surface of the testicular spermatozoon and needed for motility activation is inactive, as the surrounding medium (SFTS) contains a mixture of proteases and their inhibitors which does not allow for activation of the next hypothetical protease 'B' present in this seminal fluid. Inactive protease 'B' is not able to activate surface

protease 'A' by partial proteolysis. At the output of the testis, fluids surrounding spermatozoa are changed, anti-proteolytic activity decreases, as was shown for SFWS, and protease 'B' is activated. Active protease 'B' enables proteolytic processing of protease 'A' resulting in its activation and ability for motility initiation. This proposed sequence of events could explain observed *in vitro* maturation of TS. With soybean trypsin inhibitor added to the pre-incubation medium (SFWS), inhibition of protease 'B' precludes activation of protease 'A', and no testicular sperm maturation is observed.

A similar maturational sequence has been proposed by Miyata et al. (2012) for sperm of *Aquarius remigis* with trypsin activation of a trans-membrane protease-activated G protein-coupled receptor-2-like protein as a main trigger of the signalling pathway for motility activation. These authors also suggested the involvement of calcium ions in regulating motility, as they found that trypsin-activated motility was blocked by pre-treatment of sperm with a chelator of intracellular Ca^{2+} and was partially recovered by subsequent addition of a calcium ionophore and Ca^{2+} (Miyata et al., 2012).

4. Conclusions

This study demonstrated that the sturgeon spermatozoon maturation process is determined by the interaction of the testicular spermatozoon surface and the seminal environment, and possibly includes proteolytic processing of macromolecules located on the spermatozoon surface. Such changes in macromolecule structure can trigger a signalling cascade and, as a result, activation of flagellum motility. The participation of calcium ions and high molecular weight substances in seminal fluid in addition to proteases may be included. Sterlet testicular spermatozoa mature upon incubation in seminal fluid from Wolffian duct sperm of different sturgeon species which suggests that protease initiation of spermatozoon motility may represent a common evolutionarily conserved mechanism, as suggested by Miyata et al. (2012) for insect sperm.

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CHAPTER 4

PROOXIDANT-ANTIOXIDANT STATUS REGULATION IN RPERMATOZOON MATURATION AND MOTILITY ACTIVATION

4.1. Dzyuba, V., Dzyuba, B., Cosson, J., Boryshpolets, S., Yamaner, G., Kholodniy, V., Rodina, M., 2014. The antioxidant system of sterlet seminal fluid in testes and Wolffian ducts. Fish Physiology and Biochemistry 40 (6): 1731–1739.

4.2. Dzyuba, V., Cosson, J., Dzyuba, B., Yamaner, G., Rodina, M., Linhart, O., 2015. The antioxidant system of seminal fluid during *in vitro* storage of sterlet *Acipenser ruthenus* sperm. (manuscript)

4.3. Dzyuba, V., Cosson, J., Dzyuba, B., Rodina, M., 2015. Oxidative stress and motility in tench *Tinca tinca* spermatozoa. Czech Journal of Animal Science 60 (6): 250–255.

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The antioxidant system of sterlet seminal fluid in testes and Wolffian ducts

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Abstract Oxidative stress is a possible source of spermatozoa function deterioration. Seminal fluid (SF) protects spermatozoa against reactive oxygen species (ROS) attack during development in testes and transit through the reproductive tract. Spermatozoa curvilinear velocity and percent of motile cells as well as changes in thiobarbituric acid-reactive substance (TBARS) content, superoxide dismutase, and catalase activity, and uric acid concentration in SF were evaluated in sterlet sperm collected from testes 24 h after hormone induction of spermiation and from Wolffian ducts at 12, 24, 36, and 60 h after hormone injection (HI). While testicular spermatozoa motility was not initiated in activating medium, Wolffian duct sperm showed low motility at 12 h, significant increase at 24 and 36 h, and decrease at 60 h. Testicular SF was characterized by the highest level of TBARS and activity of studied enzymes compared with SF from Wolffian duct sperm at 24 h post-HI. In fluid from Wolffian duct sperm, a significant increase in TBARS content was shown at 36–60 h post-HI. In contrast to testicular SF, in SF from Wolffian duct sperm, this increase was not counterbalanced by changes in the studied variables of antioxidant system. This may be the source of the observed decrease in spermatozoa motility parameters 60 h post-HI. The results may confirm a dual role of ROS in fish sperm physiology. The data with respect to decrease in sturgeon spermatozoa motility parameters at 60 h post-HI should be taken into account in artificial sturgeon propagation.

Keywords Lipid peroxidation · Catalase · Superoxide dismutase · Uric acid · Spermatozoa motility

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Introduction

The process through which spermatozoa acquire the capability of motility and fertilization is called maturation (Schulz et al. 2010). This process is well investigated in many animal species, especially in

humans and domestic mammals (de Lamirande et al. 1997; Marengo 2008; Sostaric et al. 2008), because of its importance for success in assisted reproductive technologies. In mammals, spermatozoa acquire the potential for motility as they leave the testis and pass along the epididymis. Fish spermatozoa maturation has not commonly been considered a strongly limiting factor for success in aquaculture and has been investigated in only a few teleost species (Morisawa and Morisawa 1986, 1988; Miura et al. 1995; Ohta et al. 1997; Miura and Miura 2001). These studies have shown that substances in the sperm duct are required for spermatozoa to acquire the potential for motility.

We have recently established that the sperm maturation process in sturgeon takes place outside the testes and suggested that high molecular weight substances present in seminal fluid (SF) and/or urine are involved (Dzyuba et al. 2014).

Sturgeon possesses an excretory system in which sperm and urinary ducts are not completely separated. We observed significant differences between sterlet testicular sperm and sperm collected from Wolffian ducts, the latter being commonly used in aquaculture. Testicular spermatozoa did not become motile in activating medium (AM). Motility took place after in vitro incubation in urine or SF from Wolffian duct sperm in a temperature and time-dependent manner (Dzyuba et al. 2014). We found a lack of motility in testicular spermatozoa after incubation in SF from Wolffian duct sperm from which high molecular weight substances had been removed, indicating that the presence of high molecular weight substances in SF is a prerequisite for sturgeon spermatozoa maturation.

The antioxidant system of SF and spermatozoa plays an important role in maintaining the semen viability under in vivo conditions and in vitro storage (Lahnsteiner and Mansour 2010). To counteract oxidative damage, SF and spermatozoa possess low molecular weight antioxidants and antioxidant enzymes. In the present study, superoxide dismutase (SOD), catalase (CAT), and uric acid (UrAc) were chosen as representatives of enzymatic and non-enzymatic antioxidant systems. SOD and CAT are the most important elements in the enzymatic protective system, since they are, respectively, the scavengers of superoxide anion radicals and hydrogen peroxide, the latter being partially produced during enzymatic dismutation of superoxide anion radicals. For this reason, it is better to analyze the combined activity of SOD and CAT. Uric acid is probably the main non-enzymatic antioxidant in fish SF, as it is present in high concentrations in SF of a range of teleost species (Ciereszko et al. 1999; Lahnsteiner et al. 2010). There are no data available with respect to the UrAc level in SF of chondrosteans. Available data concerning the intensity of peroxidation processes and antioxidant system stability under in vitro fish sperm storage are somewhat contradictory. In brown trout, thiobarbituric acid-reactive substance (TBARS) content in spermatozoa and SF was essentially increased after 48 h of in vitro storage (Lahnsteiner et al. 2010), while the concentration of all studied non-enzymatic substances and antioxidant enzymes did not change in SF or in spermatozoa during storage. In Siberian and Russian sturgeon, significant increase in TBARS content has also been shown in spermatozoa after 3 and 6 days in vitro storage, respectively (Shaliutina et al. 2013), but an increase in SOD activity preceded the increase in TBARS concentration. Data on intensity of peroxidation processes and the antioxidant system capacity of sturgeon SF are lacking.

The primary goal of the present study was to evaluate changes in TBARS content, SOD and catalase activity, and uric acid concentration of SF of sterlet *Acipenser ruthenus* sperm collected from testes and from the Wolffian ducts at various times after carp pituitary induction of spermiation.

Materials and methods

All experiments were conducted according to the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany (based on the EU-harmonized Animal Welfare Act of the Czech Republic).

Fish rearing conditions

During the natural spawning season, 24 sterlet males (3–4 years old, 0.6–1.0 kg weight) were transferred from aquaculture ponds (water temperature 8–10 °C) into a 0.8 m^3 closed water recirculation system, located at the hatchery of South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic. Water temperature was increased to

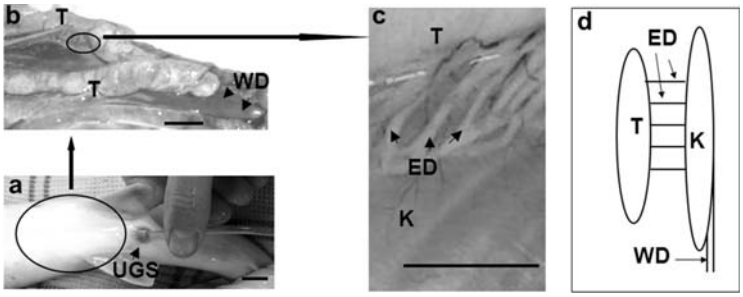

Fig. 1 Structure of sterlet male urogenital tract. *UGS* urogenital sinus, *T* testis, *K* kidney, *WD* Wolffian ducts, *ED* efferent ducts. **a** Urogenital sinus (*black arrowhead*) with sperm–urinary duct catheter inserted during urine or Wolffian duct sperm collection; *oval area* dissected to allow access to the testis–kidney junction to collect testicular sperm. **b** Overall view of body cavity after removal of skin and digestive tract; Wolffian ducts are shown by *arrowheads*; *oval* the area of the testis–kidney junction. **c** View of the testis–kidney junction. **d** Simplified schematic view of sturgeon urogenital system according to Hoar (1969). Testicular sperm was collected from incision of efferent ducts

15 °C within 24 h, and fish were held 4 days without feeding before beginning the experiments.

Sperm collection and seminal fluid processing

Sperm was collected from the Wolffian ducts (this sperm is used for artificial sturgeon propagation) and from testes. Wolffian duct sperm was collected at the urogenital sinus by aspiration using a 4-mm plastic catheter connected to a 10-ml syringe. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9 % (w/v) NaCl solution at 4 mg kg^{-1} of body weight. Fish were randomly separated into four groups. In these groups, Wolffian duct sperm was collected only once at different time after stimulation of spermiation: 12, 24, 36, or 60 h. Fish from which sperm was collected 24 h post-hormone injection (HI) were euthanized by a blow to the head and exsanguination to collect testicular sperm by incision of efferent ducts (Fig. 1).

The sperm samples were centrifuged at $300\times g$ at 4 °C for 10 min, and the supernatants were collected and centrifuged a second time for 15 min at $5{,}000\times g$. Supernatants obtained after the second centrifugation were referred to as SF and were frozen at −80 °C until use.

Motility analysis

For motility activation, 10 mM Tris–HCl buffer, pH 8.0, containing 0.25 % pluronic acid was used as AM. For triggering motility, Wolffian duct sperm was diluted in AM at 1:100, and testicular sperm was added to 50 µl AM with a tip of a dissecting needle (dilution approximately 1:1,000). Sperm suspensions were carefully mixed for 2 s. Motility was recorded for 1–2 min post-activation using video microscopy combined with stroboscopic illumination (Exposure-Scope®, Czech Republic). Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL) and percent of motile cells (motility rate) by microimage analyzer (Olympus Micro Image 4.0.1. for Windows, Japan).

Spermatozoa concentration determination

Spermatozoa concentration was determined using a Burker cell hemocytometer (Meopta, Czech Republic) and Olympus BX 50 phase contrast microscope (200× magnification; Olympus, Japan).

Spermatozoa membrane integrity assessment

Spermatozoa membrane integrity was determined using the Live/Dead Sperm Viability kit (L-7011; Molecular Probes) by the method of Flajshans et al. (2004). Briefly, the sperm samples from all groups were diluted with 150 mM NaCl into a suspension containing 5×10^6 cells/ml. Subsequently, SYBR 14 dye and propidium iodide were added to 1 ml of sperm suspension to a final concentration of 100 nM and 12 µM, respectively. After 10-min incubation in dark,

results were observed by fluorescence microscopy with an Olympus BX60 equipped with Olympus MWB filter cube (wide band, blue excitation 450–480 nm, emission above 515 nm), and images were recorded using 3CCD Sony DXC-9100P color camera and analyzed by microimage analyzer (Olympus Micro Image 4.0.1. for Windows, Japan). Sixty to 100 images per sperm sample were analyzed. Percent of live (green fluorescence) spermatozoa was used as a variable of spermatozoa membrane integrity.

Evaluation of thiobarbituric acid-reactive substance content in seminal fluid

The TBARS content was measured spectrophotometrically according to Asakawa and Matsushita (1980). Briefly, to 0.08–0.25 ml SF, 0.025 ml butylated hydroxytoluene solution (22 mg in 10 ml ethanol), 0.025 ml ferric chloride solution (27 mg of $FeCl_3$·$6H_2O$ in 10 ml water), 0.375 ml of 0.2 M glycine–hydrochloric acid buffer, pH 3.6, and 0.375 ml TBA reagent (0.5 % TBA and 0.3 % sodium dodecyl sulfate) were added. The tubes were capped and heated for 15 min in a boiling water bath. After cooling, 0.25 ml glacial acetic acid and 0.5 ml chloroform were added. The mixture was vigorously shaken and centrifuged for 10 min at 1,500×g. The absorbance of samples was determined at 535 nm against a blank with deionized water substituted for the biological sample. A molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used for calculation of TBARS content. The concentration of TBARS was expressed as nmol ml^{-1} SF.

Evaluation of enzymatic antioxidant system variables in seminal fluid

Superoxide dismutase (EC 1.15.1.1) activity was measured spectrophotometrically at 420 nm according to the method of Marklund and Marklund (1974). The inhibition of pyrogallol autoxidation by SOD-containing sample was used for the determination of the enzyme activity. The autoxidation of 0.2 mM pyrogallol in air-equilibrated 50 mM Tris–HCl buffer, pH 8.2, containing 1 mM EDTA, was inhibited by the addition of the assayed sample. One unit of the enzyme is generally defined as the amount of enzyme that inhibits the reaction (in this case, pyrogallol autoxidation) by 50 %. The results were expressed in units per ml of SF.

Catalase (EC 1.11.1.6) activity was measured spectrophotometrically at 240 nm. Reaction medium contained 10 mM K^+-phosphate buffer with 0.1 mM EDTA, pH 7.4, and 15 mM H_2O_2 according to the method of Marklund et al. (1981). Catalase activity was calculated from H_2O_2 decomposition rate, using molar extinction coefficient 39.4 M^{-1} cm^{-1}, and expressed in μmol min^{-1} ml^{-1} of SF.

Evaluation of uric acid content in seminal fluid

The uric acid content was used as an index of the non-enzymatic antioxidant system of SF. UrAc content was determined by the uricase method (Duncan et al. 1982) using absorption spectrophotometry and expressed as μmol per l of SF.

Data presentation and statistical analysis

The values of spermatozoa velocity were checked for distribution characteristics and homogeneity of dispersion using Shapiro–Wilk's and Levene's tests, respectively. As they were normally distributed with similar dispersion values, parametric one-way ANOVAs were applied and Tukey's honest significant difference test (HSD test) was used for contrasting the differences between groups. Because of the small number of observations ($n = 6$), nonparametric statistics using the Kruskal–Wallis test followed by the Mann–Whitney U test with Bonferroni correction were performed for comparison of percent of motile spermatozoa, CAT and SOD activity, uric acid and TBARS content, and membrane integrity.

Results were presented as mean ± standard deviation, and statistical significance was considered at $P < 0.05$. All analyses and plots were conducted using STATISTICA V 9.1 computer program (Statsoft Inc., USA).

Results

Spermatozoa motility parameters

Table 1 represents motility parameters of sterlet testicular spermatozoa and Wolffian duct spermatozoa. Significant differences were observed in

Table 1 Motility parameters of sterlet spermatozoa

Parameters	Testicular spermatozoa 24 h post-HI	Wolffian duct spermatozoa			
		12 h post-HI	24 h post-HI	36 h post-HI	60 h post-HI
Motility (%)	0^{a}	18 ± 8^{b}	88 ± 7^{c}	83 ± 11^{c}	31 ± 18^{b}
VCL (μm s^{-1})	0^{a}	128 ± 19^{b}	167 ± 34^{c}	178 ± 31^{c}	119 ± 35^{b}

The values in a row with different superscripts are significantly different ($P < 0.05$, Mann–Whitney U test for motility and Tukey's HSD test for velocity)

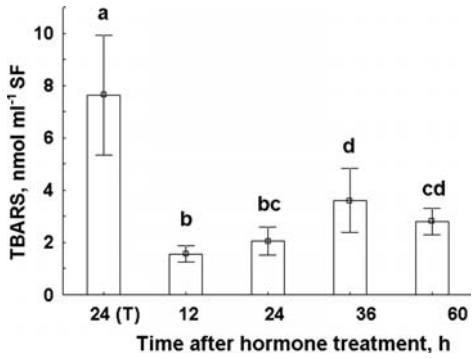

Fig. 2 TBARS content in sterlet SF, collected at various times after hormone stimulation of spermiation. *24(T)* SF from testicular sperm collected at 24 h after HI. Values with *different letters* are significantly different ($P < 0.05$, Mann–Whitney U test, $n = 6$)

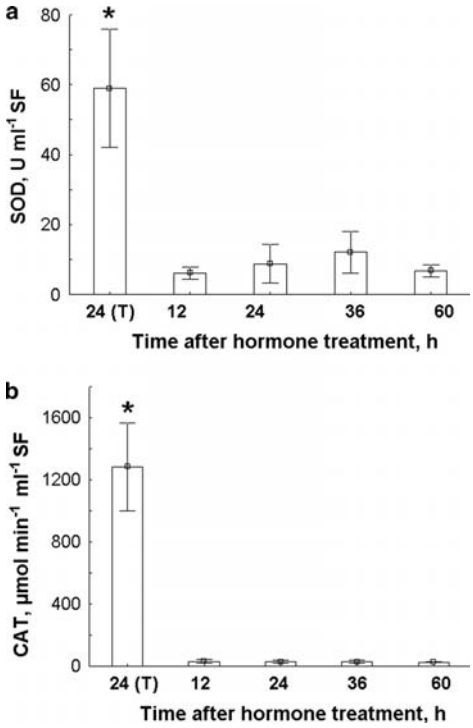

Fig. 3 Activity of SOD (**a**) and catalase (**b**) in sterlet SF collected at various times after hormone stimulation of spermiation. *24(T)* SF from testicular sperm collected 24 h after HI. Values *marked* by *asterisk* are significantly different ($P < 0.05$, Mann–Whitney U test, $n = 6$) from *unmarked*

testicular spermatozoa and Wolffian duct spermatozoa responses to AM treatment. AM did not initiate testicular spermatozoa motility, but did activate Wolffian duct spermatozoa. Wolffian duct sperm showed low motility at 12 h post-HI and a significant increase at 24 and 36 h post-HI. Wolffian duct spermatozoa at 60 h showed a decrease in motility rate. The same trend was shown for spermatozoa VCL (Table 1). Average VCL at 12 and 60 h post-HI was characterized by low values, while high, typical for sterlet (Dzyuba et al. 2012), levels were observed at 24 and 36 h post-HI.

TBA-reactive substance content in seminal fluid

Seminal fluid from testicular sperm was characterized by more than threefold (7.6 vs. 2.1 nmol ml^{-1}) content of these products compared with SF from Wolffian duct sperm collected 24 h post-HI (time at which sperm is commonly collected in aquaculture) (Fig. 2). A lower level of TBARS was found in SF from Wolffian duct sperm compared with SF from testicular sperm at all sperm collections. Content of TBARS in SF from Wolffian duct sperm was the lowest at 12 h post-HI and was significantly increased at 36 h post-HI. Sperm collected at 60 h post-HI did not show a rise in TBARS content.

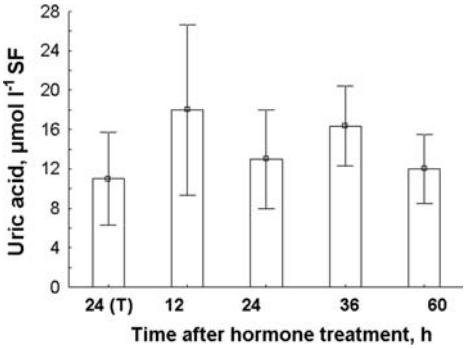

Fig. 4 Uric acid content of sterlet SF, collected at various times after hormone stimulation of spermiation. *24(T)* SF from testicular sperm collected 24 h after HI. No significant differences were found ($P > 0.05$, Mann–Whitney U test, $n = 6$)

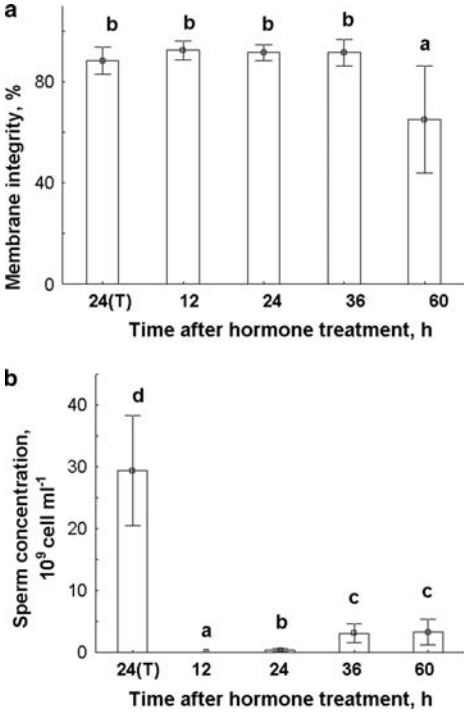

Fig. 5 Membrane integrity (**a**) and spermatozoa concentration (**b**) of sterlet sperm, collected at various times after hormone stimulation of spermiation. *24(T)* SF from testicular sperm collected at 24 h after stimulation. Values with *different letters* are significantly different ($P < 0.05$, Mann–Whitney U test, $n = 6$)

Enzymatic antioxidant activity and uric acid concentration in seminal fluid

The activities of SOD and CAT were 5–10 and 42–50 times, respectively, in SF from testicular sperm that of SF from Wolffian duct sperm at all collection times (Fig. 3a, b). The activity of the analyzed antioxidant enzymes in SF from Wolffian duct sperm was similar at all collection times.

In contrast to TBARS, SOD, and CAT, UrAc concentration in SF from testicular sperm was not different from the values in SF from Wolffian duct sperm (Fig. 4). There were no significant changes in this variable in SF from Wolffian duct sperm at any time post-HI.

Membrane integrity and spermatozoa concentration

Testicular spermatozoa and Wolffian duct spermatozoa membrane integrity at 12, 24, and 36 h post-HI were similar. Membrane integrity at 60 h post-HI was significantly reduced compared with values at 12–36 h post-HI (Fig. 5a).

The highest spermatozoa concentration was found in testicular sperm (Fig. 5b). Spermatozoa concentration was extremely low ($0.016 \pm 0.008 \times 10^9$ cell ml^{-1}) in Wolffian duct sperm collected at 12 h post-HI. Sperm collected at 24 h post-HI was characterized by increased concentration ($0.372 \pm 0.160 \times 10^9$ cell ml^{-1}). Wolffian duct sperm at 36 and 60 h post-HI showed an increase in spermatozoa concentration to $3.1 \pm 1.5 \times 10^9$ and $3.2 \pm 1.8 \times 10^9$ cell ml^{-1}, respectively. No significant difference between spermatozoa concentration at 36 and 60 h post-HI was observed.

Discussion

Results of the present study confirmed our previous observation of differences in motility of spermatozoa collected from testes and from Wolffian ducts (Dzyuba et al. 2014) and determined that motility parameters of Wolffian duct spermatozoa are altered as a function of time after induction of spermiation. Wolffian duct spermatozoa collected 60 h post-HI

were characterized by a significant decrease in velocity and motility (Table 1).

Oxidative stress is among the reasons for spermatozoa function deterioration in various animal species, including mammals and fish (Sikka et al. 1995; Agarwal and Saleh 2002; Aitken et al. 2003; Kasimanickam et al. 2007; Turner and Lysiak 2008; Shiva et al. 2011; Hagedorn et al. 2012), and a function of SF is the protection of spermatozoa from oxidative damage (Potts et al. 2000; Baumber and Ball 2005; Tavilani et al. 2008; Lahnsteiner et al. 2010), as effectiveness of spermatozoa antioxidant system is insufficient to cope with oxidative stress.

Seminal fluid from testicular sperm was characterized by a higher level of TBARS than that from Wolffian duct sperm at all collection times (Fig. 2). As we did not study the changes in TBARS content in SF from testicular sperm at different time post-HI, correct comparison can be done only for two groups of results: (1) SF from testicular sperm and Wolffian duct sperm, collected at 24 h post-HI, and (2) SF from Wolffian duct sperm, collected at different time post-HI. Content of TBARS in SF from Wolffian duct sperm changed with the time post-HI, showing a significant increase at 36 h. Intensification of lipid peroxidation at 36 h post-HI was not accompanied by deterioration of spermatozoa motility parameters. At 60 h post-HI, the level of TBARS was unchanged, with a significant decrease in spermatozoa VCL and motility rate (Table 1).

We observed that an increased amount of TBARS in SF from testicular sperm was followed by a rise in SOD and CAT activity (Fig. 3). The high level of lipid peroxidation products in SF from testicular sperm, which is immature in sturgeon, may be an indication of excessive production of reactive oxygen species (ROS). In mammals, enhancement of superoxide production by the addition of exogenous NADPH has been shown to correlate with epididymal development and peaks in immature sperm (Fisher and Aitken 1997). The involvement of ROS in mammalian sperm maturation was reviewed by Ford (2004) and Aitken et al. (2012). As fish possess cystic type of spermatogenesis, during spermiation, spermatozoa are released in the lumen of testis as a result of the breakdown of the Sertoli cell layer (Vizziano et al. 2008). It could be supposed that exactly this breakdown leads to activation of lipid peroxidation and at the same time to increase in activity of enzymatic antioxidant system. It looks like that the conditions of increased oxidative processes physiologically could be involved in sturgeon sperm maturation in a way similar to one occurring during mammalian sperm epididymal transit (Ford 2004; Aitken et al. 2012). So, ROS seem to be a factor involved in the spermatozoa maturation process. At the same time, spermatozoa are extremely sensitive to the damaging effects of these highly reactive species. In order to prevent the development of oxidative stress, spermatozoa are equipped with non-enzymatic and enzymatic antioxidants (Rhemrev et al. 2000; Chabory et al. 2010). Because of the limited cytoplasmic volume and, therefore, the low amount of endogenous antioxidant, sperm cells rely chiefly on the antioxidant capacity of SF.

Seminal fluid from testicular sperm was characterized by an extremely high level of SOD and CAT activity compared with SF from Wolffian duct sperm (Fig. 3). Uric acid of sterlet SF exhibited stable concentration in all investigated groups (Fig. 4). It is quite understandable that the testicular SF is more concentrated in the different components as it is not diluted yet by the mixing with some urine (the later explaining the homogenous uric acid concentration between samples). Uric acid concentration determined in the present study for sterlet SF was significantly lower than that has been reported for other fish species (Ciereszko et al. 1999; Lahnsteiner et al. 2010). The high activity of the enzymatic antioxidant system allowed testicular spermatozoa to cope with the deleterious effects of excessive ROS production and to retain the ability to become motile after passing through the kidneys and Wolffian ducts.

The increase observed in TBARS content with time following spermiation was not accompanied by a corresponding increase in activity of the studied antioxidant enzymes. We suggest that extended time in the Wolffian ducts resulted in spermatozoa oxidative stress and, finally, in decrease in motility parameters. The assessment of spermatozoa membrane integrity may be an additional confirmation of this. Membrane integrity was stable to 36 h (Fig. 5a), a time characterized by a significant increase in TBARS content. As this increase was not compensated for by a rise in antioxidant enzyme activity, the spermatozoa membrane lost its integrity, supposedly due to lipid peroxidation. Combined results with respect to sperm motility parameters, spermatozoa concentration, and membrane integrity (Table 1;

Fig. 5a, b) lead to the speculation that low-motility parameters observed at different time post-HI may have different sources. In testis and a short time post-HI, low motility may be due to immature spermatozoa. The cell membrane is intact, and a certain amount of time in the Wolffian duct is probably required for completion of maturation. Shortly after HI, SF and/or urine is actively produced, keeping spermatozoa concentration low. With time, spermatozoa became damaged due to oxidative stress, and this process is accompanied by decrease in SF production. As a result, at completion of the spermiation period, spermatozoa at high concentrations with compromised membrane integrity are present in the Wolffian duct.

The present study shows that sturgeon spermatozoa maturation and time in Wolffian ducts are accompanied by significant alterations in motility parameters and in SF pro-oxidant–antioxidant balance. High content of TBARS and activity of antioxidant enzymes in SF from testicular sperm may indicate a regulatory role of ROS in sturgeon sperm maturation process. Extended time in the Wolffian duct was associated with oxidative damage resulting from inadequate enzymatic antioxidant system efficacy in scavenging ROS. The obtained results may confirm a dual role of ROS in fish sperm physiology. The data concerning decrease in sturgeon sperm motility parameters 60 h after induction of spermiation should be taken into account in artificial sturgeon propagation.

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THE ANTIOXIDANT SYSTEM OF SEMINAL FLUID DURING *IN VITRO* STORAGE OF STERLET *ACIPENSER RUTHENUS* SPERM

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ABSTRACT

The role of the seminal fluid antioxidant system in protection against damage to spermatozoon during in vitro storage, often used in artificial fish reproduction, is unclear. The aim of this study was to investigate the effect of in vitro storage of sterlet Acipenser ruthenus sperm on spermatozoon motility rate and curvilinear velocity along with seminal fluid antioxidant (superoxide dismutase, catalase and uric acid) and thiobarbituric acid reactive substance levels. Sperm was collected 36 h after hormone injection and stored at 4 °C for 36 h. Spermatozoon motility parameters after storage were significantly decreased, while the level of thiobarbituric acid reactive substances, activity of assessed enzymes, and uric acid concentration did not change. Our findings suggest that the antioxidant system of sterlet sperm is effective in preventing oxidative stress during short-term storage. The observed decrease in sperm motility parameters may reflect alterations in motility signalling pathways, for example, through increase in internal Ca^{2+} concentration, or in spermatozoon plasma membrane structure via phospholipid lysis.

Keywords: *antioxidant system, in vitro storage, motility, seminal fluid, sterlet sperm*

Introduction

In vitro storage of sperm is an essential tool in gamete manipulation in artificial fish reproduction. The role of the sperm antioxidant system (AOS), as a network of enzyme and non-enzyme processes of cell protection against damage, during *in vitro* storage is not fully understood. Alterations in sperm physiology, DNA integrity, and oxidative stress indices of Russian and Siberian sturgeon sperm during short-term *in vitro* storage were studied by Shaliutina et al. (2013). They reported that the percentage of motile spermatozoa declined only after three days storage in both species, while a significant decrease in spermatozoon velocity was observed after two days storage. Shaliutina et al. (2013) found the level of thiobarbituric acid reactive substances (TBARS) to increase significantly after six days storage of Russian sturgeon *Acipenser gueldenstaedtii* sperm and after three days in sperm of Siberian sturgeon *Acipenser baerii*, with the increase in products of protein oxidative damage

preceding TBARS increase. According to these authors, the increase in superoxide dismutase (SOD) activity observed after two days of *in vitro* storage was insufficient to prevent cellular damage associated with oxidative stress. In contrast, no alterations in the level of non-enzyme antioxidants and activity of antioxidant enzymes were found in spermatozoa and seminal fluid of brown trout after 48 h storage (Lahnsteiner et al., 2010b). Such variations in sperm AOS response to storage, and sparse data on the role of sturgeon seminal fluid AOS in antioxidant defence, encourage further study. This lack of data is of key importance, as spermatozoa are characterized by low cytoplasmic volume and, therefore, low levels of endogenous antioxidants. Consequently, spermatozoa rely chiefly on the antioxidant capacity of seminal fluid for protection from oxidative damage (Lahnsteiner et al., 2010b; Potts et al., 2000; Baumber and Ball, 2005; Ciereszko, 2008; Tavilani et al., 2008). Our previous study on the effects of *in vivo* storage on sterlet sperm AOS found that extended time (up to 60 h post-hormone-injection) of sperm storage in Wolffian ducts resulted in increased TBARS content, which was not accompanied by a corresponding increase in activity of SOD and catalase (CAT) or in uric acid (UrAc) concentration (Dzyuba et al., 2014). Thus, oxidative stress was suggested to develop, leading to the decrease in spermatozoon motility parameters and loss of membrane integrity. The goal of the present study was to investigate the evolution of spermatozoa curvilinear velocity and motility rate, seminal fluid SOD and CAT activity, and UrAc concentration as AOS markers during short-term *in vitro* storage of sterlet *Acipenser ruthenus* sperm.

Materials and methods

Ethics

All experiments were conducted according to the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany, based on the EU-harmonized Animal Welfare Act of the Czech Republic.

Fish rearing conditions and sperm sampling

Five mature sterlet *Acipenser ruthenus* males (3–5 years old, weight 0.7–1.2 kg) were obtained during the natural spawning season. Fish were kept outdoors at water temperature 8–10 °C in plastic tanks located at the hatchery of the Research Institute of Fish Culture and Hydrobiology. Before hormone injection, fish were transferred to a closed recirculation system, and water temperature was increased to 15 °C within 24 h. Fish did not receive food for four days before beginning experimentation.

For initiation of spermiation, fish were injected intramuscularly with carp pituitary extract (4 mg kg^{-1}). Thirty-six h post hormone injection, sperm was collected from urogenital ducts by aspiration using a 4 mm plastic catheter connected to a 20 mL syringe. Sperm samples were divided into two groups, one of which was analysed immediately (Group 36-0) and the other stored under aerobic conditions at 4 °C for 36 h until analysis (Group 36-36). A portion of the samples was centrifuged at 10 000 x g for 10 min at 4 °C to obtain seminal fluid (SF).

Spermatozoon motility analysis

Sperm was diluted 1:50 with water from tanks in which fish were kept, and spermatozoon motility was immediately recorded until cessation, using video microscopy combined with stroboscopic illumination (ExposureScope®, Czech Republic). Video records were analyzed to estimate spermatozoon curvilinear velocity (VCL) and percent of motile cells (motility rate) by microimage analyzer (Olympus Micro Image 4.0.1. for Windows, Japan).

Evaluation of thiobarbituric acid-reactive substance content in seminal fluid

The content of TBARS in seminal fluid was measured spectrophotometrically according to Asakawa and Matsushita (1980). The reaction mixture contained 0.2 mL SF; 0.025 mL butylated hydroxytoluene solution (22 mg in 10 mL ethanol); 0.025 mL ferric chloride solution (16.2 mg of $FeCl_3$ in 10 mL water); 0.375 mL of 0.2 M glycine–hydrochloric acid buffer, pH 3.6; and 0.375 mL TBA reagent (0.5% TBA and 0.3% sodium dodecyl sulphate). Tubes were capped and heated in a boiling water bath for 15 min. After cooling, 0.25 mL glacial acetic acid and 0.5 mL chloroform were added. The mixture was shaken vigorously and centrifuged for 10 min at 1500 x g. The absorbance of samples was measured at 535 nm against a blank with deionized water replacing the SF. A molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used for calculation of TBARS content, expressed as nmol mL^{-1} SF.

Evaluation of superoxide dismutase activity in seminal fluid

Superoxide dismutase (EC 1.15.1.1) activity was measured spectrophotometrically at 420 nm according to Marklund and Marklund (1974). The inhibition of pyrogallol autoxidation by SOD in the sample was used to measure enzyme activity. The autoxidation of 0.2 mM pyrogallol in air-equilibrated 50 mM Tris-HCl buffer, pH 8.2, containing 1 mM EDTA, was inhibited by the addition of the assayed seminal fluid. One unit (U) of SOD was defined as the amount of the enzyme that inhibited the reaction by 50%. Results were expressed as U mL^{-1} of SF.

Evaluation of catalase activity in seminal fluid

Catalase (EC 1.11.1.6) activity was measured spectrophotometrically at 240 nm. Reaction medium contained 10 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, and 15 mM H_2O_2 according to Marklund et al. (1981). Catalase activity was calculated from H_2O_2 decomposition rate with the use of molar extinction coefficient 39.4 M^{-1} cm^{-1} and expressed as μmol min^{-1} mL^{-1} of SF.

Evaluation of uric acid content of seminal fluid

The UrAc content was evaluated as an index of the non-enzyme antioxidant system of seminal fluid. The UrAc content was determined by the uricase method (Duncan et al. 1982) using absorption spectrophotometry and expressed as μmol L^{-1} of SF.

Statistical analysis

A nonparametric Mann–Whitney U-test was used for comparison of studied parameters, because of the low number of observations (n = 5). Results were presented as mean ± SE. Statistical significance was accepted at $p < 0.05$. All analyses were conducted using Statistica v. 9.1 (Statsoft Inc, Tulsa, OK, USA).

Results and discussion

Immediately after collection, spermatozoa were characterized by the high motility and VCL values typical of sterlet (Dzyuba et al., 2012, 2014). After *in vitro* storage for 36 h, both parameters were significantly decreased (Fig. 1). The decrease in spermatozoon motility parameters during *in vitro* storage has been detected for diverse animal species including mammals, birds, and fishes (DeGraaf and Berlinsky, 2004; Douard et al., 2005; Kadirvel et al., 2009; Zaniboni and Cerolini, 2009; Guthrie et al., 2011; Aramli et al., 2013; Shaliutina et al., 2013; Aramli, 2014).

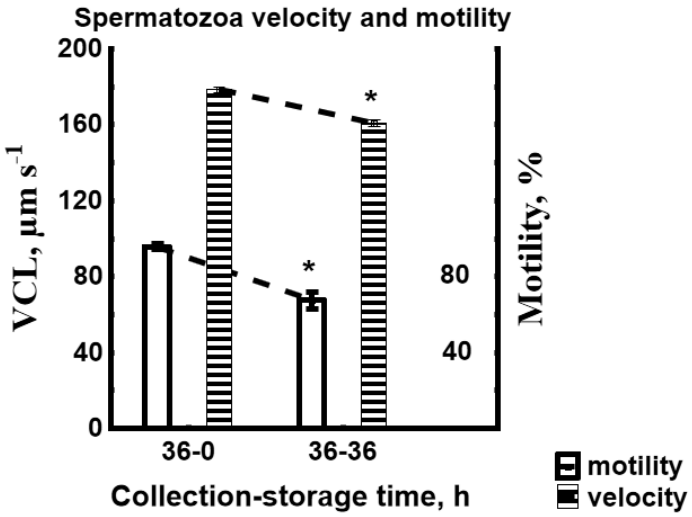

Figure 1. *Sterlet spermatozoon motility parameters before and after sperm in vitro storage. 36-0 – sperm collected 36 h after hormone injection and analyzed before storage; 36-36 – sperm collected 36 h after hormone injection and analyzed after 36 h storage; $p < 0.05$ vs. group 36-0; $n = 5$.*

Oxidative stress development during *in vitro* storage is considered to be for a major source of spermatozoon motility deterioration (Zaniboni and Cerolini, 2009; Aramli et al., 2013; Shaliutina et al., 2013; Aramli, 2014). Accumulation of lipid, protein, and DNA oxidative damage products has been reported to occur in sturgeon concurrently with reduction in motility parameters (Aramli et al., 2013; Shaliutina et al., 2013; Aramli, 2014). Notwithstanding the observed increase in SOD activity in these studies, antioxidant enzyme activity in spermatozoa has been suggested to be insufficient to prevent cellular damage (Shaliutina et al., 2013). Considering these data and the fact that the main role of seminal fluid is the maintenance of an optimal environment for storage of spermatozoa (Ciereszko, 2008), we assessed the possibility of oxidative stress development in SF of stored sterlet sperm and its impact on spermatozoon motility parameters.

The content of TBARS in seminal fluid of sterlet sperm before and after 36 h of *in vitro* storage did not show significant changes (Fig. 2). Short-term storage at 4 °C was also not associated with changes in CAT and SOD activity (Fig. 3) or UrAc concentration (Fig. 4) in seminal fluid of *A. ruthenus.* No change in SOD and CAT activity or UrAc concentration was reported in brown trout seminal fluid after 48 h of sperm storage at 4 °C (Lahnsteiner et al., 2010b).

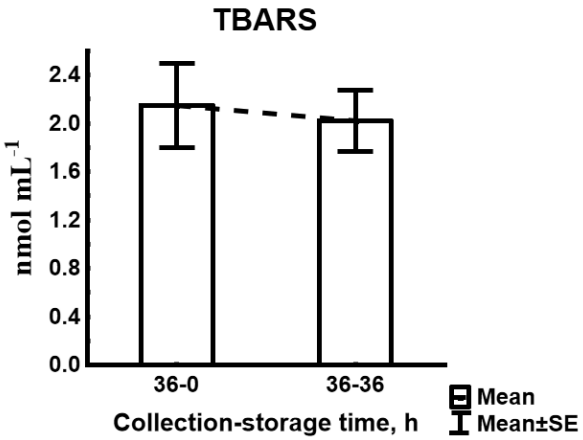

Figure 2. *Concentration of TBA-reactive substances in seminal fluid of sterlet sperm before and after in vitro storage. 36-0 – sperm collected 36 h after hormone injection and analyzed before storage; 36-36 – sperm collected 36 h after hormone injection and analyzed after 36 h storage; n = 5.*

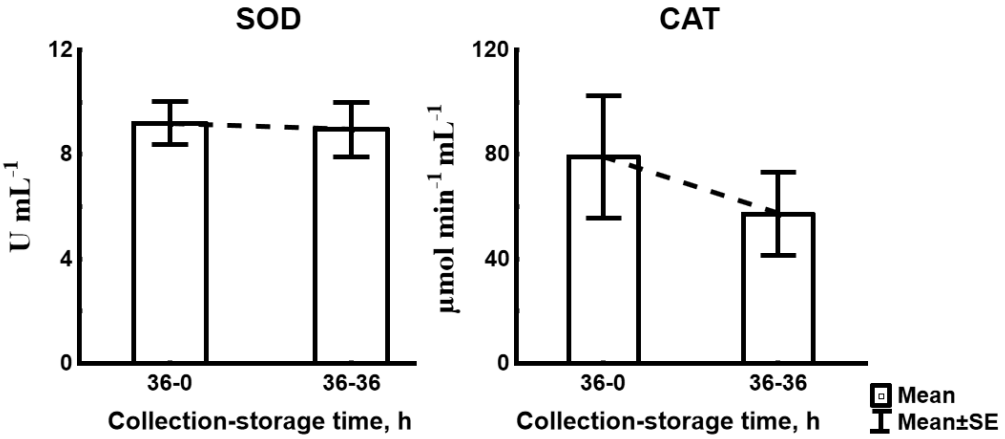

Figure 3. *Antioxidant enzymes activity in seminal fluid of sterlet sperm before and after in vitro storage. 36-0 – sperm collected 36 h after hormone injection and analyzed before storage; 36-36 – sperm collected 36 h after hormone injection and analyzed after 36 h storage; SOD – superoxide dismutase activity; CAT – catalase activity; n = 5.*

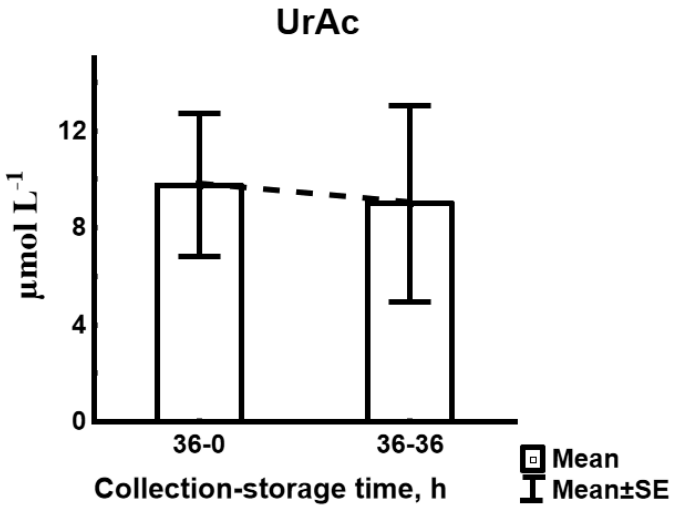

Figure 4. *Uric acid concentration in seminal fluid of sterlet sperm before and after in vitro storage. 36-0 – sperm collected 36 h after hormone injection and analyzed before storage; 36-36 – sperm collected 36 h after hormone injection and analyzed after 36 h storage; n = 5.*

As the short-term *in vitro* storage of sterlet sperm at 4 °C does not lead to significant alterations in levels of lipid peroxidation products and the studied enzyme and non-enzyme components of AOS, we hypothesize that AOS of sterlet sperm is effective in preventing the development of oxidative stress over the short-term storage. Thus, the observed significant decrease in spermatozoon motility parameters after 36 h of *in vitro* storage could be attributed to sources other than oxidative stress.

A possible source of motility decline could be storage-induced disturbance of spermatozoon calcium homeostasis, which may be detrimental to sperm activation (Guthrie et al., 2011). Guthrie et al. (2011) observed a decrease in motility activation of striped bass spermatozoon after 24 h storage at 4 °C. The decrease did not arise from oxidative damage, as there was no evidence of reactive oxygen species formation at 1 to 24 h storage. These authors found increase in internal concentration of calcium ions followed by a loss of cell viability. As the level of internal Ca^{2+} is suggested to be critical for initiation of sturgeon spermatozoon activation (Alavi et al., 2011), it is feasible that unregulated increase in internal Ca^{2+} concentration during 36 h of storage could have produced the decline in motility observed in the present study.

In contrast with the generally accepted protective role of seminal fluid in spermatozoon function, seminal fluid has been suggested to be harmful to turkey spermatozoa during storage (Douard et al., 2005). Douard et al. (2005) found that phospholipid content and membrane permeability of spermatozoa were significantly decreased by the presence of seminal plasma after 48 h storage at 4 °C. The contribution of lipid peroxidation to phospholipid lysis was not confirmed in their study; the authors proposed that phospholipid lysis may be amplified by components of seminal plasma such as phospolipases or phospholipase activators (Douard et al., 2005). Reduced sperm quality associated with extensive changes in lipid and phospholipid-bound fatty acid composition during liquid storage has also been reported by others (Zaniboni and Cerolini, 2009). In several studies concerning lipid and phospholipid composition of spermatozoa in fish, data on association of lipid and phospholipid content with spermatozoon motility, or changes in their content as a result of storage, has not been reported (Drokin, 1993; Bell et al., 1997). Lahnsteiner et al. (2010a) observed a decrease in

phospholipid and triglyceride levels of *Sparus aurata* semen during storage at 4 °C in motility-inhibiting saline solution. They concluded that *Sparus aurata* spermatozoa catabolize lipids and phospholipids during immotile storage. Motility was activated after 48 h of storage, but with decrease in motility rate and velocity, which probably related to disturbance in spermatozoon membrane composition.

Short-term storage of sterlet sperm resulted in deterioration of spermatozoon motility parameters, which could be attributed to changes in signalling pathways (e.g. through increase in internal Ca^{2+} concentration) or in spermatozoon plasma membrane structure, through phospholipid lysis, rather than to oxidative stress. The mechanism of the effect on motility characteristics of sterlet spermatozoa remains to be elucidated.

ACKNOWLEDGMENTS

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Original Paper *Czech J. Anim. Sci., 60, 2015 (6): 250–255*

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Oxidative stress and motility in tench *Tinca tinca* spermatozoa

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ABSTRACT: The attachment of the urinary bladder to the seminal duct near the anal aperture in tench constitutes a potential risk for urine contamination of sperm during collection, leading to spontaneous activation of sperm motility by urine hypotonicity. It was hypothesized that sperm hypotonic exposure can provoke oxidative stress which could be involved in sperm quality degradation. Our study aimed to describe spermatozoa motility parameters and levels of oxidative stress in activating media (AM) of differing osmolality. Tench sperm samples were collected from 6 males into Kurokura 180 immobilizing medium (IM) (180mM NaCl, 2.68mM KCl, 1.36mM $CaCl_2$ $2H_2O$, 2.38mM $NaHCO_3$, 340 mOsm/kg). Motility was recorded in AM of 0 mOsm/kg or 100 mOsm/kg using video microscopy combined with stroboscopic illumination. Video records were analyzed to calculate spermatozoa curvilinear velocity (VCL), motility rate, and motility duration. The level of thiobarbituric acid reactive substances (TBARS), measured by spectrophotometry, was used as an oxidative stress index. VCL and motility rate during the initial phase of motility (10 s post-activation) were not dependent on AM osmolality, while motility duration was significantly increased with 100 mOsm/kg AM. TBARS was significantly increased with reduction of AM osmolality. Increased TBARS was observed even at 5 s post-activation with AM of 0 mOsm/kg. These observations suggest that even a short period of sperm exposure to hypotonic conditions induces oxidative stress. Any contact of sperm with hypotonic urine during sperm collection should be avoided. The use of motility AM of moderate hypotonicity (≥ 100 mOsm/kg) is recommended for tench propagation.

Keywords: tench sperm; activating medium osmolality; motility reactivation; lipid peroxidation intensity

INTRODUCTION

The common tench, *Tinca tinca* (L.), is a freshwater cyprinid that has become important for pond aquaculture in many countries of Europe and Asia (Gela et al. 2006; Wang et al. 2006; Celada et al. 2009). Tench is utilized for food, sport fishing, and as an ornamental fish. Growing importance of tench in pond aquaculture has resulted in advances in reproduction techniques such as development of immobilizing medium (IM) for collection and preservation of spermatozoa, sperm cryopreservation, hormone induction of ovulation, and hybridization (Rodina et al. 2004, 2007; Mamcarz et al. 2006; Podhorec 2011; Targonska et al. 2012).

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Czech J. Anim. Sci., 60, 2015 (6): 250–255 Original Paper

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Because of connection of the tench urinary bladder to the seminal duct near the anal aperture in the caudal abdominal cavity, there is a risk of sperm contamination by urine during collection (Linhart et al. 2003). The lower osmolality of urine compared to seminal fluid (Linhart et al. 2003) may lead to spontaneous activation, but this can be prevented by sperm collection into IM. For tench, the best IM has been reported to be modified Kurokura solution: Kurokura180 (Rodina et al. 2004). This IM was developed taking into account the fact that hypotonicity is the main activating factor for spermatozoa motility in cyprinids (Krasznai et al. 2000). Spermatozoa in the sperm ducts are immersed in the isotonic and high K^+ environment of the seminal plasma, in which they remain immotile. Spawning into hypotonic fresh water with low K^+ induces spermatozoa motility through a Ca^{2+}-dependent and cAMP-independent cell signalling cascade.

Physical properties of the spermatozoa cell membrane control the majority of signal transduction processes. The activation of spermatozoa may involve specific stretch-activated channels, sensitive to osmotic pressure. As demonstrated by Krasznai et al. (2003), hypoosmolality results in an increase in membrane fluidity. A stretch-activated channel blocker, gadolinium, was shown to block the initiation of carp sperm motility and significantly decrease the fluidity of the spermatozoa membrane.

Fluidity of the plasma membrane is mainly determined by the presence of unsaturated fatty acids (Lenzi et al. 1996), double bonds of which are preferred targets for the attack of reactive oxygen species (ROS). High levels of unsaturated fatty acids in spermatozoa plasma membrane have been found in different animal species (Drokin 1993; Lenzi et al. 1996; Surai et al. 2001; Petcoff et al. 2008), making spermatozoa particularly vulnerable to the attack by ROS. In addition, it is known that lipid peroxidation resulting from the ROS effect leads to the lowering of membrane fluidity (Dobretsov et al. 1977).

The primary goal of the present study was determination of tench spermatozoa motility parameters and oxidative stress in AM of differing osmolalities. It was attempted to determine whether tench spermatozoa motility parameters are dependent on activating media osmolality and whether level of lipid peroxidation products changes during their motile phase.

MATERIAL AND METHODS

Sperm collection. Sperm samples were collected from six 4–6-year old tench during the natural reproductive season, mid-June 2012, at 24 h after injection with carp pituitary extract at 2 mg/kg. Sperm (0.2–0.5 ml) was collected from the genital papilla of each male into a 5 ml syringe containing 2 ml immobilizing media (IM) Kurokura 180 (K180: 180mM NaCl, 2.68mM KCl, 1.36mM $CaCl_2$, 2.38mM $NaHCO_3$, 340 mOsm/kg, pH 8.2) (Rodina et al. 2004).

Experimental design. Sperm in IM K180 was added to activating media (AM) of 100mOsm/kg (K180 diluted 1 : 2 with distilled water) or 0 mOsm/kg (distilled water), and motility was recorded. In addition, spermatozoa suspensions were diluted by the same AM and at the same dilution rate (1 : 30) and centrifuged at 10 000 *g* at 4°C for 10 min. The resulting supernatants were used for the evaluation of lipid peroxidation intensity by determination of thiobarbituric acid-reactive substance (TBARS) concentration.

The potential for tench spermatozoa motility to be reactivated was investigated using distilled water as AM. At 5 s post-activation, motility was arrested by the addition of KCl in an amount sufficient to create 300 mOsm/kg conditions. After 2 min of arrest, motility was re-activated by a second dilution (1 : 3) in distilled water. The content of TBARS after motility arrest was measured in the supernatants resulting from centrifugation as above.

Spermatozoa motility. Spermatozoa motibity was recorded in activating media of 100 mOsm/kg or 0 mOsm/kg using video microscopy (dark-field microscope (Olympus BX50F, lens UPlanF1 x20, dark field condenser, Olympus Optical Co. Ltd., Tokyo, Japan; CCD video camera, SSCDC50AP, Sony, Tokyo, Japan) combined with stroboscopic illumination (ExposureScope®, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic) AM were containing 0.25% pluronic acid to prevent spermatozoa from adhering to microscope slides. Spermatozoa motility was recorded from the bottom part of drop, no cover slip was used. Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL) at 10 and 40 s post-activation (μm/s), motility rate (% motile cells), and motility duration (s) using a micro-image analyzer (Olympus Micro Image software, Version 4.0.1., 1998, for MS Windows).

Original Paper *Czech J. Anim. Sci., 60, 2015 (6): 250–255*

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Thiobarbituric acid-reactive substance content. As an index of oxidative stress resulting from lipid peroxidation, the level of TBARS was measured spectrophotometrically according to Asakawa and Matsushita (1980). Briefly, to 0.2–0.5 ml supernatant, 0.025 ml butylated hydroxytoluene solution (22 mg in 10 ml ethanol), 0.025 ml ferric chloride solution (27 mg of $FeCl_3.6H_2O$ in 10 ml water), 0.375 ml 0.2M glycine-hydrochloric acid buffer, pH 3.6, and 0.375 ml TBA reagent (0.5% TBA and 0.3% sodium dodecyl sulphate) were added. The tubes with mixture were capped and heated for 15 min in a boiling water bath. After cooling, 0.25 ml glacial acetic acid and 0.5 ml chloroform were added. The mixture was vigorously shaken and centrifuged at 1500 *g* for 10 min. The absorbance of samples was determined at 535 nm against a deionized water blank. A molar extinction coefficient of 1.56×10^5 $M^{-1} \cdot cm^{-1}$ was used for the calculation of TBARS content. The concentration of TBARS was expressed as nmol/ml of supernatant.

Data presentation and statistical analysis. Data distribution and homogeneity of dispersion were tested by Kolmogorov-Smirnov and Levene's test, respectively. Normally distributed data on VCL were analyzed by one-way ANOVA followed by Fisher's LSD test. Due to a low number of observations ($n = 6$), a nonparametric Kruskal-Wallis ANOVA followed by multiple comparisons of mean ranks for all groups were used for comparison of motility rate, motility duration, and concentration of TBARS. Data were presented as mean ± SD. Statistical significance was accepted at $P < 0.05$. All analyses were conducted using STATISTICA software (Version 9.1, 2013).

RESULTS AND DISCUSSION

Motility rate during the initial phase of tench spermatozoa motility (10 s post-activation) was not dependent on the AM osmolality (Figure 1). There was no significant change in this parameter at 40 s post-activation. Tench spermatozoa were able to be reactivated after motility arrest. At 10 s post-reactivation, the motility rate was not different from values at initial activation in either AM, but at 40 s post-reactivation, motility rate was significantly lower than that observed with 100 mOsm/kg AM.

Reduction in AM osmolality had no effect on the initial curvilinear velocity of tench spermatozoa (Figure 2). At 40 s post-activation, VCL was significantly decreased compared to its value at 10 s in both AM solutions, being lower at activation in distilled water (0 mOsm/kg). Reactivated spermatozoa were characterized by significantly lower initial VCL compared with spermatozoa activated in AM of either osmolality. As in the

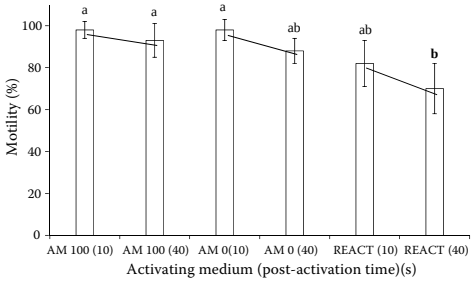

Figure 1. Motility rate (%) of tench spermatozoa at activation and reactivation

activating media (AM) 100 (10), AM 0 (10), REACT (10) = 10 s post-activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively; AM 100 (40), AM 0 (40), REACT (40) = 40 s post-activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively

[a,b]values with different letters are significantly different ($P < 0.05$, $n = 6$)

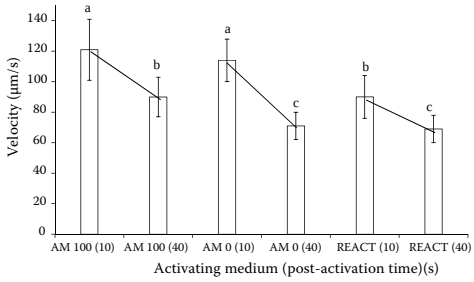

Figure 2. Curvilinear velocity (μm/s) of tench spermatozoa at activation and reactivation

activating media (AM) 100 (10), AM 0 (10), REACT (10) = 10 s post-activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively; AM 100 (40), AM 0 (40), REACT (40) = 40 s post-activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively

[a–c]values with different letters are significantly different ($P < 0.05$, $n = 6$)

Czech J. Anim. Sci., 60, 2015 (6): 250–255 Original Paper

doi: 10.17221/8238-CJAS

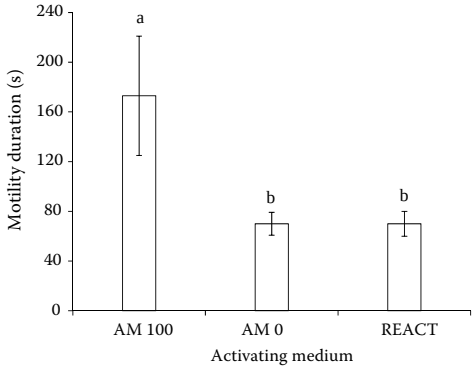

Figure 3. Motility duration (s) of tench spermatozoa at activation and reactivation

activating media (AM) 100, AM 0, REACT = spermatozoa activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively

[a,b]values with different letters are significantly different ($P < 0.05$, $n = 6$)

initial activation, VCL at 40 s post-reactivation was significantly lower than at 10 s.

Duration of tench spermatozoa motility in 100 mOsm/kg AM was more than two-fold that in AM of 0 mOsm/kg (Figure 3). There was no significant difference in this parameter in spermatozoa activated in 0 mOsm/kg AM from that reactivated in the same AM. Decrease in spermatozoa motility duration with the lowering of AM osmolality can be explained by the possibility that adenosine triphosphate (ATP) stock is needed not only to activate motility, but also to maintain numerous metabolic processes of sperm cells which could be directly involved in cell volume regulation. At reactivation (during the second motility period) spermatozoa swim slower (due to the partial use of ATP stock during the first period of motility), but for the same time period. It appears that sperm cells have developed regulatory systems which allow the duration of motility to be dependent not only on energy source.

Collection of sperm into Kurokura 180 IM preserves properties of tench spermatozoa that are typical of other cyprinids. Motility parameters were shown to depend on AM osmolality. Such dependence has been shown in the studies of other species (Morisawa et al. 1983; Perchec Poupard et al. 1997; Alavi et al. 2009). Common carp spermatozoa velocity significantly increased in AM from 60 to 140 mOsm/kg AM osmolality, with a progressive decrease when osmolality was increased to 300 mOsm/kg (Perchec Poupard et al. 1997). Motility duration of goldfish *Carassius auratus* spermatozoa at 100 mOsm/kg was significantly higher than at 0 mOsm/kg (Morisawa et al. 1983). Positive correlation of sperm motility and velocity with osmolality of AM was also reported for common barbell *Barbus barbus* (Alavi et al. 2009). In addition, as in other cyprinids, tench spermatozoa can be repeatedly activated. Common carp spermatozoa are able to be activated a second time following the transfer into a medium of high osmolality, interrupting motility, after the first phase of motility activation (Perchec et al. 1995; Linhart et al. 2008; Boryshpolets et al. 2009). The high variation in tench spermatozoa motility rate at reactivation observed in the present study was similar to that in carp spermatozoa shown by Boryshpolets et al. (2009).

Spermatozoa activation is an extremely complex and precisely orchestrated process. In general, upon reception of external activating stimuli, specific signalling pathways are switched on allowing the transduction of received signals and, ultimately, triggering motion through the activation of axonemal dyneins (Dzyuba and Cosson 2014). Initial signal transduction processes depend upon the physico-chemical properties of the cytoplasmic membrane. Similar physical changes of spermatozoa plasma membrane (e.g. changes in fluidity and membrane potential) may be also involved in physiological processes such as epididymal maturation, hyperactivation, and capacitation (de Lamirande et al. 1997; Nolan and Hammerstedt 1997).

As it appears that the loss of spermatozoa membrane fluidity would be the consequence of intensification of the processes of lipid peroxidation, the content of free radical lipid peroxidation products in spermatozoa supernatants before motility activation, at the motility activation step in media of differing osmolalities, and at motility arrest was quantitatively estimated. The concentration of TBARS during spermatozoa motility activation in 100 mOsm/kg AM was similar to that in non-activated spermatozoa (Figure 4). With reduction of AM osmolality, the TBARS content significantly increased. The level of lipid peroxidation products observed at 5 s post-activation and that following motility arrest did not differ. This may indicate that the process of lipid peroxidation was imme-

Original Paper *Czech J. Anim. Sci., 60, 2015 (6): 250–255*

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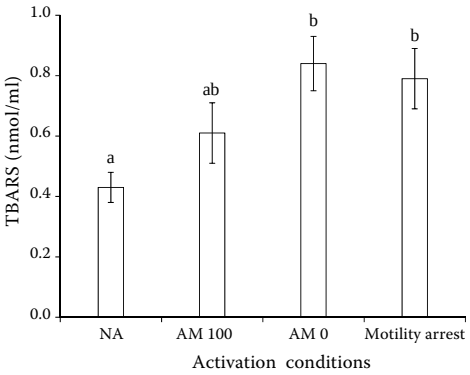

Figure 4. Concentration of thiobarbituric acid-reactive substances (nmol/ml) at activation and motility arrest of tench spermatozoa

NA = before activation, AM 100 and AM 0 = spermatozoa activation in 100 mOsm/kg activating media (AM) and 0 mOsm/kg AM, motility arrest = motility stopped by the addition of KCl up to osmolality of 300 mOsm/kg at 5 s post-activation

[a,b]values with different letters are significantly different ($P < 0.05$, $n = 6$)

diately enhanced upon activation in AM of low osmolality. The high concentration of TBARS seen at activation in medium with low osmolality and immediately following motility arrest may be the source of decreased motility duration at activation and reactivation of tench spermatozoa.

Data on decreased spermatozoa motility duration with activation and reactivation in distilled water, decreased VCL at reactivation, and increased TBARS content at 5 s post-activation in distilled water and after motility arrest are evidence that even a short exposure of tench sperm to hypotonic conditions can provoke oxidative stress.

CONCLUSION

The results indicate that correctly collected tench spermatozoa, implying the use of immobilizing media, possess the properties typical of cyprinid sperm, including dependence of motility parameters on AM osmolality and potential for reactivation after the initial motile phase.

During tench artificial propagation, the application of AM of moderate hypotonicity (≥ 100 mOsm/kg) is preferable. During sperm collection even a minimal contact of spermatozoa with hypotonic urine should be avoided in order to prevent oxidative stress. It can be assumed that the addition of antioxidants to immobilizing media may be useful for tench sperm preservation. The latter assumption and the endogenous antioxidant system in tench sperm are topics for further research.

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CHAPTER 5

GENERAL DISCUSSION

Proteins are an extremely diversified group of substances with very important roles for every cell types, including spermatozoa. They are essential components of all living organisms, and play an important role in cell viability. Proteins carry out metabolic processes, take part in organization of the intracellular structures, catalyze various chemical reactions, serve as signal substances, regulate many intracellular processes, participate in the transport of ions and small molecules, and carry out receptor, motor and several types of protective functions. Careful analysis of the composition and functions of proteins in the seminal plasma and spermatozoa of chondrostean and teleostean fishes allowed Li and coauthors (2009) to summarize the main functions conducted by proteins in fish sperm. These proteins are involved in regulation of spermatogenesis and maturation of spermatozoa, initiation and duration of sperm motility, energy supply, regulation of fertilization events, and protection from several types of damaging factors (proteolytic attacks, microbes, oxidative stress). The representatives of all of these groups of regulatory proteins, except the one participating in fertilization regulation, were the main topics for the present study.

Energetic aspects of spermatozoon motility implementation

While it is generally accepted that the source of energy for flagella activity is the hydrolysis of ATP (Cosson, 2013), the metabolic pathways involved in its generation/regeneration and distribution along the length of the flagellum may be species-specific and are not fully understood. The main pathways for energy generation in fish sperm were exhaustively reviewed by Ingermann (2008) who suggested the existence of differing metabolic strategies for storage and generation of ATP. In our study we focused on the functioning of two systems taking part in ATP regeneration in the motility phase of spermatozoa: adenylate kinase (AK) which can catalyze the formation of ATP from motility-generated ADP, and creatine kinase (CK) which can also convert ADP into ATP in the presence of phosphocreatine (PCr). The investigation was carried out using demembranated spermatozoa of sterlet and common carp, taxonomically distant fish species in which spermatozoa differ in structure (Psenicka et al., 2009a, 2009b), mode of motility activation (Perchec Poupard et al., 1997; Alavi and Cosson, 2006), and motility duration (Billard et al., 1995; Perchec Poupard et al., 1997; Lahnsteiner et al., 2004).

In the present study, it was shown for the first time that the PCr/CK phosphagen system is present in sturgeon spermatozoa and plays an essential role in regeneration of ATP. As a result of this investigation, we found that spermatozoa of two taxonomically distant fish species, common carp and sterlet, possess similar systems for ATP regeneration at the motility stage. The results are in agreement with the data of Saudrais et al. (1998) who studied the relative contribution of AK and CK to ATP regeneration in demembranated rainbow trout spermatozoa, showed the presence of a creatine-phosphocreatine (Cr/PCr) shuttle and found that AK was less effective in ATP regeneration than CK.

At the same time, the efficacy of the studied ATP regeneration systems differs in carp and sterlet sperm. Using AK and CK inhibitors, we found that in carp sperm, CK was the main energy supplier, as its inhibition alone or together with AK inhibition, resulted in a more pronounced decrease of beat frequency, while AK inhibition alone had no effect. A slightly different situation was shown in case of sterlet: in this case, inhibition of AK appears mostly crucial for the maintenance of flagella movement.

Among the studied soluble enzymes, CK activity showed the highest absolute values in both carp and sterlet. Sterlet spermatozoon extracts displayed approximately three-fold (224 vs. 80 U/10^9 spermatozoa) the CK activity and two-fold (1.03 vs. 0.48 U/10^9 spermatozoa)

the ATPase activity observed in carp spermatozoa. Conversely, sterlet spermatozoon extracts showed significantly lower AK activity, at approximately one quarter of the level observed in carp spermatozoa. The low baseline activity of CK in carp and AK in sterlet could be the source of the most pronounced effects of their inhibition on energy supply for flagella movement in the respective species. At the same time, it can be assumed that longer average spermatozoon motility period in sterlet (4 min) compared to carp (40 s) could be partially explained by the higher CK activity.

Additionally, apparent Michaelis constant for ATP was found to be 180 ± 8 μM for carp and 79 ± 4 μM for sterlet. This suggests that sterlet axonemal dynein ATPases have a higher degree of affinity for ATP than do those of carp. Such striking difference in the value of Michaelis constant in sterlet and carp spermatozoa, and, correspondingly, in their axonemal dynein ATPase affinity to ATP, and possible existence of other ATP regeneration system(s) in sterlet sperm, which will lead to more efficient energy supply, are discussed but still remain to be clarified.

Sturgeon sperm maturation: involvement of proteolysis regulators

The physiologic process by which morphologically completely formed spermatozoa acquire the ability for motility initiation and fertilization is called maturation and has been well investigated in many mammalian species (de Lamirande et al., 1997; O'Flaherty et al., 2005; Marengo, 2008; Sostaric et al., 2008) and several insect species (Friedlander et al., 2001; Miyata et al., 2012). In this maturation process, the involvement of calcium ions, reactive oxygen species, cyclic AMP, different protein kinases, protein phosphatases and proteolysis regulators are now generally recognized.

In addition to rainbow trout, chum salmon and Japanese eel (Morisawa and Morisawa, 1988; Miura et al., 1995: Ohta et al., 1997) in which spermatozoa acquire the potential for motility activation due to the presence of various factors (pH, bicarbonate level) in their sperm ducts, we found that sperm maturation also occurs in sturgeon (Dzyuba et al., 2014c). The main evidence for this assertion was obtained from experiments with *in vitro* pre-incubation of immotile testicular sperm (TS) in urine and seminal fluid (SF) from Wolffian duct sperm (WS) that enabled testicular spermatozoa to initiate their motility. It allowed us to suggest that under *in vivo* conditions, maturation takes place during the passage of testicular sperm through the kidney and Wolffian duct and results from dilution of testicular sperm with urine and SFWS. Incubation of TS in artificial SF also led to the maturation of spermatozoa, but much longer incubation time was needed (more than 2 h in the artificial SF, compared to 10 min in urine or SFWS) to acquire the ability to activate motility, and in addition they had a significantly lower motility rate and curvilinear velocity than those incubated in SFWS or urine. In contrast to salmonids, extracellular pH was not involved in the induction of TS maturation in sturgeons, but removal of calcium ions from incubating media or blocking of sperm Ca^{2+} channels completely inhibited the process of maturation.

While investigating proteolysis regulators (proteases and their inhibitors) involvement in sterlet spermatozoon maturation, we did not find significant differences between caseinolytic and gelatinolytic activities in SF from sterlet TS and WS of various sturgeon species. This means that the majority of proteases present in SFWS originate from the testis. The only exception is a protease with molecular weight 269 kDa, which was present in almost every sample of SFWS but never in SFTS. Evaluation of amidase and anti-proteolytic activities in seminal fluids from sterlet TS showed that there was a significant decrease in activity during spermatozoa transit through kidneys and Wolffian ducts. Considering our observation that trypsin activity inhibition during *in vitro* TS maturation resulted in prevention of TS to become activated (Dzyuba et al., 2014c), we propose that a decrease in anti-proteolytic activity of spermatozoa

surrounding fluids during their transit through kidneys and Wolffian ducts could be needed for them to become mature. We further believe that it is not the levels of proteolytic and anti-proteolytic activities *per se*, but rather changes in their inter-relations and triggered by such a way removal of some kind of blockage on a spermatozoon surface that could be a switch for the maturation process.

With regard to spermatozoa extracts, casein zymography revealed that proteolytic profiles of sterlet WS and TS extracts were absolutely different: Wolffian ducts spermatozoa extracts were characterized by the presence of broad proteolytic spot with molecular weight ranging from 48 to 41 kDa, while TS extracts did not reveal such activity: thus, as a result of *in vitro* maturation, TS extracts acquired the same activity as WS extracts. The observed changes were supported by the measurement of total amidase activity, which showed that this activity of TS extracts was significantly lower compared to the level in WS extracts, and that it increased after TS *in vitro* maturation.

So, in the present study we confirmed that the sturgeon maturation process is realized due to the interplay between testicular spermatozoon surface and seminal environment, and probably includes proteolytic processing of some macromolecules located on the spermatozoon surface. Such changes in macromolecular structure can trigger a signaling cascade and, as a result, the activation of flagellum motility. The exact signaling pathways initiated by proteolysis regulators (proteases and their inhibitors) remain to be further elucidated. Considering that the capacity to induce TS maturation by seminal fluid from Wolffian duct sperm is not species specific among sturgeons, the mechanism of protease initiation of sperm motility may represent a common and evolutionarily conserved mechanism as has been proposed for insects (Miyata et al., 2012). Participation of calcium ions and other high molecular weight substances of seminal fluid (not only proteases) in the process of sturgeon spermatozoa maturation is not excluded.

Following *in vitro* maturation, sterlet testicular spermatozoa showed a freeze–thaw survival and fertilizing ability similar to that of Wolffian duct sperm (Dzyuba et al., 2014a). These results served as a basis for the elaboration of a method to use sturgeon testicular sperm in aquaculture (Dzyuba et al., 2014b). This is important, considering the fact that the use of TS can be required in artificial reproduction for several reasons such as low quality of ejaculated sperm, oligospermia as in many siluroid species (Legendre et al., 1996), and accidental death of valuable broodstock, and the fact that sturgeon are more critically endangered than any other group of species (IUCN, 2010). However, the shorter motility duration found for *in vitro* matured testicular spermatozoa compared to Wolffian duct spermatozoa suggests that further studies are needed on the bioenergetic pathways, which probably affect sperm maturation.

Prooxidant-antioxidant status regulation in spermatozoon maturation and motility activation

As spermatozoa are known to be highly redox active cells (Aitken et al., 2014), the importance of redox status regulation cannot be overemphasized. The dual role of reactive oxygen species (ROS) is generally accepted in mammalian spermatology (Aitken and Curry, 2011). The level of ROS is governed by the numerous systems in their production and scavenging. The imbalance between generation of ROS and their removal accounts for the development of oxidative stress leading to the accumulation of damaged macromolecules and the deterioration of cell functions. On the other hand, a certain level of ROS should be present as redox modifications of a great diversity of molecules intervene in almost every signaling pathway described to date (Covarrubias et al., 2008).

The data on the role of ROS in fish sperm physiology are much poorer. Most information indicates that intensification of free radical oxidation activated by environmental contaminations, cryopreservation, sperm storage and some other factors leads to sperm motility deterioration. Addition of different antioxidants into activating, storage and cryopreservation media has been shown to be essential for spermatozoa functioning. However, the data of Morita et al. (2011) describing redox and phosphorylation pathways in the regulation of osmotic tolerance and motility of euryhaline tilapia (*Oreochromis mossambicus*) could be considered as support for the importantce of redox-based signaling for spermatozoa physiology, and supporting the need for further investigation of the role of redox status regulation for different aspects of spermatozoon functioning.

Our present study showed that maturation of sturgeon spermatozoa and different times of storage (up to 60 h) in Wolffian ducts (*in vivo* storage) were accompanied by significant alterations in motility parameters and in SF redox balance. While TS were immotile upon dilution in activating medium, WS motility parameters were changed as a function of time after induction of spermiation, significantly decreasing by 60 h after hormone stimulation. During the study of prooxidant-antioxidant system, we observed that an increased amount of TBA-reactive substances (TBARS, products of lipid peroxidation) in SFTS was followed by a rise in superoxide dismutase (SOD) and catalase (CAT) activity. The high level of lipid peroxidation products in SF from immature testicular sperm may be an indication of excessive production of ROS, which is known to peak in immature mammalian sperm (Fisher and Aitken 1997). From our point of view, high content of TBARS followed by increased activity of antioxidant enzymes in SF from testicular sperm may indicate a regulatory role of ROS in the sturgeon sperm maturation process similar to that occurring during mammalian sperm epididymal transit (Ford, 2004; Aitken et al., 2012). The high activity of the enzymatic antioxidant system allows testicular spermatozoa to cope with the deleterious effects of excessive ROS production and to retain the ability to become motile after passing through the kidneys and Wolffian ducts or after *in vitro* maturation.

The increase in TBARS in SFWS at 36 h following spermiation was not accompanied by a corresponding increase in activity of the antioxidant enzymes. We suggest that an extended time in the Wolffian ducts results in spermatozoa oxidative damage from inadequate enzymatic antioxidant system efficacy in scavenging ROS and, finally, a decrease in motility parameters. To investigate this possibility, we assessed membrane integrity and found that it was stable up to 36 h, a time characterized by a significant increase in TBARS content. As this increase was not compensated for by a rise in antioxidant enzyme activity, the spermatozoa membrane lost part of its integrity, probably due to lipid peroxidation.

Alongside with *in vivo* storage, the effect of short-term *in vitro* storage on the evolution of sperm motility parameters (curvilinear velocity and motility percentage) and seminal fluid enzymatic (activity of SOD and CAT) and non-enzymatic (uric acid concentration) antioxidant system markers were studied using sterlet *Acipenser ruthenus* sperm. It was found that 36 h of *in vitro* sperm storage at 4°C resulted in a significant decrease in percentage of motility and spermatozoon curvilinear velocity, which were not followed by changes of any studied parameters of seminal fluid antioxidant system. The results suggest that the antioxidant system of sterlet sperm is effective enough to prevent the development of oxidative stress during short-time sperm storage. Thus, the observed decrease in sperm motility parameters after 36 h of *in vitro* storage could rather be caused from some changes in signaling pathways or in spermatozoon plasma membrane structure than from the development of oxidative stress.

The possible involvement of oxidative stress that can be provoked by different factors from spermatozoon environment in sperm quality decline was also studied using tench *Tinca tinca*.

The connection of the urinary bladder with the seminal duct near the anal aperture in tench constitutes a potential risk for urine contamination of sperm during collection, leading to spontaneous activation of sperm motility by urine hypotonicity. We hypothesized that sperm hypotonic exposure could be the reason of oxidative stress development and consequent sperm quality degradation. It was found that curvilinear velocity and motility rate of tench spermatozoa during the initial phase of motility (10 s post-activation) were affected by media osmolality, while motility duration was significantly increased in 100 mOsm kg^{-1} activating medium. In addition, TBARS level was significantly increased with reduction of activating medium osmolality. Increased level of TBARS was present even at 5 s post-activation with activating medium of 0 mOsm kg^{-1} osmolality. These observations suggest that even a short period of sperm exposure to hypotonic conditions induces oxidative stress. Spermatozoon plasma membrane is characterized by high level of unsaturated fatty acids (Drokin, 1993; Surai et al., 2001; Petcoff et al., 2008) that makes spermatozoa particularly vulnerable to the effects of ROS. Lipid peroxidation resulting from this leads to the lowering membrane fluidity (Dobretsov et al., 1977). Probably, such physical changes of the spermatozoon plasma membrane can complicate the reception of external activating stimuli taking place at its level and, as a result, disarrange signaling pathways triggering activation of axonemal dyneins.

The combined results of the present study may confirm a dual role of reactive oxygen species in fish sperm physiology and emphasize the importance of regulation of their level in spermatozoa and seminal fluid.

As a general conclusion to the present study: proteins of fish sperm certainly possess multifaceted regulatory roles which are essential for the implementation of different processes: maturation, energy supply for activation and maintenance of motility, and redox state controlling. Many aspects of protein involvement in the above mentioned processes (especially in fish sperm) still need clarification through further investigations.

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ENGLISH SUMMARY

Role of regulatory proteins in fish sperm motility

Viktoriya Dzyuba

The investigation of the energetic aspects of spermatozoon motility implementation (Chapter 2) was carried out using demembranated spermatozoa of taxonomically distant fish species (common carp and sterlet). Special attention was given to the functioning of ATP regeneration systems: adenylate kinase (AK), and creatine kinase (CK). It was shown for the first time that the phosphocreatine/CK system is present in sterlet spermatozoa and plays an essential role in ATP regeneration. Spermatozoa of carp and sterlet were shown to have similar systems for ATP regeneration, while the efficacy of the studied systems differs in these species. Among the studied soluble enzymes, CK activity had the highest absolute values in both carp and sterlet. Sterlet spermatozoon extracts had significantly higher CK and ATPase activity, and lower AK activity compared to carp. The low baseline activity of CK in carp and AK in sterlet suggests these to be the source of the most pronounced effects of their inhibition on energy supply for flagella movement in the respective species.

The presence of a maturational process during the post-testicular transit of sperm in sturgeon was recently ascertained in our laboratory (Chapter 3). This discovery prompted investigation of the factors that regulate this process including the involvement of proteolysis regulators and prooxidant-antioxidant system. As a result of this study (Chapter 3.3.), we found that there was no significant difference between proteolytic profiles of seminal fluids (SF) of testicular sperm (TS) and Wolffian duct sperm (WS). It suggests that the majority of proteases present in SF of mature sperm originate in the testis. Measure of amidase and anti-proteolytic activities in the SF of sterlet sperm showed significant decrease in activities as the sperm passed through the kidneys and Wolffian ducts. For spermatozoon extracts, casein zymography revealed that proteolytic profiles of sterlet WS and TS extracts were significantly different: WS was characterized by the presence of broad proteolytic smear with molecular weight ranging from 48 to 41 kDa, while TS extracts did not show such activity, but as a result of *in vitro* maturation they acquired the same activity. Considering our observation that trypsin inhibition during *in vitro* TS maturation blocked the maturation process (Chapter 3.1.), and based on zymography, amidase and anti-proteolytic activity determination, we think that the decrease in anti-proteolytic activity of spermatozoa surroundings during their post-testicular transit could be needed to prepare them for maturation. We believe that it is not certain levels of proteolytic and anti-proteolytic activities *per se*, but rather changes in their inter-relations that initiate the removal of some kind of blockage on a spermatozoon surface, and thereby provide a switch for the maturation process.

The present study showed that maturation of sturgeon spermatozoa and different times of storage in Wolffian ducts (*in vivo* storage) are accompanied by significant alterations in motility parameters as well as in SF redox balance (Chapter 4.1.). A high level of TBA-reactive substances (TBARS) and a high activity of antioxidant enzymes were found in immature TS compared to those in WS. The high activity of the enzymatic antioxidant system (AOS) allows TS to cope with the deleterious effects of excessive reactive oxygen species production and to retain the ability to become motile after post-testicular transit, or after *in vitro* maturation. The increase in TBARS content during *in vivo* storage was not accompanied by a corresponding increase in activity of AOS. We suggest that extended time in the Wolffian ducts resulted in sperm oxidative damage resulting from inadequate AOS efficacy and, finally, in decreases in motility parameters.

Short-term hypothermic *in vitro storage* of sterlet sperm resulted in a significant decrease in motility and velocity without changes of AOS activity (Chapter 4.2.). It means that AOS of sterlet sperm is effective enough to prevent the development of oxidative stress during short-term storage.

Short period of tench sperm exposure to hypotonic conditions was shown to induce oxidative stress and, as a result, sperm quality decline (Chapter 4.3.).

The combined results of the study concerning the regulation of sperm prooxidant-antioxidant status (Chapter 4) during spermatozoa maturation, motility activation and sperm *in vivo* and *in vitro* storage may confirm a dual role of reactive oxygen species (regulatory or damaging depending from the levels of their formation and elimination) in fish sperm physiology.

CZECH SUMMARY

Význam regulačních proteinů pro pohyblivost rybích spermií

Viktoriya Dzyuba

Stanovení energetických aspektů motility spermií (kapitola 2) bylo provedeno za použití demembranovaných spermií taxonomicky vzdálených druhů – kapra obecného a jesetera malého. Zvláštní pozornost byla věnována funkci ATP regeneračního systému: adenylát kináze (AK) a kreatin kináze (KK). Podařilo se nám poprvé prokázat, že ve spermatu jesetera malého je přítomen fosfokreatin/KK systém a hraje nezastupitelnou roli v regeneraci ATP. Spermie kapra i jesetera malého obsahuje obdobné systémy pro regeneraci ATP, přičemž účinnost těchto systémů se u daných druhů liší. Mezi studovanými enzymy vykazovala nejvyšší absolutní hodnoty aktivita KK jak u kapra, tak u jesetera malého. Extrakty ze spermií jesetera malého vykazovaly ve srovnání s extrakty ze spermií kapra signifikantně vyšší hodnoty aktivity KK a ATPazy, a naopak nižší míru aktivity AK. Nízká bazální aktivita KK u kapra a AK u jesetera malého by mohla být zdrojem nejvýraznějšího efektu inhibice energetických zdrojů pro pohyby bičíku spermie u příslušného druhu.

V naší laboratoři byla recentně prokázána existence procesu dozrávání spermií během post-testikulárního transportu spermatu u jesetera (kapitola 3). To vedlo ke zkoumání různých faktorů regulujících tento proces, které zahrnují např. regulátory proteolýzy či prooxidační-antioxidační systém. V naší studii (kapitola 3.3.) jsme nezjistili žádné signifikantní rozdíly mezi proteolytickými profily seminální plazmy (SP) testikulárního spermatu (TS) a spermatu z Wolfových vývodů (SWV). To by mohlo naznačovat, že většina proteáz přítomných v SP dozrálého spermatu pochází z varlat. Stanovení amidázové a anti-proteolytické aktivity v SP ukázalo, že prokazatelné snížení sledovaných aktivit se odehrává během transportu spermií přes ledviny a Wolfovy vývody. U extraktu spermií odhalila kaseinová zymografie, že proteolytický profil extraktu SWV a TS jesetera malého se signifikantně lišil: SWV bylo charakterizováno přítomností širokého proteolytického bandu s molekulární hmotností v rozmezí 48 až 41 kDa, zatímco extrakt TS neodhalil žádnou takovou aktivitu, a potom jako výsledek *in vitro* dozrávání TS dosáhl stejné aktivity. Na základě našich pozorování, že inhibiční aktivita trypsinu během *in vitro* dozrávání TS blokuje proces dozrávání (kapitola 3.1.) a na základě výsledků zymografie a stanovení amidázové a anti-proteolytické aktivity předpokládáme, že snížení anti-proteolytické aktivity okolní tekutiny spermií během post-testikulárního transportu by mohlo být potřebné pro jeho dozrání. Zdá se, že spíše než konkrétní hladina proteolytické a anti-proteolytické aktivity sama o sobě by mohly být spouštěčem procesu dozrávání spíše změny v jejich vztazích a odstranění některých typů blokád na povrchu spermie.

Tato studie dokazuje, že dozrávání spermatu jesetera a rozdílný čas jeho uchování ve Wolfových vývodech (*in vivo* uchovávání) jsou doprovázeny signifikantními změnami v parametrech motility a redoxní rovnováhy SP (kapitola 4.1.). V nezralém TS byla zjištěna vysoká míra TBA-reaktivních látek (TBARL) a vysoká aktivita antioxidačních enzymů ve srovnání s SWV. Vysoká aktivita enzymatického antioxidačního systému (AOS) umožnila TS vyrovnat se se škodlivými účinky nadměrné produkce volných kyslíkových radikálů a zachovat tak schopnost motility po post-testikulárním transportu nebo po dozrávání *in vitro*. Zvýšení obsahu TBARL během *in vivo* uchování nebylo doprovázeno odpovídajícím zvýšením aktivity AOS. Předpokládáme, že zvýšená doba zadržování spermií ve Wolfových vývodech má za následek oxidativní poškození vyplývající z nedostatečné účinnosti AOS, a v konečném důsledku tak vede k poklesu v parametrech motility.

Krátkodobé uchovávání spermatu jesetera malého *in vitro* vedlo k signifikantnímu poklesu motility a rychlosti pohybu spermií beze změn v aktivitě AOS (kapitola 4.2.). To znamená, že AOS spermatu jesetera malého je dostatečně efektivní v prevenci rozvoje oxidativního stresu během krátkodobého uchovávání.

Krátkodobé vystavení spermatu lína hypotonickým podmínkám indukovalo oxidativní stres a ve výsledku vedlo k poklesu kvality spermií (kapitola 4.3.).

Kombinované výsledky studie týkající se regulace prooxidačního-antioxidačního stavu (kapitola 4) během dozrávání spermií, aktivace motility, *in vivo* a *in vitro* uchovávání mohou potvrdit dvojí roli reaktivních kyslíkových radikálů (regulační nebo naopak poškozující v závislosti na míře jejich utváření či odstraňování) ve fyziologii rybích spermií.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Dzyuba, V., Cosson, J., Dzyuba, B., Rodina, M., 2015. Oxidative stress and motility in tench *Tinca tinca* spermatozoa. Czech J. Anim. Sci. 60, 250–255.

Dzyuba, B., Boryshpolets, S., Cosson, J., **Dzyuba, V.,** Fedorov, P., Saito, T., Psenicka, M., Linhart, O., Rodina, M., 2014. Motility and fertilization ability of sterlet *Acipenser ruthenus* testicular sperm after cryopreservation. Cryobiology 69, 339–341.

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Dzyuba, B., Boryshpolets, S., Shaliutina, A., Rodina, M., Yamaner, G., Gela, D., **Dzyuba, V.,** Linhart, O., 2012. Spermatozoa motility, cryoresistance, and fertilizing ability in sterlet *Acipenser ruthenus* during sequential stripping. Aquaculture 356–357, 272–278.

Application of methodologies, patents, pilot plants, verified technologies

Dzyuba, B., Boryshpolets, S., Cosson, J., Rodina, M., Psenicka, M., Linhart, O., Praskova, E., **Dzyuba, V.,** Fedorov, P., 2014. Použití testikulárního spermatu jeseterovitých ryb. Edice Metodik, FROV JU Vodňany, CZE, No. 151, 13 pp. (in Czech)

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Bondarenko, O., Dzyuba, B., Cosson, J., Prokopchuk, G**., Dzyuba, V.,** Rodina, M., Linhart. O., 2013. The role of Ca^{2+} in sterlet (*Acipencer ruthenus*) spermatozoa *in vitro* maturation. In: Book of Abstracts 4th Int. Workshop on the Biology of Fish Gametes. Albufeira, Portugal, 17–20 Sept., 2013, pp. 24–25. (oral)

Bondarenko, O., Dzyuba, B., Cosson, J., Prokopchuk, G., **Dzyuba, V.,** Rodina, M., Linhart, O., 2013. Spermiogenesis in sturgeons: improvement of testicular sperm motility for application in aquaculture / AQUA2013

Cosson, J., Fedorov, P., **Dzyuba V.,** 2013. Energetic aspects of fish sperm motility. In: Book of Abstracts 4th Int. Workshop on the Biology of Fish Gametes. Albufeira, Portugal, 17–20 Sept., 2013, pp. 36–37. (oral)

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Cosson, J., Prokopchuk, G., Bondarenko, V., **Dzyuba, V.,** Dzyuba, B., Boryshpolets, S., Rodina, M., Fedorov, P., Bondarenko, O., Linhart, O., 2013. More secrets about fish sperm flagella revealed by high-speed video-microscopy and stroboscopy. B In: Book of Abstracts Int. Conf. Diversification in Inland Finfish Aquaculture II (DIFA II). Vodňany, Czech Republic, 24–26 Sept., 2013, p. 77. (poster)

Dzyuba, B., Cosson, J., Boryshpolets, S., Bondarenko, O., **Dzyuba, V.,** Rodina, M., Linhart, O., 2013. Sperm maturation in sturgeon: extending the efficiency of male exploitation in aquaculture. In: The 7th International Symposium on Sturgeon (ISS7). Nanaimo, British Columbia, Canada, 21–25 July, 2013, Abstract N 5_O_096. (only abstract)

Dzyuba, B., Cosson, J., Boryshpolets, S., Bondarenko, O., Prokopchuk, G., Gazo, I., **Dzyuba, V.,** Rodina, M., Linhart, O., 2013. Testicular sperm *in vitro* maturation in sterlet *Acipencer ruthenus*. In: Book of Abstracts 4th Int. Workshop on the Biology of Fish Gametes. Albufeira, Portugal, 17–20 Sept., 2013. pp. 104–105. (poster)

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Linhart, O., Alavi, S.M.H., Hatef, A., Psenicka, M., Rodina, M., Li, P., Hulak, M., Boryspolets, S., Dzyuba, B., Gela, D., Shalutina, A., Bondarenko, O., **Dzyuba, V.,** Gazo, I., Prokopchuk, G., Linhartova, P., Linhartova, Z., Cosson, J., 2013. Sperm biology of chondrostean fish species, a review. In: The 7th International Symposium on Sturgeon (ISS7) (Nanaimo, British Columbia, Canada, 21–25 July, 2013), Abstract N 1_O_172. (oral)

Dzyuba, B., Cosson, J., Boryshpolets, S., Shaliutina, A., Bytyutskyy, D., Flajshans, M., **Dzyuba, V.,** Rodina, M., Linhart, O., 2012. Sperm fertilizing ability and sensitivity to hypotonic stress after cryopreservation in sterlet (*Acipenser ruthenus*). In: Book of Abstracts Domestication in Finfish Aquaculture. Olsztyn-Mragowo, Poland, 23–25 October, 2012, pp. 43–47. (oral)

Dzyuba, V., Cosson, J., Dzyuba, B., Rodina, M., 2012. Motility and oxidative stress in tench, *Tinca tinca*, spermatozoa. In: Book of Abstracts VIth International Workshop on Biology and Culture of the Tench (*Tinca tinca* L.), 17–20 September 2012, Písek, Czech Republic, p. 19. (oral)

Dzyuba, V., Cosson, J., Dzyuba, B., Yamaner, G., Rodina, M., Linhart, O., 2012. Antioxidant system during *in vitro* storage of sterlet *Acipenser ruthenus* sperm. In: Book of Abstracts Domestication in Finfish Aquaculture. Olsztyn-Mragowo, Poland, 23–25 October, 2012, p. 76. (poster)

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