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Fish spermatozoa metabolites content in various physiological conditions

Pavlo Fedorov

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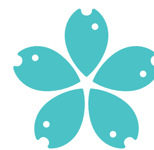

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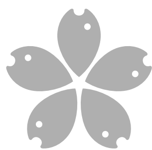

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CHAPTER 1

GENERAL INTRODUCTION

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Energetics of fish spermatozoa: The proven and the possible

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ABSTRACT

Main features specific to sperm of fish with external fertilization are 1—the seminal fluid osmolality, and in several species, some ions prevent sperm motility in the genital track; 2—transfer from seminal fluid into sea- or freshwater triggers full motility due to osmotic and/or ionic signal; 3—immediately after activation, fish sperm swim with very high efficiency; 4—the motility period is limited to a very short duration for freshwater sperm and not for much longer in case of marine fish spermatozoa; 5—most motility parameters are decreasing and wave shape is changing during the motility period; 6—the regulation of axonemal motility by ionic concentration, as well as by ATP concentration and other substances, can be observed *in vitro* by using membrane-deprived spermatozoa; 7—the chemical energy available in fish spermatozoa is rapidly exhausted during the swimming period; 8—the membrane potential of the spermatozoon represents an important aspect of the cell electro-chemical energy homeostasis; 9—the ultimate task for sperm, before fertilization, is to meet an egg, and this task is facilitated by chemotaxis.

The present chapter will tentatively deal with some of these salient features such as a—osmolality and ionic signals controlling fast but short-lasting motility in connection with ATP and other energetic compounds management; b—role of sperm electro-chemical potential in the *trans*-membrane management of this complementary aspect of sperm energetics; c—considerations on physical energy needed by moving fish flagella; and finally, d—how oriented motility governs sperm behavior while swimming toward egg. Finally, recent studies on applied aspects of fish sperm bioenergetics are summarized.

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1. Introduction

Fish spermatozoa are simple “aquasperm” consisting of a head that is composed of a nucleus and a motility device, called flagellum, and are quite similar to that of sea urchin, a classical model from which most understanding of flagellar beating arises. The ultra-structure of sperm flagella is simple: mostly a scaffold of 9 + 2 microtubules strung with molecular motors—dynein ATPases, which are in charge of generating mechano-chemical forces. The biochemical composition of fish sperm flagella is nevertheless complicated: there are more than 500 regulatory protein components used by flagellum for its operation.

Fish males during spawning deliver sperm in surrounding water at the same time as female's ova. In most fish species, these minute unicells have to reach the egg micropyle within a very brief period (seconds to minutes), meaning that their highly efficient flagellum must become fully activated immediately on contact with water and propel the sperm cell at a very high initial velocity. The cost of this “hyperactivity” is a very rapid consumption of intracellular energy accumulated as energy of macroergic phosphates (ATP, ADP, and CrP), which outstrips the supply. It is important to recall that ATP hydrolysis is the only source for flagella mechanical motion that is why ADP and CrP should be converted into ATP for utilization of stored energy. The spermatozoa become quickly exhausted since mitochondria cannot compensate for such fast flagellar energy consumption, with the result that spermatozoa cease moving. Within this brief period following activation, various successive events occur from full motility until complete arrest of flagellar activity.

Elucidation of interrelationship between the sperm bioenergetics processes and flagellum mechanical motion leading to successive gamete fusion, even being previously intensively studied, nowadays is still needed. In this chapter, we are updating previously reviewed knowledge with recent data in the field following summary of recent investigation of fish sperm bioenergetics in relation to fish reproduction studies.

Abbreviations: AK, Adenylate kinase; ADP, Adenosine di-phosphate; AMP, Adenosine mono-phosphate; AQP, Aquaporin; ATP, Adenosine tri-phosphate; cAMP, Cyclic-adenosine mono-phosphate; CK, Creatine phospho-kinase; CrP, Creatine-phosphate; GTP, Guanosine tri-phosphate; HSAPs, Herring sperm activating peptides; ROS, Reactive oxygen species; SMIF, Sperm motility initiating factor; TRPs, Transient receptor potential channels.

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2. Chemical energy sources for fish sperm motility

2.1. ATP and other phosphate chemicals as basic energetic compounds

Spermatozoa of most fish species with external fertilization are maintained immotile in the genital ducts, due to the osmolality and ionic composition of the seminal fluid, but initiate motility immediately after dispersion into water environment (Morisawa and Suzuki, 1980). Just after release, spermatozoa need energy to launch and sustain flagellar activity, which is responsible for sperm propulsion. The major role in the motile spermatozoa bioenergetics is assigned to adenylate– and creatine–phosphate metabolisms. Flagella mechanical movement is energetically supported exclusively by dynein ATPase activity. Nevertheless, during motility, ATP level in flagella can be regenerated from ADP by the AK activity or from CrP via CK activity (for review, see Cosson, 2013; Inaba, 2008). Metabolic pathways involved in processes of ATP generation occurring outside of the flagellum, its transport along flagellum, and regeneration near the sites of consumption were comprehensively reviewed by Ingermann (2008) and, until recently, this book chapter could be considered as a description of modern paradigm of fish sperm bioenergetics. From that review, it is clear that ATP level alone is not sufficient as a sole descriptor of the bioenergetic state of spermatozoa and that other metabolites (ADP, CrP) as determinants of motility should be studied. Until recently, just a few studies have been performed in simultaneous quantification of macroergic phosphates pool in relation to fish sperm physiology (Table 1). From data summarized in Table 1, it is clear that species specificity of sperm metabolic pathway do not allow an elaboration of common general rules on description of sperm bioenergetics based on macroergic phosphates content even if the energy consuming process of sperm motility is considered (see Dreanno et al., 1999b; Lahnsteiner and Caberlotto, 2012). Recently, the macroergic phosphates content as a biomarker for semen quality was proposed by several authors (Cabrita et al., 2009; Cabrita et al., 2014; Hatef et al., 2013). However, quite probably this parameter could be used as a marker of sperm quality, only in cases when general species-specific description of macroergic phosphates content is performed. Otherwise, there is a risk of incorrect recognition of the cause of sperm impairment as was shown in striped bass *Morone saxatilis* sperm by Guthrie et al. (2011).

It is important to mention that for comparative studies performed in different laboratories and on different species, the uniformity in data presentation (e.g. content of metabolites) should adopt a same unit, such as mole per number of spermatozoa.

Remark: in column "conditions," B means before motility activation and A means after motility stop.

Another adenosyl compound, cAMP, was shown to play an important role at motility initiation of fish spermatozoa (Morisawa, 1985), even though it is not directly involved in the generation of energy used by flagellar mechanics (Saudrais et al., 1998) but rather through protein phosphorylation signaling (see Zilli et al., chapter in this book). Nevertheless, cAMP also controls the flagellar activity *during the motility period* because it has been shown to be strongly interactive with ATP itself (Cosson et al., 1995).

One important point relates to the diffusion rate of energetic compounds along the restricted internal volume of a flagellum. Studies by Takao and Kamimura show that diffusion is a rate-limiting factor for small molecules as predicted in Fig. 1 (Takao and Kamimura, 2008), even though the head-tail junction does not represent a major bottleneck for such diffusion (Takao and Kamimura, 2010). The respective roles of ATP and ADP in the energetic/regulation of flagellar beating are far from being fully understood. Results from a series of studies by Shingyoji's group (see synthesis in Shingyoji, 2009) on sea urchin flagella lead to the unexpected hypothesis that relatively high ATP concentration (above 0.1 mM) partly inhibits the dynein ATPase activity, but this inhibition can be overcome when ADP is present at quite high concentrations. These results indicate that in addition to its role as the source of chemical energy, the physiological concentration of ATP plays a role as an inhibitory factor to prevent dynein arms from causing microtubule sliding, probably by maintaining cross-bridges between certain dynein arms and microtubules. These aspects deserve careful investigation in fish spermatozoa, where the concentrations of both ATP and ADP are subjected to important changes during the motility period.

2.2. Short motility duration of fish spermatozoa

Generally, sperm motility duration in fish is much shorter in comparison to that in mammals and differs greatly among fish species: this suggests that ATP can be generated and stored according to

Table 1
Creatine–phosphate and adenylate–phosphates content in fish sperm before and after motility activation.

Species	ATP	ADP	AMP	CrP	Conditions	References
Siberian sturgeon *Acipenser baerii*	7.6 nmol per 10^8 spermatozoa				B	Billard et al. (1999)
	4.0 nmol per 10^8 spermatozoa				A	
Turbot *Psetta maxima*	≈24 nmol per 10^8 spermatozoa	1.4 nmol per 10^8 spermatozoa	0.6 nmol per 10^8 spermatozoa		B	Dreanno et al. (1999b)
	≈6 nmol per 10^8 spermatozoa	8 nmol per 10^8 spermatozoa	6.0 nmol per 10^8 spermatozoa		A	
Turbot *Psetta maxima*	15.4 nmol per 10^8 spermatozoa	9.9 nmol per 10^8 spermatozoa		72.7 nmol per 10^8 spermatozoa	B	Dreanno et al. (2000)
Sterlet *Acipenser ruthenus*	5.7 nmol per 10^8 spermatozoa	1.3 nmol per 10^8 spermatozoa		0.7 nmol per 10^8 spermatozoa	B	Fedorov et al. (2015)
	4.5 nmol per 10^8 spermatozoa	1.7 nmol per 10^8 spermatozoa		0.15 nmol per 10^8 spermatozoa	A	
Sea bass *Dicentrarchus labrax*	11.49 nmol per 10^8 spermatozoa	1.08 nmol per 10^8 spermatozoa	0.57 nmol per 10^8 spermatozoa		B	Dreanno et al. (1999c)
	3.04 nmol per 10^8 spermatozoa	4.03 nmol per 10^8 spermatozoa	8.03 nmol per 10^8 spermatozoa		A	
Common carp *Cyprinus carpio*	1.29 ± 0.09 nmol/1 µl dry semen	0.058 ± 0.019 nmol/1 µl dry semen	0.024 ± 0.012 nmol/1 µl dry semen	9.64 ± 0.72 nmol/1 µl dry semen	B	Zietara et al. (2009)
African catfish *Clarias gariepinus*	0.64 ± 0.09 nmol/1 µl dry semen	0.052 ± 0.012 nmol/1 µl dry semen	0.020 ± 0.005 nmol/1 µl dry semen	8.45 ± 0.49 nmol/1 µl dry semen	B	
Gilthead seabream *Sparus aurata*	16.28 ± 1.35 µmole per ml			26.94 ± 3.28 µmole per ml	B	Lahnsteiner and Caberlotto, (2012)
	20.46 ± 3.77 µmole per ml			32.14 ± 2.21 µmole per ml	A (40% motile cells)	

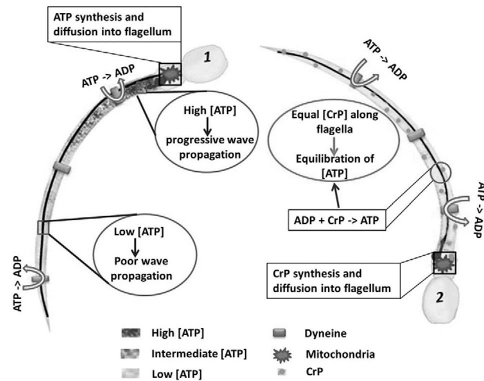

Fig. 1. Predictive schematic representation of macroergic phosphates conversion in relation to spermatozoon structure. Site of ATP synthesis is mid-piece, and flagellum is site of chemical energy transport via diffusion of CrP (so called "creatine–phosphate shuttle"), and ATP consumption by dynein ATPase. ATP also could be regenerated from ADP in axoneme by adenylate kinase reaction (not shown in the figure). Drawn by analogy with the results of Tombes et al. (1987) on sea urchin sperm and Kaldis et al. (1997); for details, see Cosson (2013). 1 = situation with non-operating CrP shuttle, 2 = with operating shuttle.

different metabolic strategies present in sperm cells (Cosson, 2013; Ingermann, 2008). The rapid decrease in ATP concentration during the motility period was described in many fish species such as carp, trout, turbot, sea bass, catfish, sturgeons (Cosson, 2010, 2013). But it is remarkable that ATP level remains significantly high at the cessation of motility. An estimation of the intracellular concentration leads to values ranging in mM that are sufficient to sustain flagellar dynein ATPase at a high activity level (Cosson, 2013).

Recent studies on perch spermatozoa showed that total motility duration and, thus, total sperm ATP content consumption could be prolonged by a stepwise decrease in osmolality of the swimming medium (Boryshpolets et al., 2009c). This observation suggests the existence of sperm sub-populations differing in sensitivity to the level of hypotonicity required for their motility activation. As was shown by Billard et al. (1999), and recently reinvestigated by Fedorov et al. (2015), the total ATP content in sturgeon sperm drastically decreases shortly after activation (within 10 s) and remains unchanged until motility is arrested, while a decline of flagella beat frequency and motility percentage occurs at later motility phases (starting from 30 s post-activation) and permanently decrease with motility period. In contrast to sturgeons, recent results of Butts et al. (2010) on relationship between ATP content and motility parameters in Atlantic cod *Gadus morhua* clearly show the parallel decrease of spermatozoa velocity, but not motility percentage during 2 min motility period. These results suggest that the regeneration of ATP must be activated immediately after spermatozoa motility activation. Recently, the presence of CK and AK systems and their essential role in ATP regeneration were confirmed for spermatozoa of sterlet *Acipenser ruthenus* which has a relatively long motility duration (2–6 min), as well as common carp *Cyprinus carpio* with a short motility period (1–2 min) (Dzyuba et al., 2016). This study indicated that spermatozoa of these two taxonomically distant fish species with different structure, mode of motility activation (ionic in sturgeon and osmotic in carp) and motility duration, possess similar systems for energy supply of flagella motility. It is worth noting that for sturgeon spermatozoa, the observation of such a CrP/CK phosphagen system and establishment of its essential role in regeneration of ATP level is innovation.

The efficacy of the ATP regeneration systems differs in the investigated fish species. AK and CK activities provide demembranated carp spermatozoa with sufficient energy to allow flagellar beat frequency to reach essentially the same level as in the untreated spermatozoa. In contrast, functioning of the AK and CK systems in demembranated sterlet spermatozoa was suggested as less effective in energy maintenance for motility, although observed beat frequencies were similar to those found in native, or untreated, spermatozoa (Dzyuba et al., 2016). Nevertheless, recent investigations have also revealed that in rare cases, the ATP dynamics during the motility period was absent (Lahnsteiner and Caberlotto, 2012).

A large variety of energetic metabolites need a more detailed description of their implication for better understanding of metabolic processes in fish spermatozoa (Ingermann, 2008). In this respect, the information about topology of the energy distribution from the place of its synthesis (mitochondria) to the place of its consumption (axoneme) remains to be elucidated (Fig. 1).

2.3. Fast intracellular ATP consumption is associated with low respiration rate

It is known that mitochondria in fish spermatozoa consume oxygen that constitutes basal metabolism connected to an ATP production. The rate of sperm respiration depends on pH and carbon dioxide (Ingermann et al., 2002; Inoda et al., 1988). Probably, sustaining a steady state of ATP level in quiescent spermatozoa is the main function of oxidative phosphorylation. The tricarboxylic acid cycle and oxidative phosphorylation are the key metabolic pathways that sustain basal metabolism in spermatozoa (Lahnsteiner et al., 1993, 1999; Mansour et al., 2003; Zietara et al., 2004). For instance, quiescent state and motility period of rainbow trout *Oncorhynchus mykiss* spermatozoa strongly depend on oxidative phosphorylation and on tricarboxylic acid cycle, and subsequently on aerobic conditions (Lahnsteiner et al., 1999). It is also worth noting that lipid metabolism is very important because of positive correlation between intracellular lipid levels and successful fertilization in rainbow trout (Lahnsteiner et al., 1998). In relation to glycogen, importance of its storage in fish spermatozoa has not been sufficiently described. Sperm motility and viability of quiescent spermatozoa can be stimulated by pyruvate and coenzyme A, but the character of stimulation seems to be species specific for Danube bleak *Chalcalburnus chalcoides* and rainbow trout. Pyruvate is generally formed during glycolysis and during catabolism of the amino acids alanine, serine, cysteine, and threonine, and enters the citric acid cycle after oxidative decarboxylation (Rawn, 1983). Coenzyme A is a pantothenic acid serving as an acyl-group carrier during oxidative decarboxylation of pyruvate and during oxidation of fatty acids (Rawn, 1983). Pyruvate and coenzyme A occurred in limited concentrations in spermatozoa because of their stimulating effect. There is a slight effect on sperm motility and viability of the inhibitor of glycolysis 2-deoxy-*D*-glucose and several indications that glycolysis occurred in spermatozoa of Danube bleak and rainbow trout. There are significant decreases of glucose levels during motility (Lahnsteiner et al., 1992, 1993) and during immotile storage of spermatozoa (Lahnsteiner et al., 1993, 1997), whereas lactate levels increase when inhibiting the respiratory activity. Also, an increase of lactate levels was observed in the cyprinid spermatozoa during anaerobic conditions (Belova, 1982). Spermatozoa highly depend on respiratory activity, probably because glycolysis is much more effective under aerobic conditions than under anaerobic conditions (Rawn, 1983).

As summarized by Ingermann (2008), fast intracellular ATP consumption during motility phase cannot be compensated rapidly enough by mitochondrial synthesis of ATP via respiration. Moreover, the possibility to increase sperm respiration rate by application of an "uncoupler" could indicate the presence of substrate stores available for mitochondrial ATP production. In addition to previously summarized knowledge on diversity of metabolic pathways associated with respiration in fish spermatozoa, recent suggestions have speculated that sperm oxidative phosphorylation plays a physiological role during the period which

precedes the motility phase of ejaculated spermatozoa. Results obtained on carp sperm (Boryshpolets et al., 2009a) experimentally support this speculation and show that respiratory rate of immotile spermatozoa can depend on physiological state, not related to motility itself. Additionally, it has been shown that motility activation provokes an increase of sperm respiration rate which reaches maximal level quite soon after activation and remains at this level until spermatozoa immotility. A recent study by Fedorov et al. (2015) on sterlet testicular spermatozoa clearly indicates that respiration is needed during a final process of sperm maturation occurring outside of testes (Dzyuba et al., 2014).

2.4. Relationship between energetics and reactive oxygen species

Various factors affect spermatozoa movement in the external environment; these include water pollutants, temperature, pH, and osmotic conditions. Numerous studies have shown that these factors induce reactive oxygen species (ROS) production and, consequently, oxidative stress in different cell types, including sperm. Our recent results indicate that exposure of sterlet spermatozoa *in vitro* to environmentally relevant concentrations of xenobiotics (biphenol A, vinclozolin, or duroquinone) leads to development of oxidative stress, reduced spermatozoa motility, and ATP content (Gazo et al., 2013; Hulak et al., 2013; Linhartova et al., 2013). One of the possible effects of increased ROS production in fish spermatozoa is ROS-mediated inhibition of one or more enzymes of oxidative phosphorylation and/or glycolysis, which limit the generation of ATP. It is well known that kinases and phosphatases, the two types of enzymes involved in the regulation of protein phosphorylation, are susceptible to redox-regulation (de Lamirande et al., 1997). Phosphatase activity in carp spermatozoa has been shown to decrease in the presence of ROS (Gazo et al., 2015). Therefore, it can be assumed that increased ROS production in spermatozoa alters intracellular signaling pathways.

In fish spermatozoa, as described in Section 2.2, numerous metabolic enzymes, including CK, were shown to participate in sperm motility activation and sustaining (Tombes and Shapiro, 1989). As shown by Zilli et al. (see in this volume), the role of protein phosphorylation in fish sperm motility appears more and more obvious. Recent data obtained by our group indicate that involvement of metabolic enzymes in fish sperm motility is regulated by protein phosphorylation/dephosphorylation (Gazo, 2015). Therefore, excessive production of ROS in spermatozoa could result in membrane and DNA damage, but also can affect regulation of energy production in spermatozoa. Thus, studies on CK in myofibrils showed that its activity could be inhibited by ROS, probably by the oxidation of its essential sulfhydryl groups (Mekhfi et al., 1996). Abundance of CK and enolase b in spermatozoa of sterlet suggests their importance in energy production pathways (Li et al., 2010c). Hence, suppression of CK activity by ROS could lead to decreased intracellular ATP content in spermatozoa exposed to xenobiotics and oxidative stress.

Support for the involvement of ROS in the mitochondrial ATP homeostasis in fish sperm comes from a study on a marine teleost, the gilthead seabream *Sparus aurata* (Chauvigné et al., 2015). This study revealed that upon activation of spermatozoa in hyperosmotic conditions, the teleost orthologue of aquaporin-8, termed Aqp8b, is rapidly phosphorylated and inserted into the inner mitochondrial membrane. Aquaporins (AQP) are integral membrane proteins from a larger family of major intrinsic proteins that form pores in the membrane of biological cells and facilitate water transport across the membrane. Chauvigné et al. (2015) showed that this AQP mediates H_2O_2 efflux from mitochondria and mitigates cellular oxidative stress. Thus, ROS signaling exhibits a complex relationship with protein phosphorylation, where redox-regulation is involved in modification of balance between kinases and phosphatases, and, *vice versa*, protein phosphorylation can regulate ROS production. The involvement of ROS and redox-regulation in conjunction with proteins phosphorylation pathway was documented in sperm of mammals (Ecroyd et al., 2003) and in fish during the osmotic adaptation process of tilapia spermatozoa (Morita et al., 2011). The latter study identified a 18 kDa protein as a superoxide–dismutase as a central regulator of the osmotic tolerance.

2.5. Capacity for fish spermatozoa to be revived

Several observations on various species demonstrate that previously activated fish spermatozoa can be activated for a second time after a certain period of rest, meaning that after the first round of motility, spermatozoa are still metabolically active. To restore near initial levels of available energy, spermatozoa are briefly placed in a non-swimming medium at 20 °C. A second activation of motility has been induced in spermatozoa of channel catfish *Ictalurus punctatus* (Guest et al., 1976) but recently has been more thoroughly illustrated in common carp (Linhart et al., 2008). In case of marine species, like turbot, spermatozoa can be revived for a second round by settling of these cells at 20 °C in an artificial seminal fluid with osmolality lower than in seawater (Cosson, 2004). After subsequent transfer into seawater, spermatozoa reinitiate motility and swim in a fashion similar to that observed during the first activation in seawater. A second round of activation can be accomplished only within a certain time period, during which cells are able to reload their ATP concentration to a “normal” level (Cosson, 2013; Dreanno et al., 1998). This reloading time interval allows both mitochondrial respiratory and ion pumping activities, the latter also being ATP-dependent.

During sperm movement, the mitochondria and vacuole volumes of the mid-piece undergo changes in morphology (Suquet et al., 1998), but such morphological alterations can be reversed by the reviving process. Ability for fertilization is also recovered during this second round of motility of turbot sperm (Cosson et al., 1999) as well as in the case of common carp (Linhart et al., 2008). It was found by Boryshpolets et al. (2009a) that during recovery, carp spermatozoa are in maximum respiration conditions, which probably determines ATP generation required for next motility phase. Further, the same principle of ATP recovery underlays the ionic implications to recover ability for motility activation in case of carp sperm samples of poor quality, as described by Redondo-Muller et al. (1991).

3. Electro-chemical *trans*-membrane potential as a source of energy for sperm activity

Flagella are mostly considered as motile cells but one should also see them as sensory organelles (Bloodgood, 2010). Historically (and still basically), flagella were probably a sensory organelle that evolved a motile function (Mitchell, 2004). It is currently recognized that flagellar membrane is sensitive to physical contact such as in case sea urchin flagellum (Kambara et al., 2011), this physical reaction being ion-connected through membrane potential and internal Ca^{2+} signaling as shown by use of various blockers (Gadolinium, Verapamyl) and emphasizing the role of flagellasialin.

Membrane potential mostly relates to an ionic balance across the sperm membrane but, one way or another, it is connected to osmotic potential, i.e. solute concentration difference between inside and outside a compartment which is separated by the membrane.

Therefore, osmosis should be seen as a physical force and thus, as a potential source of energy for a sperm cell. How can flagella be sensitive to osmotic pressure? Electro-chemical gradients essentially act as batteries that create voltage across the membrane. A crucial mean by which the energy in electro-chemical gradients is collected is either by ATP-synthase (using proton motive force, such as in mitochondria), or by transport proteins that pump other substances across membranes. The latter process, so-called co-transport, can use any excess of ions to drive what is also termed secondary active transport, since ATP is not directly driving the active transport of those other substances. Therefore, an electro-chemical gradient has a direct relationship with ATP stores, because any active transport of ions needs ATP. The ion pump most

relevant to the action potential is the Na^+-K^+ pump, which transports three sodium ions out of the cell in exchange for two potassium ions. There is increasing evidence for Na^+–K^+ ATPase of sperm cells having a crucial role (Gatti and Christen, 1985; Woo et al., 2000).

3.1. The connection with osmotic gradient: The Na^+–K^+ pump maintains the osmotic balance of sperm cells

Movement of water from a region of low solute concentration (high water concentration) to a region of high solute concentration across a membrane is called osmosis. The driving force for the water movement is equivalent to a difference in water pressure, called osmotic pressure. Any difference of physical pressure represents a potential source of energy for the cell. In the absence of any counteracting pressure, the osmotic movement of water into a cell will cause it to swell. Such effects can cause a severe problem for animal cells, which have no rigid external wall that prevents them from swelling. Placed in water, such cells will generally swell until they may burst. At any given moment, there are two factors for an ion that determine how much influence that ion will have over the membrane potential of a cell: the driving force is the net electrical force available to move that ion across the membrane, while the permeability is a measure of how easily an ion can cross the membrane. Both factors ultimately contribute to the osmotic force (or gradient). These general considerations are well summarized and accepted nowadays (Alberts et al., 2015). Therefore, it appears that both the electro-chemical gradient and the osmotic gradient have a crucial role in energy homeostasis of the sperm cell. The interaction between osmotic gradient and electro-chemical gradient was recently demonstrated in sturgeon sperm by Prokopchuk et al. (2016). In that study, it was shown that even short-term pre-incubation in hypertonic solutions prepares sturgeon spermatozoa to become fully motile even in the presence of high concentration of K^+ ions, which is inhibiting motility before the treatment (Linhart et al., 2002).

Regarding sperm cell homeostasis, the movement of materials against a gradient, visualized as "pumping" a substance from a region of low concentration to a region of even higher concentration, comes at an energetic cost. Conversely, movement of materials along a gradient (from a region of high concentration to a region of low concentration) is energetically favored.

3.2. Sensors and controllers as cell volume regulatory networks

How do fish sperm use stored energy in the *trans*-membrane gradients? It is crucial for cell function that osmolality, membrane tension, and hydrostatic pressure are tightly controlled (Zonia and Munnik, 2007). Thus, early in evolution, cells were confronted with the problem of how to control their volume and regulate the flow of water across the membrane. Solutions to this problem must have arisen in the earliest protocells which formed the basis of volume regulation during the development of cellular complexity (Morris, 2002).

Many cell functions have been attributed to ion channels (Darszon et al., 2006) and more specifically to mechano-sensitive ion channels. It has been shown in bacteria that milli-osmolar changes in water concentration are sufficient to shift the osmotic pressure across the plasma membrane and generate stretch and compression forces along the plane of the lipid bilayer (Kung, 2005). These forces are gating mechano-sensitive ion (Chaumont et al., 2005). It has been proposed that AQPs (Martinac, 2004) might function as osmosensors and volume regulators in cells of animals, plants, fungi, and bacteria (Hill et al., 2004). In fish sperm, several molecular species of AQPs have been characterized in the recent past (Chauvigné et al., 2015).

3.3. Activation of ion fluxes during cell volume perturbation and recovery

The volume of sperm cells change in solutions of different osmolalities and also vary differently during motility period among fish species having either osmotic (carp) or ionic (sterlet and brook trout *Salvelinus fontinalis*) modes of motility activation (Bondarenko et al., 2013). It was demonstrated that an increase of carp sperm volume occurs during motility period in hypotonic condition. In contrast, no indication of sperm volume change was observed in sterlet and brook trout spermatozoa in relation to environment osmolality modifications occurring during motility period (Bondarenko et al., 2013).

In some freshwater fish species, hypo-osmotic induction of sperm motility is accompanied by a reorganization of the membrane structure and by hyperpolarization of the cell membrane (Krasznai et al., 2003). It is predicted that sperm swelling at the moment of activation could result from water transport through AQPs (see Zilli et al., this volume) because water diffusion, without intervention of AQPs, is a much slower process (Chen and Duan, 2011). Thus, activation of stretch-dependent channels could constitute a second step in motility activation of carp spermatozoa that would follow the water transport step through AQPs (see chapter by Zilli et al., this volume).

In anadromous fish species (migrators from marine- to freshwater for spawning) such as salmonids and acipencerids, spermatozoa motility possesses ionic mode of activation (Alavi and Cosson, 2006). As previously discussed, spermatozoa of some species change in volume during their motility period (Bondarenko et al., 2013). Thus, activation of stretch-dependent channels seems to be impossible because of the absence of detected stretch during motility activation and whole period of motility. How sperm can maintain a constant volume under hypotonic conditions remains unclear. It was previously described that AQPs are involved in motility of sea bream and trout sperm (Zilli et al., 2011; Takei et al., 2015). However, it is still unknown if AQPs transport water according to or against the concentration gradient. Additionally, water influx, as a result of osmosis, could be partially overcome by activity of co-transporters (Chen and Duan, 2011).

Euryhaline fishes like tilapia (fish that can acclimate to wide range of salinities, from freshwater to seawater or even higher) possess unique sperm osmotic sensitivity. It was shown that tilapia *Sarotherodon melanotheron heudelotii* sperm could modulate their regulatory mechanism to suit fertilization in either low- or high- or even hyper-salinity (Legendre et al., 2008). Seawater acclimated tilapia sperm requires Ca^{2+} for motility activation in hypertonic media (seawater), whereas in freshwater acclimated tilapia, sperm do not swim in hypertonic conditions even in the presence of Ca^{2+} (Morita et al., 2003). Linhart et al. (1999) reported that during acclimation of tilapia from fresh- to seawater, their sperm adapt to conditions of high salinity by losing their Ca^{2+}-independent pathways of motility activation.

3.4. Activation of signaling cascades

Hypo-osmosis and cell swelling induce increases in phosphatidic acid in unicellular green algae and erythrocytes. Hyperosmosis and cell shrinkage induce increases in phosphatidylinositol-bis-phosphates in animal and plant cells. Osmotic stress also activates mitogen-activated protein kinase cascades and induces gene expression. Cell volume changes impact cytoskeletal organization, and Rho GTPases are a major point of convergence to integrate membrane signals and cytoskeletal organization. In animal cells, there is increasing evidence that Rho GTPases are regulated by cell volume and intracellular ionic strength (Di Ciano-Oliveira et al., 2006). The Rho family of GTPases is a family of small (~21 kDa) signaling G-proteins and is a subfamily of the Ras superfamily. All G-proteins act as "molecular switches," and Rho proteins play a role in organelle development, cytoskeletal dynamics, cell movement, and other common cellular functions.

This family of proteins deserves interest in case of fish spermatozoa: when the latter are submitted to osmotic stress, flagellar morphology is strongly affected (Perchec et al., 1996; Perchec-Poupard et al., 1997).

Also, in fish spermatozoa, bleb formation on membranes represents a serious handicap for flagellar motility when it occurs during the motility period as a response to osmotic stress, even though it is reversible

process (Cosson, 2010; Dreanno et al., 1998). In other cell types, blebs form to develop extensions of the cell as a physiological situation (Langridge and Kay, 2006). This may be the case in fish sperm that need to expand their total cell surface so as to increase their intracellular volume.

In case of sperm cell membranes, it is important to emphasize the role of some membrane proteins such as transient receptor potential channels (TRPs) (Kung, 2005) and AQPs (Martinac, 2004). TRPs are members of an ancient class of ion channels (Darszon et al., 2006) that are present in most animal tissues. Consistent with their wide tissue distribution, TRPs are capable of influencing diverse physiological processes including adipocyte function, energy intake, and energy expenditure (Kung, 2005). TRPs function as transduction channels downstream of G-protein-coupled receptors and receptor tyrosine kinases, and some can also be direct sensors of chemical irritants that influence food intake or regulate body temperature and thermogenesis. It is worth mentioning that, recently, some thermo-sensitive ion channels were detected in sperm and are involved in the sperm motility of a cyprinid (Majhi et al., 2013).

All these examples represent numerous new avenues to be explored so as to better understand global energy homeostasis that fish sperm cells must afford when confronted with osmotic/ionic gradients. In any case, the ability of membrane regionalization should be better documented due to the possibility this offers to modulate a signal received by the sperm cell (Toowicharanon and Shapiro, 1988) in connection with the sub-compartments that are present in cilia and flagella (Lee and Chung, 2015).

4. Considerations on physical energy needed by moving fish flagella

The behavior of the flagellum determines the motility (guideline) of the spermatozoon. Any sperm swimming in a fluid needs both to overcome the liquid viscosity as well as resistance to wave displacement: those two processes require energy (Cosson and Prokopchuk, 2014). Fluid mechanics as applied to the nano-scale universe of flagella offers the estimation of the energy requirements for flagellar parameters by application of the resistive force theory initially developed by Gray and Hancock (1955). Several flagellar parameters can be quantified, such as wave amplitude or wave length.

4.1. Parameters of beating flagella

In order to observe the detailed pattern of live flagellar motion or of their major components, it has been proposed to use phase-contrast or dark-field optical microscopy with high magnification (40×–100×) objective lenses, which, if applied with oil immersion, results in a bright image of the very small flagellum. To achieve complementary assessment, additional methods, such as stroboscopic illumination or high-speed video techniques, allow the recording of sperm during its motion and especially the flagellar images of high quality and resolution. Multi-flash stroboscopic illumination allows visualization of each frame of well-defined, successive positions of a single moving spermatozoon at time intervals of millisecond (Cosson, 2008a). Alternatively, high-speed video recording provides higher spatial and temporal resolution (up to several thousand images per second). Serial frames, individually selected from such video records, permit following successive positions (every millisecond or so) of flagellum waves covering one or several full beat cycles (Cosson et al., 1997).

During brief swimming periods of fish spermatozoa, their flagellar motility characteristics change in many respects which contribute to changes in forward velocity. Such behavior is interrelated with flagellar energy consumption; its bending results from active sliding between axonemal microtubule force exerted during ATP hydrolysis by dynein (Brokaw, 1989; Gibbons and Rowe, 1965; Lindemann and Lesich, 2010; Satir, 1968; Shingyoji et al., 1977; Summers and Gibbons, 1971). To characterize all aspects of moving flagella and their variations during the time period after motility activation, parameters are quantified from a series of video images, such as waves beat frequency, wave length, wave amplitude, wave velocity, curvatures of waves, bend angle, and number of waves developed along the length of the flagellum, etc. (Cosson et al., 1999; Cosson, 2015). It has been well documented that depletion of intracellular ATP content during the motility phase contributes to a decrease in flagellum beat frequency and subsequently sperm velocity and thus fertilizing ability (Denehy, 1975; Dreanno et al., 1999a, 1999b; Gibbons, 1981; Lahnsteiner et al., 1998; Perchec et al., 1995; Zilli et al., 2004). Initial motility period of fish sperm is characterized by the high beat frequency up to 70–100 Hz, which corresponds to a high swimming velocity (Cosson, 2008b) and a consequentially rapid exhaustion of the ATP stores. An ATP consumption rate that is higher than the synthesis rate is considered as the limiting factor for sustaining sperm motility (Dreanno et al., 1999b; Perchec et al., 1995). Nevertheless, it has been shown that the initial beat frequency is not well related to motility duration, probably because sperm velocity represents a global combination of several variables in addition to beat frequency but also wavelength or wave amplitude, which contribute differentially to energetic exhaustion (Cosson, 2010). As a complement, by delaying motility activation, our recent measurements of beat frequency in sturgeon spermatozoa showed that right after motility triggering, sperm flagella beat at a low frequency but rises very rapidly, so that after a few seconds, the value is two-fold higher (Prokopchuk et al., 2015). We also suggested that during sperm motility initiation, hydrolysis of ATP within the cell occurs and a shivering stage when spermatozoa disruptively tremble with only low amplitudes corresponding to the early period of microtubule sliding (more details in Prokopchuk et al., 2015). However, further studies on correlation between ATP consumption and flagella beat pattern at the sperm motility initiation in different fish species are required.

4.2. Energy needed by flagellum for swimming in a fluid and efficiency of movement

Simple considerations of fluid mechanics for sperm flagella are introduced below. When moving through a fluid, a fish sperm is submitted to two types of force that result from fluid pressure: inertial and viscous forces (Holwill, 1977). The relative values of the two forces is given by R_e, the Reynolds Number:

$$R_e = lv\rho/\eta = \text{inertial force/viscous force.}$$

$$v = \text{velocity}, l = \text{length of the flagellum}, \rho = \text{density, and } \eta = \text{viscosity.}$$

In case of flagella, inertial force is very low while viscous force is predominant, therefore R_e value ranges from 10^{-3} to 10^{-4}. According to Holwill (1977), an approximate value of R_e can also be obtained by the product of f (the beat frequency) by a (the wave amplitude).

Specialists of fluid mechanics have attempted to predict sperm forward velocity by using physical parameters of the flagellum. For example, Rikmenspoel (1966) provided a quite empirical relationship:

$$v = \varepsilon b^2,$$

wherein v is velocity, b is the wave amplitude, coefficient ε is equal to $1.2\ \mu^{-1}\ s^{-1}$.

The quantified flagellar parameters, described in Fig. 1, can be incorporated in a more elaborated formula that results from the

resistive force theory of Gray and Hancock (1955), already abovementioned:

$$v_{calc} = 2f\pi^2b^2/\lambda\left\{1/1+4\pi^2b^2/\lambda^2-\left(1+2\pi^2b^2/\lambda^2\right)^{1/2}3a/n\lambda[(lnd/2\lambda)+1]\right\} \quad (1)$$

v_{calc} is the calculated velocity of propulsion of the spermatozoon, b is the amplitude of wave, λ is the length of wave, n is the number of simultaneous waves, f is the beat frequency of waves, a is the radius of head, and d is the radius of flagellum.

One advantage of the above considerations is that one can compare the value of predicted velocity with the actual value experimentally obtained v_{exp}. For turbot spermatozoa, when velocity is measured at steady state (see below) and above flagella parameters evaluated in both cases very early in the motility period (6 s post-activation), v_{calc} ranges value of 231 μm/s, a value which, as compared to that of v_{exp} (220 μm/s), is in good agreement between experiment and theory (unpublished results). Ishijima (2012b) studied sperm of 35 different marine fish species, where the same flagella parameters as used in Eq. (1), were evaluated; Cosson more recently enlarged this list of species (unpublished, see Fig. 2). Several important conclusions can be drawn and these are briefly summarized:

— the swimming velocity (v_{exp}) is linearly proportional to f, the flagellar beat frequency;
— the smaller the flagellum length, the higher the swimming velocity;
— the number of waves along the flagellum varies linearly with the flagellum length;
— the power output of the flagellum varies in proportion with the flagellar beat frequency (see below);
— the swimming efficiency increases in proportion with the swimming speed.

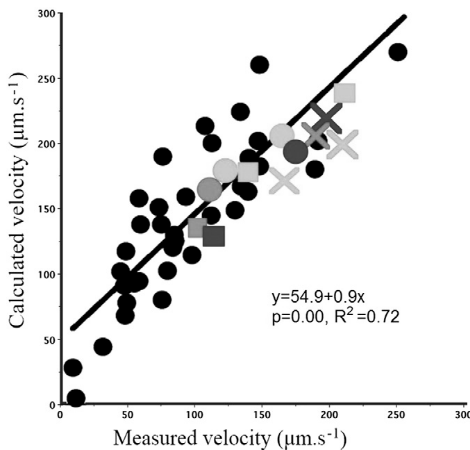

Fig. 2. Measured velocity and velocity values calculated from Eq. 1. Blue cross = turbot (*Scophthalmus maximus*); red square = sea bass (*Dicentrarchus labrax*); light blue cross = sturgeon (sterlet, *Acipenser ruthenus*); red cross = paddlefish (*Polyodon spathula*); green cross = tuna (*Thunnus thynnus*); blue square = cod (*Gadus morhua*); light blue square = hake (*Merluccius merluccius*); light blue circle = halibut (*Hippoglossus hippoglossus*); red circle = perch (*Perca fluviatilis*); blue circle = pike (*Esox lucius*); green circle = carp (*Cyprinus carpio*); black circles = data from Ishijima (2012a). Eq. .(1): $v_{calc}=2f\pi2b2/\lambda\{1/1+4\pi2b2/\lambda2-(1+2\pi2b2/\lambda2)1/23a/n\lambda[(lnd/2\lambda)+1]\}$ (see text for details).

As seen in Fig. 2, v_{exp} and v_{calc} are compared for a single spermatozoon at each successive stage following initiation of the first flagellar wave (upper panel). Values of velocity, v_{exp}, are measured from head displacement between two successive positions, while a v_{calc} value at each position is derived from Eq. (1) above.

Some correlations were also established experimentally such as those published by Rikmenspoel (1966):

— the wave amplitude and the swimming velocity are linearly related;
— the swimming velocity is minimally dependent of the medium viscosity, even though the latter greatly affects the wave shape;
— waves of helical shape generate rotation of the sperm cell at a frequency that is in proportion with the flagellar beat frequency.

In a way similar to that leading to calculation of the velocity (v_{calc}) from values of flagellar parameters, it is possible to evaluate the value of power (P) needed to sustain steady-state motility by Eq. (2) as proposed by Holwill (1977) and Rikmenspoel (1966). It is remarkable that the Eq. (2) for evaluation of P shows proportionality to the square of the frequency value, f^2, as well as proportionality to the square of the wave amplitude value, b^2.

$$P = 4\pi^3\mu f^2b^2L/\{0.62-\ln(2\pi d/\lambda)\} \quad (2)$$

Interestingly, the power value changes dramatically when flagella switch from inactive to active. This was observed and video-recorded at wave initiation (Cosson, unpublished), when turbot sperm initially were exposed to motility arrest by CO_2 application: upon cessation of CO_2 application, motility is reinitiated within a fraction of a second (Inaba et al., 2003). In such a situation, the values of flagellar parameters at successive time points were used to evaluate the corresponding power developed by the flagellum (see Fig. 3).

The notion of swimming efficiency and its formula (Rikmenspoel, 1966) involves the values of the same parameters, as already mentioned above.

$$\eta^{-1} = P/[\{6\pi\mu a+2\pi\mu L/2L/d\}v^2{}_{exp}] \quad (3)$$

It should be noted that swimming efficiency differs from propulsive efficiency, the latter expresses the distance covered by the sperm cell during each beat cycle (μm per beat). As determined by Ishijima (2012a), values of η^{-1}, the swimming efficiency, range 20–80 (arbitrary units) for the series of fish sperm investigated in his study. In case of turbot sperm, values of η^{-1} range 25–100, while in sea bass, the range is 35–75 (unpublished results). Altogether, this set of results shows that the swimming efficiency increases with the swimming velocity.

Another way to evaluate flagellar efficiency is to compare sperm velocity relatively to the total cell length (sum of flagellum plus head lengths). This is illustrated in Fig. 4 and shows considerable heterogeneity among fish species. Specifically in fish spermatozoa, this species specificity could result from an inter-dependency between the total chemical energy (stored and produced) relative to the energy needed to generate flagellar waves, which are governed by physical laws.

A simple energetic estimate can be obtained from an entirely different starting point. The axoneme can be considered as a classical rigid beam and the amount of energy required to bend this axoneme into a roughly sinusoidal shape estimated. Each beat consumes 2.3×10^5 ATP which corresponds to 1.84×10^{-14} J of energy considering free energy for each ATP hydrolyzed (Howard, 2001). Axonemal bending stiffness of 16.6×10^{-21} Nm^2 was estimated for demembranated *Lytechinus pictus* (sea urchin) axonemes in the absence of ATP but this stifness value decreases by an order of magnitude in presence of 10 mM ATP (Okuno and Hiramoto, 1979). A similar approach as above (in sea urchin flagella) will be interesting to develop in case of fish spermatozoa of various species.

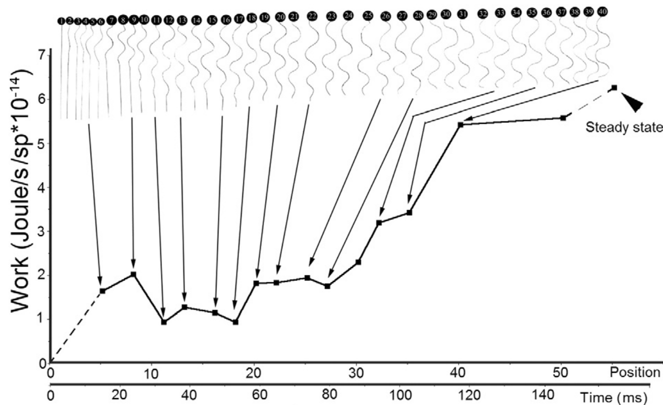

Fig. 3. Evaluation of work developed by turbot sperm while initiating waves after CO_2 arrest. Each position (1, 2, 3, etc.) is labeled on the head, and an arrow indicates correspondence with the time point where work value was evaluated, such as 5 s, 8 s, 12 s, 14 s, 17 s, etc.

These series of preliminary results show that a similar approach can be applied to sperm of various fish species so as to lead to an evaluation of the swimming efficiency as well as the power developed by sperm flagellum during the period of motility or at motility initiation.

4.3. Waves shape of fish sperm flagella evolves during the motility period

Efficient forward movement of spermatozoa relies on the ability of flagella to generate waves; these initiate at the head–tail junction and propagate toward the tip of the flagellum. In a recent series of investigations dealing with sperm activation of various fish species, we established that in the large majority of cases where sperm motility initiation was described, the first bend occurs in a proximal region of the flagellum at the head–tail junction (Prokopchuk et al., 2015 and unpublished data). Subsequently, this first bend progresses toward the

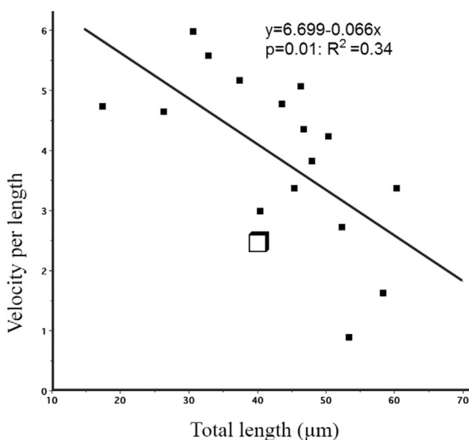

Fig. 4. Velocity per length (the ratio between velocity and sperm length) is plotted versus the total sperm length (head + tail) for a large range of fish species (see names of species in legend of Fig. 2) for which data are available (black squares). For comparison, pooled data from the Ishijima (2012a) are included as the large open square.

flagellar tip and is immediately followed by the appearance of other curvatures. When most of the flagellar length is covered by waves, the progression of the sperm cell can occur, resulting in forward motion. We also estimated that the total time needed to switch from resting to fully efficient forward motility, ranges 0.4–1.2 s in all freshwater species investigated (Prokopchuk et al., 2015).

After initiation, during the steady-state cruising motion, fish sperm flagella generally describe a pseudo-sine wave shape composed of linear segments interposed between two curvatures (Brokaw, 1991). This original flagella waveform can be modified by several factors, for instance, energetic content (ATP) affects amplitude of waves, but also internal ionic concentration, namely calcium (Ca^{2+}), influences symmetry and constancy of the wave amplitude along the flagella (Alavi et al., 2009; Inaba, 2015; Wargo et al., 2004).

When intracellular or in case of *Acipenser ruthenus* (Alavi et al., 2008), *Perca fluviatilis* (Alavi et al., 2007) or *Esox lucius* (Alavi et al., 2009) extracellular Ca^{2+} level transiently increases, the flagellar beat alternates the bending shape, from symmetrical to asymmetrical, where two successive bends are of different amplitude; thus the spermatozoa move in circular tracks (Guerrero et al., 2010; Kaupp et al., 2008; Shiba et al., 2008). Such a switching pattern of the sperm cells suggests that this might be a mechanism by which their swimming direction is responsponding to chemotaxis to specific molecules released by eggs (see paragraph on chemotaxis in chapter 5).

Another example of wave shape variability controlled by Ca^{2+} ion concentration refers to their flatness. It is assumed that flagellar waves are almost planar, but, in reality, the flatness of waves is not perfect, so waves slightly deviate out of a single plane. This was shown in sperm flagella of several fish species where a slight distortion was related to the ability of sperm cells to remain swimming in the vicinity of any surface (Cosson et al., 2003). Apart from it, sperm flagella can also develop helical waves (Ishijima, 2012b), which were described in European eel spermatozoa possessing of corkscrew shape (Gibbons et al., 1985; Woolley, 1998). Not strictly planar waves also appear, consequently spermatozoa may roll as they swim, described in turbot (Dreanno et al., 1999a, 1999b) or sturgeon species (Billard et al., 2000; Cosson et al., 1997; Prokopchuk et al., 2015). However, according to Gray (1953), this helical propulsion reduces the efficiency of forward velocity, this is why swimming in 2D is more advantageous.

Right after activation and during the first phase of motility of fish spermatozoa, waves are propagated along the whole length of the flagellum, but later the wave pattern rapidly evolves: bends become restricted to

that portion of the flagellum close to the head and much later, only to a very restricted portion of the flagellum in the vicinity of the head (Cosson et al., 1997, 1999). The cessation of movement of the distal region prior to total arrest of flagellar movement seems to be a general feature for fish species studied so far (Cosson et al., 2008ab; Cosson, 2010; Fauvel et al., 2010). It is obvious that this dampening effect results in a rapid drop in the swimming efficiency. This process has been hypothetically attributed to an uneven energy distribution in the flagellum at the end of the motile period: low oxidative phosphorylation relative to the rapid consumption of ATP would lead to a lack of ATP in the distal portion (Chauvaud et al., 1995; Cosson, 2013; see paragraph on energy shuttle in chapter 2). A complementary and non-exclusive explanation could be the uneven distribution of the dynein ATPase sub-species (Piperno and Ramanis, 1991; Yagi et al., 2009) whose affinity to energetic nucleotides may be different.

In another example, damping of waves and increasing of curvature number was observed after sperm exposure to a drastic environmental osmotic conditions encountered when fish sperm in the seminal fluid is abruptly changed upon entry into the external aquatic environment (Cosson et al., 1999; Hulak et al., 2008). Alteration of extracellular osmolality perceived by spermatozoa causes the rapid changes in intracellular ionic concentration and, as a consequence, the development of waves by flagellar axonemes first slow, then fully stop, largely due to curling of the flagellar tip (Perchec et al., 1996). The wave shape of demembranated flagella (axonemes) is also modulated by ion concentration as demonstrated in turbot (Cosson, 2008b; Dreanno et al., 1999a); this ionic strength effect is reminiscent of the conditions that occur *in vivo* in the membranated sperm during the motility period, where external osmolality is responsible for internal ionic concentration.

Wave shape of fish spermatozoa also partly results from the physical constraints imposed by the external milieu. One such constraint is viscosity, which affects sperm cells in the egg proximity, i.e. ovarian fluid or jelly-like layers surrounding eggs of some fish species. It has been shown that an increase of viscosity results in an increase in the number of waves and, at the same time, reduces the bending velocity and wavelength and hence of sperm velocity (Brokaw, 1977; Cosson, 2008b). In fish spermatozoa with a ribbon-shaped flagella, such as sturgeon sperm (Gillies et al., 2013; Psenicka et al., 2007, 2011), the viscosity effects are increased due to a greater increase in the surface of viscous interaction with the surrounding medium (Cosson et al., 1999). When viscosity effect becomes that of a nearby surface, it has been shown that a rigid surface such as a glass slide differentially influences the sperm velocity as compared to a more flexible surface such as that of an air/water interface (Boryshpolets et al., 2013).

Altogether, energy developed by fish flagella to actuate sperm cell forwardly is highly dependent on physical conditions imposed by the surrounding fluid.

4.4. Toward a connection between CASA and flagellar parameters

As already stated, the behavior of the flagellum determines the motility guideline of the spermatozoon that is reflected by CASA analysis. Characteristics dealing with velocity, linearity, *et cetera*, on the one hand and amplitude of flagellar wave, wave length, etc., on the other hand lead to two complementary sets of data that can be grouped so as to obtain a better global interpretation of the quality and performances of a sperm sample. This is the goal developed in a recent publication by Duffy et al. (2013) where authors propose a “glyph” analysis generating in a same schematic image of each individual spermatozoon where are included both CASA results and flagellar parameters. Application of such approach in case of fish spermatozoa is promising.

5. Chemotaxis: Optimization of energy consumption by sperm?

In fish species so far investigated, there are very few observations of spermatozoa attraction by egg (Cosson, 1990; Hart, 1990). Earliest studies are probably those on bitterlings *Rhodeus ocellatus* and fathead minnows *Pimephales promelas* in several papers published by Suzuki (1958), and next papers, but the issue of chemo-attraction was not directly addressed for these species. Sperm guidance to the micropyle area of the egg in freshwater fishes was observed in a cyprinid fish, the rosy barb *Pethia conchonius* (Amanze and Iyengar, 1990), but this study did not provide any clear indication of chemical guidance.

Among fish species, a clear demonstration of chemotaxis was reported for the pacific herring, *Clupea pallasi*, a seawater fish species (Cherr et al., 2008; Yanagimachi and Kanoh, 1953; Yanagimachi et al., 1992; Yoshida et al., 1999). Several egg substances were included in this process, among which the herring sperm activating peptides (HSAPs) that are rapidly dissipated from the eggs at spawning and the sperm motility initiating factor (SMIF), a 105-kDa glycoprotein that was localized in the micropylar region of the herring egg. It was shown that SMIF induces calcium influx, sodium efflux, and a membrane depolarization in herring sperm. Motility initiation by SMIF depended on decreased extracellular sodium (<350 mM): it is remarkable that activation could be induced in the absence of SMIF in very low sodium seawater (Vines et al., 2002). The latter demonstrates the importance of *trans*-membrane ionic potential in the control of fish sperm motility (Cosson, 2004).

In salmonids, sperm motility is activated at spawning by contact with the surrounding freshwater but briefly (25–35 s): in the vicinity of egg, sperm swim along the chorion surface (*in situ* or isolated from the egg itself) and many spermatozoa enter the micropylar opening (Yanagimachi et al., 1992). Absence of Ca^{2+} prevented the attraction behavior but the latter did not occur if eggs were mildly treated by trypsin, again leading to suspect a protein nature of the attractant. More recently, by using a new chemo-attraction assay, Yeates et al. (2013) demonstrated the participation of ovarian fluid, a jelly surrounding eggs when they are shed, to the sperm attraction process in salmonids and its importance in situation of sperm competition. Modulation of sperm motility parameters was shown recently to be highly influenced and dependent on inter-population variation of ovarian fluid (Beirao et al., 2015). Fast-evolving reproductive proteins, especially those hypothetically present at sperm surface, are likely candidates to allow species-specific signaling between ovarian fluid and sperm, switching the behavior of conspecific sperm via changes in ion channels that modify flagellar beat and therefore swimming direction toward the “right” egg (Vacquier, 1995).

Yanagimachi et al. (2013) recently described the phenomenology of sperm attraction to micropyle in several fish species, such as black flounder, barfin flounder or herring. Tracking the sperm cells on video records clearly illustrates how individual spermatozoa orient the direction of their swimming path toward the egg micropyle.

The abovementioned information indicates that in terms of energy economy of fish spermatozoa, the location of an egg by sperm cells would represent a way to prevent superfluous dispersal of male gametes. In contrast, chemo-attraction would facilitate gathering sperm in the vicinity of an egg cell. This probably could be a reason that chemotactic guidance mechanisms would have developed during evolution so as to minimize energy expended to reach the egg. However, bioenergetics aspects of sperm chemotaxis must be further studied.

6. Fish sperm bioenergetics in relation to applied studies

As fish sperm motility is requisite for fertilization, it is generally accepted that study of sperm bioenergetics is required for understanding the causes of loss in sperm quality, occurring under various conditions (Ingermann, 2008). Among others, these factors are sperm *in vitro* storage (both in liquid state and during cryopreservation procedure), condition of fish brood stock holding or reproductive seasonality, sperm collection techniques, and toxicological studies. These needed studies could be considered as applied for artificial fish reproduction for elaboration of media for prolonged sperm storage in unfrozen state or sperm cryopreservation improvement.

It has been shown in several studies that content of macroergic phosphates in spermatozoa decreases together with sperm motility parameters to the end of spawning period, or within *in vitro* sperm storage under hypothermic conditions (for review, see Fauvel et al., 2010) or under influence of different contaminants (Hatef et al., 2013). That leads to suppose that the content of macroergic phosphates constitutes a potent indicator of fish sperm quality. However, that speculation give a little chance to use this parameter for the practical purposes until the species-specific metabolic pathways are described in term of metabolite content (for review see discussion by Suquet et al., 1998). However, in case efforts toward the understanding of the processes are made, the results became really applied. As was shown by Zietara et al. (2009), the study of macroergic phosphates content during sperm incubation with different metabolites (glucose, pyruvate) was successful for elaboration of storage medium for African catfish *Clarias gariepinus*. In rainbow trout, fertilization rate can be best described by sperm motility rate, seminal plasma pH and spermatozoal respiration activity as these parameters explain a high percentage of variance in fertilization rate (Lahnsteiner et al., 1998). Interesting enough that in another Salmonidae species, brook trout, impaired sperm motility to the end of spawning season quite probably was not related to changes in bioenergetics parameters as activation medium supplementation with Ca^{2+} was enough to restore high motility parameters (Bondarenko et al., 2014). As example of not related to bioenergeitics sperm quality decrease could be found in recent study of Guthrie et al. (2011) performed in striped bass *Morone saxatilis*. In this study, it was shown, that it is Ca^{2+} ions internal concentration increase, rather than ATP content changes is responsible for significant sperm motility deterioration.

As fish sperm cryopreservation has great practical potential, numerous studies have been performed to understand whether information on fish sperm bioenergetics could be useful for cryobiological studies (Cabrita et al., 2010). During freezing and thawing, spermatozoa are exposed to influences from numerous physical and chemical factors, which appear as nonviable spermatozoa in thawed samples. That mixture of live and cryodamaged spermatozoa are objects of routine studies in cryobiology. It has been shown that a decrease in sperm quality after freezing/thawing is related to a decrease in macroergic phosphates content (Billard et al., 2004), especially ATP (Dreanno et al., 1997) or changes in sperm protein profiles (Li et al., 2010b), the latter being also related to bioenergetics (Dietrich et al., 2014). However, the occurrence of irreversibly damaged spermatozoa, biases the results. Future study of changes in spermatozoa survival during cryopreservation is required to understand whether cryopreservation influences bio-energy pathways of spermatozoa. Separation of the live cell fraction before analysis is a promising direction for progress in this field (Li et al., 2010a). Recently described energy-dependent processes of fish sperm motility cryoactivation represents an additional aspect of bioenergetics consideration required for elaboration of effective sperm cryopreservation methods (Boryshpolets et al., 2009b; Dzyuba et al., 2010). Positive effect of mild uncoupling of mitochondrial respiration to cryopreservation success was recently discovered (Fang et al., 2014). This process quite probably is related to decreased level of lipid peroxidation and can be considered as good example of application of knowledge on fish sperm bioenergetics for cryopreservation methods improvement.

7. Conclusions

This review clearly emphasizes the crucial role of energetics in the elaboration and maintenance of the quality of fish male gametes, during spermatogenesis, maturation, and motility period. From recent studies on fish sperm cells, it emerges that ATP is involved not only in a crucial fashion to sustain rapid flagellar movement, especially during the early phase of the brief motility period, but also in other functions such as homeostasis or ionic and osmotic balance of fish sperm cells. Another emergence coming from recently published papers is the importance of energetic compounds other than ATP, such as creatine-phosphate and indirectly ADP; both contribute to supplementing ATP level so that its concentration decreases more slowly and is maintained relatively uniform along the intra-flagellar length. Future studies should contribute to broaden these concepts to more species because they are based on results from a very limited number of fish, mostly cultured species.

It should be emphasized that future studies oriented on monitoring of bioenergetical processes at the level of a single sperm cell will benefit for both basic and applied aspects of fish spermatology. Nowadays, only first steps are made in this area (Chen et al., 2015).

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Fish spermatozoa metabolism and influence of the macroergic metabolites on sperm parameters

Description of fish spermatozoa metabolism by evaluation of metabolites content is one of methodological approaches in fish spermatology (Ingermann, 2008). This approach provides valuable information about the processes occurring in spermatozoa under such physiological conditions as sperm motility (Cosson, 2010), maturation (Lahnsteiner et al., 1998) and *in vitro* manipulation (Lahnsteiner and Mansour, 2010).

It is known that sufficient sperm motility is a prerequisite for successful fertilization. Therefore, cell metabolism mostly involves the activation and maintenance of movement and the protection of the genome from harmful environmental effects. Spermatozoa are immotile while in the testes and are activated after release into the environment. Their movement usually lasts a short time, up to several minutes. Motility depends on the time after release of fish spermatozoa into water and on the content of intracellular energy substrates.

The most important intracellular spermatozoan energy resources are adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine phosphate (CP), so called „macroergic" metabolites which may represent the outcomes of glycolysis, lipid catabolism, beta-oxidation of fatty acids and Krebs cycle. In addition, a large variety of other metabolites require more detailed description of metabolism in fish spermatozoa (Ellington and Kinsey, 1998). Currently, it is poorly known how macroergic metabolites are distributed from the place of synthesis (mostly mitochondria) to the place of its consumption (axoneme, the active motor of flagellum). In case of a few fish species, it is known that the ATP consumption process involves creatine phosphokinase shuttle. It remains questionable whether this process is species-specific. So far, the information on spermatozoa energetic status remains limited and this represents a lack which limits detailed understanding of how the dynamics of metabolite content is associated with the motility parameters and, thus, their fertilization capability.

Access to a large variety of fish species gives us the potential to examine the physiology of sperm and understand differences of metabolic processes among several species: different duration of the mobility, different metabolic pathways, and different mechanisms of the motility activation (Ingermann, 2008). Development of a rapid method for quantitative assessment of a large number of metabolites provides us a good opportunity to achieve a better understanding of fish specific metabolic energy pathways.

It is well known that flagellar movement needs hydrolysis of ATP along the entire length of the flagellum. ATP synthesis occurs mostly in mitochondria, which are located at the „base" of the flagellum that is also the head/tail junction (Cosson, 2012).

Many metabolites must have a more detailed description for better understanding of the process of metabolism in fish spermatozoa (Lahnsteiner and Caberlotto, 2012; Lahnsteiner et al., 1993; Mansour et al., 2003). The lack of information about energy distribution from the place of its synthesis (mostly mitochondria) to the place of its consumption (axoneme, the active motor of flagellum) is one of the important questions of fish spermatology (Ellington and Kinsey, 1998). This is the most limiting factor in the description of spermatozoa energetic status, which limits detailed understanding of how the dynamics of metabolite content is associated with the motility parameters and, thus, their fertilization ability (Boryshpolets et al., 2009; Fauvel et al., 2010).

Fertilization of fish eggs depends on the metabolism of spermatozoa. Metabolic activity of fish spermatozoa differs during each stage of their life, so its description will help us understand various processes, for example, quiescence of spermatozoa during storage in testes and reproductive tracts and high activity and motility after entry into the water environment. In addition, the process of activation of fish sperm involves significant

changes in sperm physiology. Spermatozoa of species with internal fertilization metabolize extracellular substrate. Spermatozoa released in the female fluid can use some components. For example, extracellular glucose supports glycolysis occurring in spermatozoa of Guppy (*Poecilia reticulata*) and Surfperch (*Cymatogaster aggregata*) (Shaw and Allen, 1977). However, the medium cannot provide energy supply for spermatozoa of species with external fertilization. In contrast, there are non-fish species with external fertilization which can swim very long periods (days) using only internal substrates such as lipid metabolism, for example sea urchins or oysters. Probably, these cells cannot metabolize extracellular substrate like species with internal fertilization. Spermatozoa of rainbow trout cannot transport extra cellular glucose (Cosson, 2012). On the other hand, incorporation of tricarboxylic acids can compensate weak glucose metabolism, this has been demonstrated for several species with extracellular pyruvate, lactate or glyoxylate (Dreanno et al., 2000; Gardiner, 1978). Difficulties in metabolizing extracellular glucose are connected with a poor membrane permeability to this substrate (Dreanno et al., 1999). After contact with water, spermatozoa need a large amount of energy to start and sustain flagellar movement supported by dynein ATPase activity. It causes the rapid ATP consumption described in most species such as: rainbow trout (*O. mykiss.*) (Christen et al., 1987), common carp (*Cyprinus carpio*) (Perchec et al., 1995), sea bass and turbot (*Scophthalmus maximus*) (Spiropoulos et al., 2002; Terner and Korsh, 1963a,b). Creatine phosphate and monosaccharides support ATP level during motility in the chub (*Leuciscus cephalus*) (Lahnsteiner et al., 1992; Terner and Korsh, 1963b; Turman and Mathews, 1996).

Mitochondria, in fish spermatozoa consume oxygen that constitutes basal metabolism connected to ATP production. The rate of sperm respiration depends on pH and carbon dioxide (Ingermann et al., 2003; Inoda et al., 1988). Probably, oxidative phosphorylation is responsible for sustaining of the ATP steady state level in quiescent spermatozoa. The tricarboxylic acid cycle and oxidative phosphorylation are the key metabolic pathways that sustain basal metabolism in spermatozoa (Lahnsteiner et al., 1999, 1993; Mansour et al., 2003; Zietara et al., 2004). During motility, the spermatozoa of *O. mykiss* strongly depend on oxidative phosphorylation and on tricarboxylic acid cycle, and subsequently on aerobic conditions (Lahnsteiner et al., 1999). Also lipid metabolism is very important because of positive correlation of intracellular lipid levels with successful fertilization in the trout (Lahnsteiner et al., 1998). The importance of stored glycogen in fish spermatozoa is still not adequately described. Sperm motility and viability of quiescent spermatozoa can be stimulated by pyruvate and coenzyme A, but the character of stimulation is species-specific for *Chalcalburnus chalcoides* and *O. mykiss*. Pyruvate is generally formed during glycolysis and during catabolism of the amino acids alanine, serine, cysteine, and threonine, and enters the citric acid cycle after oxidative decarboxylation (Rawn, 1983). Coenzyme A is a pantothenic acid serving as an acyl-group carrier during oxidative decarboxylation of pyruvate and during β-oxidation of fatty acids (Rawn, 1983). Pyruvate and coenzyme A occurs in limited concentrations in spermatozoa because of their stimulating effect. There is a slight effect on sperm motility and viability of the inhibitor of glycolysis 2-deoxy-D-glucose and several indications that glycolysis occurred in spermatozoa of *Chalcalburnus chalcoides* and *O. mykiss*. Significant decrease of glucose levels is observed during motility (Lahnsteiner et al., 1992, 1993) and during immotile storage of spermatozoa (Lahnsteiner et al., 1993, 1997) increasing the lactate levels when inhibiting the respiratory activity. In addition, an increase of lactate levels was observed in the cyprinid spermatozoa during anaerobic conditions (Belova, 1982). Spermatozoa are highly depend on respiratory activity probably, because glycolysis is much more affective under aerobic conditions than under anaerobic conditions (Rawn, 1983).

Catabolism of the amino acids alanine, serine, cysteine, and threonine was investigated only recently in teleostean spermatozoa and there are no indications about existence of these pathways.

The high variability of protein levels in seminal plasma is still incomprehensible (Lahnsteiner et al., 1995, 1994; Morisawa et al., 1979). However, there is a suggestion that seminal proteins prolong and stabilize sperm viability (Lahnsteiner et al., 2004).

Sperm maturation is defined as a process that results in the acquisition of potential for motility and fertilization in morphologically fully developed spermatozoa; it is considered as a final physiological stage of spermiogenesis and is well known in taxonomically distant groups of animals. In fish with external f fertilization, mature spermatozoa initiate full motility immediately after their release into an aqueous environment while immature spermatozoa are not capable of initiating efficient motility under the same conditions. Therefore, the acquisition of the potential for motility initiation by environmental signals represents a feature particularly crucial for functionality of fish spermatozoa. In sturgeons, testicular spermatozoa are not able to initiate motility after dilution with water because of their immature state. Recently the existence of a maturation phase in sturgeon has been discovered (Dzyuba et al., 2014). Testicular sturgeon sperm maturation can be simulated *in vitro* only by pre-incubation with urine-containing solution such as seminal fluid obtained from Wolffian duct semen. Some portion of energy in sturgeon spermatozoa supplies the physiological state, another for movement. It was shown that the switch (release) from iso- to hypotonic conditions is physiologically important for sturgeons.

Three fish species (sturgeon, whitefish, and European eel) were considered in this study. Release of sturgeon and whitefish spermatozoa into hypotonic conditions can occur without motility activation. The motility of their spermatozoa can be inhibited (prevented) by ionic composition of an activation medium. The knowledge of European eel spermatology is valuable for artificial reproduction of eels. Nowadays, eel spermatozoa have been fairly well studied from various aspects except bioenergetics.

The question, how energy is consumed during motility and by environmental response was not investigated before. It is impossible to investigate without new suitable and convenient approaches. It is important to find the appropriate fixation and extraction of metabolites because of very fast processes of their dynamics after motility activation. In addition, it is important to measure the main metabolites simultaneously to get more information.

Methodological approaches in fish sperm bioenergetics and motility analyses

The determination of macroergic metabolites in fish spermatozoa is of high importance. Most frequently used approaches (colorimetric and chemiluminescent assays) allow quantifying only one single substrate (for example ATP or CP), therefore limited data could be obtained (Lahnsteiner and Caberlotto, 2012; Perchec et al., 1995). To monitor general energy status of spermatozoa it is necessary to measure all the substrates. Therefore, simultaneous analysis of several interrelated metabolites (especially macroergic phosphates) is of great interest as it allows acquisition of more information from a single measurement, which consequently can be used for the assessment of the role of macroergic phosphates in sperm motility (Ingermann, 2008). The application of conventional method is laborious and it was carried out only for few fish species (Saudrais et al., 1998). Multi-compounds analysis based on high resolution nuclear magnetic resonance (NMR) has been applied for sperm samples of several mammals (Lin et al., 2009; Smith et al., 1985). However, only one article using this approach was dedicated to analysis of fish sperm (Dreanno et al., 2000). The high requirements for sample preparation, expensive instrumentation and skilled personnel, together with relatively

high limits of quantification (LOQ) result in relatively low use of NMR methods for fish samples. Liquid chromatography coupled with mass spectrometry (LC/MS) can be a technique of choice. Novel LC/MS analytical methods allow sensitive and selective identification and quantification of biomolecules in complex biological matrices (de Jong et al., 2010; Higashi et al., 2008). Analytical methods using different LC/MS configurations have been successful for the quantification of nucleotides in different cells in various biological matrices (Buchholz et al., 2001; Qian et al., 2004; Witters et al., 1996). To our knowledge, there are no published papers reporting LC/MS analysis of adenine nucleotides in sperm. Proper extraction is required to apply this technique for the quantification of adenine nucleotides, creatine phosphate, and creatine in sperm cells. The most frequent procedure for the fixation of sperm cells is based on perchloric acid, which prevents alterations of target compounds after extraction (Lahnsteiner and Caberlotto, 2012). This method has been shown to be appropriate for LC/MS analysis (Klawitter et al., 2007). However, the influence of the fish sperm extraction by perchloric acid on the content of creatine- and adenylate-phosphates is still not known. Description of fish spermatozoa by measurement of metabolites cannot give us enough information about very important characteristic – their motility. Nowadays the using of computer-assisted sperm analysis (CASA) systems in the analysis of fish spermatozoa motility is becoming more and more popular. Efficiency of CASA has been demonstrated for use with a variety of species in assessing male reproductive quality as well as the impact of various treatments on sperm motility; however, many investigations continue to rely on manual analytical methods.

CASA systems provides many parametric measures of motion or morphology of individual spermatozoon and for subpopulations (clusters) of spermatozoa in the microscopic field of view. There are several commercial CASAs used for work with animal sperm. All are based on slightly different principles and provide to customer (researcher) only final values without the preliminary data about all calculation steps, which could be a big source of errors. The real problem now is to standardize methodology of the CASA application in the evaluation of sperm parameters of different species (mammals, fish, etc.) (Abaigar et al., 1999; Gallego et al., 2013, 2015; Martinez-Pastor et al., 2005). Moreover, spermatozoa populations from the same ejaculate may consist of several subpopulations, which may differ in several parameters (Gallego et al., 2015; Kanuga et al., 2012). Therefore, the cluster analysis is required to obtain valuable information about the motility parameters and morphology of spermatozoa.

Directions of the present study

The main objective of this study was to evaluate creatine- and adenylate phosphates content in fish spermatozoa by measuring prior to and during the spermatozoa motility period. Special attention was paid to the analytical method development for the simultaneous quantification of creatine- and adenylate phosphates (Chapter 2). Little work has been done in this field, thus, development of new methods is a challenge. Most existing methods do not cover a wide range of compounds and use costly and time-consuming measurement techniques (such as NMR), which are considered to be the main limitation of the analysis. Taking into account the number of metabolites which are involved in physiological processes of spermatozoa prior and after the initiation of motility, it is of great importance to develop methods for simultaneous quantification, which enable us significantly reduce time and costs of the analysis. To date, significant shortcomings of this novel approach are lacking for compounds of interest. There is no other report about simultaneous determination of creatine- and adenylate phosphates by LC/MS methods in fish spermatozoa prior to and during the post-activation period, which emphasizes the advantages of the proposed LC/HRPS method, which is much more accessible to laboratories than NMR. (Chapter 2).

The maturation of sturgeon spermatozoa was studied. Quantification of creatine- and adenylate phosphates in sturgeon spermatozoa during maturation is of great importance because it gives us valuable information about energetic changes during this period and can provide relevant information for further studies. On the other hand, data on the content of creatine- and adenylate phosphates in sturgeon spermatozoa describe a general energetical status (Chapter 3). For instance, it can be used to evaluate the spermatozoa ability to be matured, if they have appropriate content of aforementioned compounds at the initial stage of maturation (Chapter 3).

Taking into account, that European eels are of high interest for aquaculture, its sperm is currently studied fairly well in different aspects. These aspects are: cryopreservation; morphology and viability of eel spermatozoa; effects of different agents on eel's spermatozoa cryoresistance; aspects of the initiation of eel sperm motility; role of calcium, sodium and potassium in the maintenance of spermatozoon's volume; intracellular pH and the initiation of eel spermatozoa motility; the presence of different subpopulations of spermatozoa after the activation of eel sperm. Nevertheless, the bioenergetical side of eel spermatozoa metabolism is poorly investigated by now. Therefore, estimation of creatine- and adenylate phosphates content during the motility period in European eel spermatozoa at the beginning and peak of spermiation induced by hCG hormonal treatment is currently of high interest. Additionally, the influence of *in vitro* sperm storage on content of creatine- and adenylate phosphates was studied (Chapter 4).

Another important issue is the influence of osmolality on the spermatozoa bioenergetics and movement (subpopulations of spermatozoa during the motility period). Unreasonably, low attention is paid to this factor. Nevertheless, contrarily to the initial idea that all spermatozoa from the same ejaculate activate similarly and would have a similar motility pattern, recent researches have shown that different sperm subpopulations with different motility parameters could coexist within the sperm of the same male (Beirão et al., 2011; Holt and Van Look, 2004). In our study of whitefish spermatozoa, four sperm subpopulations with different motility characteristics were identified after cluster analysis of CASA data (Computer-Aided Sperm Analysis) (Chapter 5).

The aims of the study were:

- The development of analytical approach for description of the creatine- and adenylate phosphates content prior to and during the motility period of fish spermatozoa;
- The study of bioenergetic aspects of sturgeon sperm maturation;
- The study of creatine- and adenylate phosphates content during the motility period in European eel spermatozoa at different phases of hormonal treatment and during the short-term storage;
- The study of osmolality effect on whitefish sperm motility and creatine- and adenylate phosphates content.

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CHAPTER 2

DEVELOPMENT AND APPLICATION OF LC/HRPS FOR QUANTIFICATION OF ADENINE NUCLEOTIDES, CREATINE PHOSPHATE, AND CREATINE IN STURGEON SPERMATOZOA

Fedorov, P., Grabic, R., Fedorova, G., Dzyuba, B., Cosson, J., 2017. Development and application of LC/HRPS for quantification of adenine nucleotides, creatine phosphate, and creatine in sturgeon spermatozoa. Czech J. Anim. Sci. 62, 67–74.

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Development and Application of LC/HRPS for Quantification of Adenine Nucleotides, Creatine Phosphate, and Creatine in Sturgeon Spermatozoa

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ABSTRACT

Fedorov P., Grabic R., Fedorova G., Cosson J., Boryshpolets S., Dzyuba B. (2017): **Development and application of LC/HRPS for quantification of adenine nucleotides, creatine phosphate, and creatine in sturgeon spermatozoa**. Czech J. Anim. Sci., 62, 67–74.

The objective of this study was to investigate the applicability of liquid chromatography coupled with high resolution product scan (LC/HRPS) mass spectrometry for simultaneous quantification of adenine nucleotides, creatine phosphate, and creatine levels after fixation of sturgeon sperm by perchloric acid. This approach has been used for the determination of adenine nucleotides, creatine, and creatine phosphate in biological tissues, but no applications to sperm cells have been reported to date. The results of method validation showed that specific correction factors for the calculation of macroergic phosphate content in sperm cells extracted by perchloric acid should be used to get accurate concentration values. The proposed LC/HRPS method is beneficial for the analysis of adenine nucleotides because it allows simultaneous quantification of all target analytes at low concentrations in a single run. This is an advantage compared to conventional methods based on colorimetric or chemiluminescent assays and even compared to sophisticated methods based on high resolution nuclear magnetic resonance.

Keywords: macroergic phosphates; high resolution mass spectrometry; extraction; method validation; sperm; chondrostean fishes

The spermatozoa of most fish species with external fertilization are maintained immotile in the seminal duct but initiate motility immediately after dispersion into water (Morisawa et al. 1983); this motility initiation is crucial for fertilization. Spermatozoa motility is primarily powered and regulated by creatine phosphate (CP) and adenylate phosphate metabolism. This metabolism is a very

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fast process, especially within the first 10 seconds post-activation, when the concentrations of CP and adenylate phosphates change up to several times (Dreanno et al. 1999). Adenosine triphosphate (ATP) production and CP synthesis occur in the middle part of the flagella, CP transport occurs along the flagellum, and ATP regeneration occurs, using CP and adenosine diphosphate (ADP), in the flagellum, which is why consideration of the contents of these components is important for the description of metabolic strategies (Cosson 2012). The content of each metabolite depends on the content of others: ATP could be regenerated from ADP in the axoneme by the adenylate kinase reaction (Cosson 2004), and CP also supports ATP levels during motility via the creatine–phosphokinase reaction (Lahnsteiner et al. 1992). Sperm motility highly depends on the energy of ATP hydrolysis catalyzed by dynein ATPase that is coupled to the sliding of adjacent microtubules, leading to flagellar movement (Morisawa and Okuno 1982; Christen et al. 1987; Saudrais et al. 1998).

Sturgeon spermatozoa are characterized by the elongated head commences with an acrosome with 8–12 posterolateral projections. The flagellum consists of an axoneme with a typical "9 + 2" structure of microtubules and presents a ribon-like structure due to two lateral membranous fins (Psenicka et al. 2008). Normal density of sturgeon spermatozoa (spz) is $0.46 \pm 0.25 \times 10^9$ spz/ml. Egg water, Ca^{2+}, and Mg^{2+} can trigger acrosome reaction. Released spermatozoa are immotile in seminal plasma and become motile only after dilution with water or specifically designed activating media (Ca^{2+} and K^+ ions are the main participants of cyclic adenosine monophosphate (cAMP) dependent pathways of signalling for sperm motility activation), suggesting the ionic mechanism of motility activation (Dzyuba et al. 2013). The duration of motility period is longer (up to several minutes) in comparison to some other species and supported by adenylate kinase and creatine phosphokinase reactions as the main sources of energy (Fedorov et al. 2015; Dzyuba et al. 2016).

To understand the processes involved in fertilization of fish eggs, it is important to accurately measure macroergic compounds in the spermatozoa. The most frequently used approaches (colorimetric and chemiluminescent assays) allow quantification of only one substrate (e.g. ATP or CP), limiting the amount of obtainable data (Perchec et al. 1995; Lahnsteiner and Caberlotto 2012). To monitor the general energy status of spermatozoa, it is necessary to measure all the substrates. Therefore, simultaneous analysis of several interrelated metabolites (especially macroergic phosphates) is of great interest, as it allows us to obtain more information from a single measurement, which consequently can be used for the assessment of the role of macroergic phosphates in sperm motility (Ingermann 2008). The application of conventional methods is laborious and was carried out for only a few fish species (Saudrais et al. 1998). Multi-compound analysis based on high resolution nuclear magnetic resonance (NMR) has been applied to the sperm samples of several mammals (Smith et al. 1985; Lin et al. 2009); however, we have found only one article using this approach for the analysis of fish sperm (Dreanno et al. 2000). The high requirements for sample preparation, expensive instrumentation, and skilled personnel, together with relatively high limits of quantification (LOQ), result in a limited use of NMR methods for fish samples.

Novel liquid chromatography/mass spectrometry (LC/MS) analytical methods allow selective identification and quantification of low concentrations of biomolecules in complex biological matrices (Higashi et al. 2008; de Jong et al. 2010). Analytical methods using different LC/MS configurations have already been successfully applied to the quantification of nucleotides in different cells in various biological matrices (Witters et al. 1996; Buchholz et al. 2001; Qian et al. 2004). Up to date, no papers reporting the LC/MS analysis of adenine nucleotides in sperm have been published. To utilize this technique for the quantification of adenine nucleotides, CP, and creatine in sperm cells, proper extraction is required. The most common procedure for the fixation of sperm cells is based on the use of perchloric acid (PCA), which prevents alterations of target compounds after extraction (Lahnsteiner and Caberlotto 2012). This method is appropriate for LC/MS analysis (Klawitter et al. 2007); however, the influence of PCA on the extraction of CP and adenylate phosphates from fish sperm is still insufficiently investigated while it is widely used in studies of bacteria, plant, and mammalian cells (Chida et al. 2012).

The aim of the present work was to evaluate the applicability of the liquid chromatography/high resolution mass spectrometry in product scan mode (LC/HRPS) method for the simultaneous quanti-

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fication of ATP, ADP, adenosine monophosphate (AMP), cyclic adenosine monophosphate (cAMP), CP, and creatine after fixation of sturgeon sperm by PCA. Elaborating this method could open new possibilities for the study of sperm bioenergetics in comparison to existing methods.

MATERIAL AND METHODS

Sperm sampling and quality parameters evaluation. Sterlet, *Acipenser ruthenus*, was selected as a model sturgeon species. During the natural spawning season (April–May), 6 sterlet males (3–4 years, 0.6–1.0 kg body weight) were transferred from aquaculture ponds (water temperature 8–10°C) into a 0.8 m^3 closed water recirculation system. The system water temperature was increased to 15°C within 24 h, and, prior to experimentation, fish were held four days without feeding. Spermiation was stimulated by an intramuscular injection of carp pituitary powder (Rybníkářství Pohořelice a.s., Czech Republic, www.rybnikarstvi-pohorelice.cz) dissolved in 0.9% (w/v) NaCl solution (4 mg/kg body weight (BW)). 24 h post-stimulation, milt was collected from the urogenital (Wolffian) ducts by aspiration using a 4-mm plastic catheter connected to a 10 ml syringe.

To initiate the motility, sperm was diluted in activation medium (1 : 50) containing 5 mM Tris-HCl buffer, 5 mM NaCl, and 0.25% Pluronic (Sigma-Aldrich Chemie GmbH, Germany), pH 8.5. Spermatozoon motility was recorded from 10 s post-activation until cessation, using video microscopy combined with stroboscopic illumination (ExposureScope, Czech Republic). Sperm motility video records were analyzed using CASA-automated plugin (http://www.ucs.mun.ca/~cfpurchase/CASA_automated-files.zip) for ImageJ software (National Institutes of Health, USA) to assess motility percentage and curvilinear velocity (VCL) (Purchase and Earle 2012).

For the evaluation of sperm concentration, sperm was diluted in 0.9% NaCl solution at a ratio of 1 : 2000 (sperm/saline). Sperm concentration was calculated after counting the number of spermatozoa in 12 cells of a Burker hemocytometer (Meopta, Czech Republic) at ×200 magnification with an Olympus BX 50 phase-contrast microscope (Olympus, Japan).

Experimental design. Initially the LC/HRPS method for quantification of adenine nucleotides, creatine phosphate, and creatine in sturgeon spermatozoa was validated. Sperm sample from one male was used for the LC/HRPS method validation. Twenty replicates were used for this purpose (10 were fortified and 10 measured as is). After that samples from six males were used for the study of macroergic phosphates content during sperm motility. Samples were taken and fixed by PCA at 10, 60, 120, 240, and 480 s post-activation.

Preparation of samples for adenine nucleotides, CP, and creatine analysis. Three molar PCA was added at 1 : 1 v/v to sperm samples (Lahnsteiner and Caberlotto 2012). Samples were subsequently frozen in liquid nitrogen and held at −80°C for 14 days. The samples were thawed at +4°C and then stored on ice. The amount of standard mixture corresponding to 1 µg of the target compounds (ATP, ADP, AMP, cAMP, CP, and creatine) was added to each fortified sample prior to extraction. The sample preparation for LC/HRPS analysis was carried out as follows: (1) centrifugation at 17 000 *g* for 14 min to collect the protein-free supernatant to avoid possible interference; (2) adjusting the pH range to 4.0–8.0 by addition of 12M KOH to the supernatant; (3) centrifugation at 3000 *g* to separate the precipitate from the $KClO_4$ solution; (4) dilution of the supernatant with 1M Tris-Cl (pH = 7.0) at 1 : 1 v/v to adjust the pH to 7.0; and (5) filtering through 0.45 µm regenerated cellulose filters (LABICOM, Czech Republic) into autosampler vials.

LC/HRPS quantification of adenine nucleotides, CP, and creatine. Adenine nucleotides of interest, creatine, and CP were detected using a hybrid Q Exactive™ Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA) coupled to an Accela 1250 liquid chromatography (LC) pump (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Switzerland). The LC method previously published by Jiang et al. (2012) was adapted to the LC/HRPS method for the analysis of target compounds. In brief, a Hypercarb column (50 mm × 2.1 mm ID × 3 µm particles; Thermo Fisher Scientific) was used to separate the target analytes. The mobile phases used for the separation consisted of ultrapure water (aqua-MAX-Ultrasystem; Younglin Instrument Co., South Korea) and acetonitrile (LiChrosolv Hypergrade; Merck, Germany). Both were buffered with 2 mM ammonium acetate and the pH of water was adjusted to 10.0 using am-

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monia solution (measured off bottle with a pH meter). The same amount of ammonia used for water was used initially for acetonitrile, but some extra addition was necessary (see below in "Results and Discussion"). The LC gradient is reported in Supplementary Table S1.

Heated electrospray (HESI-II), in both positive/negative ion modes, was used for the ionization of target compounds. The analysis was performed using product scan acquisition with the mass inclusion list, optimized collision energies, and expected retention times of the target analytes. Precursor ions were isolated at the first quadrupole, operated with 0.7 mass units extraction window, and collision products were analyzed in the orbital trap, operated at resolution of 17 500 full width at half maximum (FWHM) (m/z 200). The automatic gain control (AGC) target was set to 1 000 000 with a maximum injection time of 50 ms. Collision energy was optimized for all analytes of interest. The m/z ratios for the precursor and product ions of target compounds, their collision energies, and their retention times are presented in Supplementary Table S2.

The performance of the method was assessed in terms of its linearity, repeatability, matrix effect, limit of quantification (LOQ), and trueness. For the quantification of target compounds, external calibration, ranging from 1 to 500 ng/ml, was used. The lowest standard concentration in the calibration curve was considered the LOQ (LOQ response should be identifiable and reproducible within a precision of 20%). Validation of the method was performed using 10 replicates. The matrix effect was evaluated by preparing a matrix-matching standard using sperm extract.

Ethical clearance. 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 (USB), Research Institute of Fish Culture and Hydrobiology (RIFCH), Vodňany, Czech Republic. The Faculty of Fisheries and Protection of Waters of the USB and the RIFCH ran all experiments under the authorization for the use of experimental animals, reference No. 53100/2013-MZE-17214, and the authorization for breeding of experimental animals and delivery of experimental animals, reference No. 53103/2013-MZE-17214, accredited by the Ministry of Agriculture of the Czech Republic. Scientists were trained to work with animals according to the Act No. 246/1992 on the protection of animals against cruelty. Technical workers were regularly trained according to the Public notice of the Ministry of Agriculture of the Czech Republic No. 419/2012.

Statistical analysis. Statistical analysis was conducted on ATP, ADP, AMP, CP, and creatine, expressed as nmol/10^9 spz, taking into account dilutions of the sperm sample during activation and extraction. Due to a low number of observations (n = 6), a nonparametric Kruskal–Wallis ANOVA, followed by multiple comparisons of mean ranks for all groups, was used to compare ATP, ADP, AMP, CP, and creatine content. Data were presented as medians with percentiles (25%). Motility percentage and VCL values for each individual sample were presented as mean ± SEM.

Statistical significance was accepted at $P < 0.05$. Analyses and graphing were conducted using STATISTICA software (Version 12, 2013).

RESULTS AND DISCUSSION

Chromatography. Although adapting a previously published method (Jiang et al. 2012), the preparation of the mobile phase was not straight forward. Particularly problems arose with the reproducibility (day-to-day repeatability) of the chromatography. It was difficult to find the exact ammonia amount to achieve acceptable chromatography, as illustrated in Figure 1. It is obvious from Figure 1 that pH is very important for successful chromatographic separation of adenine nucleotides. Another complication was observed when the acetonitrile phase stood for some time (about 1 day) and was then reused: the same phenomenon of peak shift and broadening was observed. We assumed that this problem could be caused by ammonia volatilization or by ammonia reacting with atmospheric CO_2. Filling the head space above the mobile phase with argon seemed to be a useful solution for this problem; however, the final adjustment of the ammonia content in newly prepared acetonitrile had to be performed empirically – as a sequence of ammonia additions and high performance liquid chromatography (HPLC) analysis of the standard solution.

Method performance. Method performance parameters are presented in Table 1. Good linearity ($r \geq 0.9975$) was obtained for all six analytes. LOQs were determined in the low ng/ml range, which

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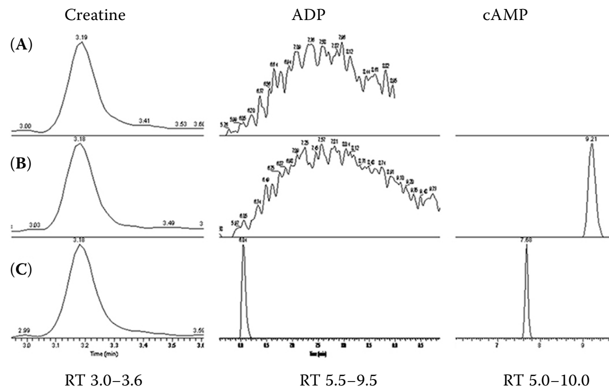

Figure 1. Shift in retention time (RT) of selected compounds under different additions of ammonia to acetonitrile solution acetonitrile solution with the same amount of ammonia as was added to the water phase (**A**), the same acetonitrile solution + 2 ml of 10% ammonia solution (**B**), the same acetonitrile solution + 5 ml of 10% ammonia (**C**)
ADP = adenosine diphosphate, cAMP = cyclic adenosine monophosphate

allowed the quantification of target compounds in sperm at low concentrations.

The trueness of the method was evaluated by calculating the analyte recoveries from fortified sterlet spermatozoa samples. One fortification level (1 μg/ml) was tested in ten replicates. Average recoveries ranged from 52 to 86% in these samples. Relative standard deviations (RSDs) ranged from 2 to 20%.

The above-mentioned results show that even correction for the matrix effect (especially relevant for CP, where significant ion suppression was found) did not lead to acceptable recoveries for all the analytes in the range given by the Commission Decision 2002/657/EC on analytical methods performance (80–110%).

To compensate for the low recoveries of most of the target compounds, correction factors were established. Using these correction factors for measured concentrations, calculations improved the quantification accuracy and compensated for probable analyte loss during sampling and sample

Table 1. Method performance parameters

Analyte	Linearity (R^2)	LOQ (ng/ml)	Repeatability (% RSD)[1] (non-spiked samples)	Matrix effect (%)		Absolute recovery (%)[2]	Correction factor[3]
				ion suppression	ion enhancement		
CP	1	5	20	54	–	53 (13)	1.9
Creatine	0.9999	5	4	1	–	86 (2)	1.2
AMP	0.9999	1	12	3	–	54 (2)	1.9
ADP	0.9996	1	2	–	1	60 (4)	1.7
ATP	0.9975	1	2	–	4	73 (20)	1.4
cAMP	0.9997	5	nd	4	–	52 (3)	1.9

LOQ = limits of quantification, RSD = relative standard deviation, CP = creatine phosphate, AMP = adenosine monophosphate, ADP = adenosine diphosphate, ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate, nd = not detected
[1]ten replicates were analyzed
[2]accounted for matrix effect; RSD (%) of ten replicates are given in brackets
[3]to calculate concentrations, multiply by the following factor

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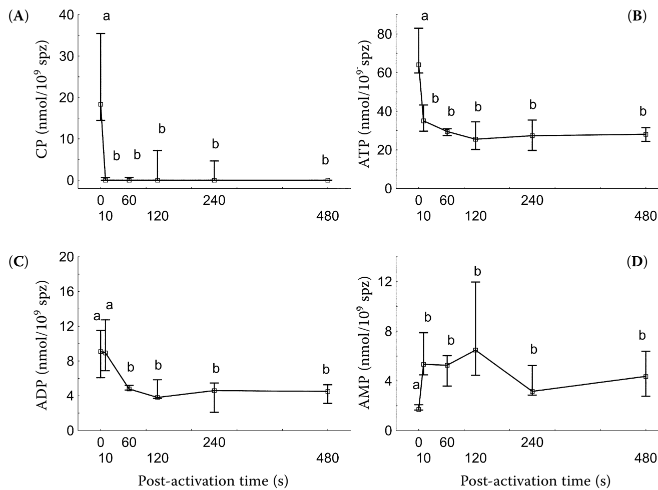

Figure 2. Creatine phosphate (CP) (**A**), adenosine triphosphate (ATP) (**B**), adenosine diphosphate (ADP) (**C**), and adenosine monophosphate (AMP) (**D**) content of sterlet spermatozoa (spz) after dilution with activation medium (n = 6)
[a,b]values with different letters differ significantly, as determined by the nonparametric Kruskal–Wallis ANOVA test ($P < 0.05$)
data are presented as medians with percentiles (25%)

handling. The study performed by Klawitter et al. (2007) with rat kidney tissue extracted in the same way reported acceptable recovery rates for ATP (76%) and ADP (80%), indicating that compound losses could be related to specific mechanisms in certain cells (Billard et al. 1999). However, the extraction efficiency of adenine nucleotides from sperm cells by PCA still requires to be estimated (even though it is the most frequently used approach). We believe that this is an important finding, as correct biological interpretation of the results must be based on the "true" proportions of energy metabolites. The correction of measured data with this factor is crucial; otherwise it can lead to misinterpretation of the real physiological state of the tested sperm.

Application of LC/HRPS for the quantification of adenine nucleotides, CP, and creatine in sterlet sperm. The method was successfully applied for the determination of CP, ATP, ADP, AMP, and creatine in sperm samples from six sterlet males under conditions of motility activation (Figure 2). The obtained dynamics of ATP content during the post-activation period (Figure 2b) were similar to those obtained for Siberian sturgeon (*Acipenser baerii*) using a bioluminescence method (Dreanno et al. 2000). The median creatine content was 419 nmol/10^9 spz (95% range, 254–520 nmol/10^9 spz) without a statistically significant difference during the entire post-activation period. cAMP was not detected, possibly due to dilution during the collection and preparation of samples. To avoid the influence of these factors, a 15-fold pre-concentration was used, but no cAMP was found. In any case, we obtained a complex picture of macroergic phosphates and their metabolites using this method. Motility percentage and velocity curvilinear (VCL) of spermatozoa were gradually decreased from 10 s (86.4 ± 3.9%, 139.7 ± 9.3 μm/s) to 120 s (50.2 ± 11.1%, 101.5 ± 14.8 μm/s) and finally to 480 s (5.3 ± 2.2%, 32.9 ± 7.6 μm/s).

There was an attempt to measure ATP, ADP, CP, and other substances simultaneously in the spermatozoa of turbot (*Psetta maxima*) with the help of NMR analysis, but data for the content of ATP, ADP, and CP were obtained only before motility activation, and not during the post-activation period. Possibility of simultaneous determination of ATP, ADP, AMP, CP, and creatine in fish spermatozoa during the motility period demon-

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strates the advantages of the proposed LC/HRPS method. Additionally, it is much more accessible to laboratories than NMR.

CONCLUSION

The developed method shows good performance for the target compounds in terms of linearity, LOQ, trueness, and repeatability. The obtained results revealed the necessity of using correction factors to account for the probable losses of target compounds during sample preparation. Currently, this is the first application of LC/MS for the quantification of all target analytes in fish sperm cells in a single run. Despite some problems with the liquid chromatography, the method promises a simple and fast multi-component determination of low levels of marcoergic phosphates and their metabolites.

While this method was successfully applied to simultaneous determination of ATP, ADP, AMP, CP, and creatine in spermatozoa from sterlet, it can be proposed for a study of sperm energy metabolism in other fish species.

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Development and Application of LC/HRPS for Quantification of Adenine Nucleotides, Creatine Phosphate, and Creatine in Sturgeon Spermatozoa

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Supplementary Online Material (SOM)

Table S1. Liquid chromatography gradient for the separation of target analytes

Time (min)	A (%)	B (%)	Flow (µl/min)
0.00	98.0	2.0	200
2.00	98.0	2.0	200
4.00	85.0	15.0	300
8.00	50.0	50.0	400
10.00	50.0	50.0	400
10.01	98.0	2.0	250
13.00	98.0	2.0	250

A = water + 2 mmol/l ammonium acetate + ammonia, B = acetonitrile + 2 mmol/l ammonium acetate + ammonia

Table S2. Mass spectrometry/high resolution mass spectrometry parameters

Analyte	Mode	Parent ion (*m/z*)	Quantification transition (*m/z*)	Confirmation transition (*m/z*)	NCE (%)	RT (min)
CP	–	210	78.9591	96.9696	35	1.11
Creatine	–	130	88.0404	*	35	3.76
AMP	–	346	78.9591	134.0472	30	6.09
ADP	–	426	134.0472	328.0453	30	6.31
ATP	–	506	158.9253	408.0124	30	6.35
cAMP	+	330	136.0618	312.0489	30	7.91

CP = creatine phosphate, AMP = adenosine monophosphate, ADP = adenosine diphosphate, ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate, RT = retention time, NCE = normalized collision energy

*no confirmation ion

CHAPTER 3

THE STUDY OF STURGEON SPERM MATURATION

3.1. Fedorov, P., Dzyuba, B., Fedorova, G., Grabic, R., Cosson, J., Rodina, M., 2015. Quantification of adenosine triphosphate, adenosine diphosphate, and creatine phosphate in sterlet *Acipenser ruthenus* spermatozoa during maturation. J. Anim. Sci. 93, 5214–5221.

It was allowed by the publisher on 28th April, 2017 to include the published paper in this Ph.D. thesis.

My share on this work was about 70%.

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, 339–341.

According to the publishing agreement between the authors and publisher, it is allowed to include the paper in this Ph.D. thesis

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My share on this work was about 20%.

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Quantification of adenosine triphosphate, adenosine diphosphate, and creatine phosphate in sterlet *Acipenser ruthenus* spermatozoa during maturation[1]

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ABSTRACT: Sturgeon spermatozoa maturation during their passage through the kidney is a prerequisite for initiation of motility. Samples of sterlet (*Acipenser ruthenus*) testicular sperm (TS) were matured in vitro by incubation in seminal fluid (SF) or in SF supplemented with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; a respiration uncoupling agent). Sperm was diluted in activation medium (AM) containing 10 m*M* Tris-HCl buffer (pH 8.5) and 0.25% Pluronic, and spermatozoon motility was assessed. Samples were taken and fixed in 3 *M* perchloric acid at 3 points in the incubation process. Quantification of ATP, ADP, and creatine phosphate (CrP) was conducted using liquid chromatography/high-resolution mass spectrometry. We observed a significant decrease in CrP during artificial maturation of TS in SF. In contrast, ATP and ADP were not significantly affected. Addition of CCCP to SF halted maturation and led to significantly lower CrP whereas ADP significantly increased and ATP was unaffected. Dilution of matured and immature TS with AM led to a significant decrease of ATP and CrP and an increase of ADP compared with their levels before dilution, although immature TS were not motile. Energy dependency of TS maturation in sturgeon was confirmed, which suggests that mitochondrial oxidative phosphorylation is needed for maturation of sturgeon TS.

Key words: *Acipenser ruthenus*, adenosine triphosphate, adenosine diphosphate, chromatography, creatine phosphate, testicular sperm

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INTRODUCTION

Sturgeon testicular spermatozoa cannot become motile, because they have not undergone the critical maturation during passage through the kidney. Upon this passage, testicular spermatozoa are mixed with hypotonic urine and stay in the Wolffian duct, where they acquire the ability for motility activation after release into the aquatic environment. This process can be simulated in vitro by mixing testicular spermatozoa with hypotonic fluids such as urine or seminal fluid (**SF**) obtained from Wolffian duct sperm (**WS**). Interestingly, during this in vitro maturation, spermatozoa velocity and motility percentage gradually increase (Dzyuba et al., 2014). Cellular processes determining sperm maturation in sturgeon are unknown. However, transition of spermatozoa from isotonic conditions (in testes) to hypotonic conditions during maturation can lead to ionic channels activation (Okada, 2004), which, in turn, could be the main source of energy consumption during maturation.

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Spermatozoon motility depends primarily on the energy released from ATP hydrolysis catalyzed by dynein adenosine triphosphatase that is coupled to the sliding of adjacent microtubules generating flagellar movement (Christen and Gatti, 1987). Increase in ATP consumption after motility activation stimulates increase of oxidative phosphorylation (Ingermann, 2008) and activation of ATP regeneration using ADP and creatine phosphate (**CrP**) via adenylate and creatine kinases (Cosson, 2012). However, the role of oxidative phosphorylation and the ATP regeneration system in the maturation process is not clear.

In the present study, we hypothesized that sturgeon sperm maturation is an energy-dependent process caused by changes of sperm environment tonicity and that it could be associated with respiration and macroergic phosphate regeneration pathways. To test this hypothesis, we aimed our study to compare ATP, ADP, and CrP levels in immature and in vitro–matured testicular spermatozoa of *Acipenser ruthenus* in response to environmental osmolality changes.

MATERIALS AND METHODS

Fish and Sperm Sampling

Sterlet (*A. ruthenus*) was selected as a model sturgeon species. 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. Experiments were conducted in the 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), 5 sterlet males (3–4 yr and 0.6–1.0 kg BW) were transferred from aquaculture ponds (water temperature 8–10°C) into a 0.8-m^3 closed water recirculation system. The system water temperature was increased to 15°C within 24 h, and before experimentation, fish were held 4 d without feeding.

Spermiation was stimulated by an intramuscular injection of carp pituitary powder (Rybníkářství Pohořelice a.s., Pohořelice, Czech Republic; http://www.rybnikarstvi-pohorelice.cz) dissolved in 0.9% (wt/vol) NaCl solution (4 mg/kg BW). Twenty-four hours after stimulation, milt was collected from the urogenital (Wolffian) ducts by aspiration using a 4-mm plastic catheter connected to a 10-mL syringe. Wolffian duct sperm is commonly used in fisheries for artificial sturgeon propagation. Immediately after WS collection, the fish were euthanized by striking the cranium followed by exsanguination. The digestive tract was removed, and testicular sperm (**TS**) was collected with a micropipette from an incision in the efferent ducts.

Sample Preparation

Seminal fluid was obtained from 2 successive centrifugations of WS for 10 min at 300 × g at 4°C and then (only collected supernatant) 10 min at 10,000 × g at 4°C.

For in vitro maturation, TS samples were incubated in SF for up to 15 min at 20°C at a dilution rate specific to each male, based on its initial TS concentration, to obtain 1×10^9 spermatozoa (**spz**)/mL. The incubation time required to obtain the maximum percent of activated spermatozoa for an individual fish ranged from 5 to 15 min. This time was designated full maturation time, corresponding to 1 arbitrary unit (**AU**).

To assess the role of respiration in spermatozoon maturation, TS samples were incubated for up to 15 min at 20°C in SF containing carbonyl cyanide *m*-chlorophenyl hydrazone (**CCCP**) as an uncoupler of mitochondrial respiration and oxidative phosphorylation (Guthrie et al., 2008). Carbonyl cyanide *m*-chlorophenyl hydrazone dissolved in dimethyl sulfoxide (**DMSO**) was added to SF (final concentration of DMSO was 0.5%) to a final CCCP concentration of 50 μ*M*. To evaluate the influence of DMSO on maturation, TS samples were also incubated for up to 15 min at 20°C in SF containing 0.5% DMSO.

Spermatozoa concentration was estimated using a Burker cell hemocytometer (Meopta, Prague, Czech Republic) at 200x magnification of an Olympus BX 50 phase contrast microscope (Olympus, Tokyo, Japan; Perchec and Jeulin, 1995).

Activation of Spermatozoa

The activation medium (**AM**) used was 10 m*M* Tris-HCl buffer, pH 8.5, containing 0.25% Pluronic (Sigma-Aldrich, St. Louis, MO) to prevent sperm sticking to the microscope slides. Wolffian duct sperm and in vitro matured sperm samples were diluted 1:50 in AM, and motility parameters were determined at 10 s after activation. The dilution rate was selected according to requirements of the motility assessment procedure previously described (Dzyuba et al., 2012).

Motility Analysis

Motile spermatozoa were recorded for 2 min after activation using video microscopy combined with stroboscopic illumination (ExposureScope; University of South Bohemia in České Budějovice, Faculty of

Fisheries and Protection of Waters, Vodňany, Czech Republic). Video records were analyzed to estimate spermatozoon curvilinear velocity (**VCL**) and percent of motile cells (motility rate) by micro-image analyzer (Olympus Micro Image 4.0.1. for Windows; Olympus), which allows overlapping of 5 successive video frames. Overlapping tracks of spermatozoon heads became visible, permitting calculation of VCL, defined as total point-to-point distance traveled by the spermatozoon in 0.16 s (time the separating the first and fifth frames) and motility rate. Motility duration was defined as the period of time to cessation of movement in 95% of spermatozoa visible in the microscopic field.

Preparation of Samples for ATP, ADP, and Creatine Phosphate Assay

To investigate differences in concentration of ATP, ADP, and CrP over the course of maturation, aliquots of TS diluted in SF were fixed in 3 *M* perchloric acid (**PCA**) at 0, 0.2, and 1.0 AU of time as previously determined. Quantification of ATP, ADP, and CrP content was conducted using liquid chromatography/mass spectrometry/high-resolution mass spectrometry (**LC/MS/HRMS**).

Three molar PCA was added at 1:1 (vol/vol) to sperm samples prepared according to individual fish maturation periods (0 and 1.0 AU) at 10, 20, 60, and 120 s after activation. Addition of PCA prevents alterations in ATP content and metabolites extracted from cells (Lahnsteiner and Caberlotto, 2012) and has been shown to be appropriate for liquid chromatography/mass spectrometry analysis (Klawitter et al., 2007). Samples were subsequently frozen in liquid nitrogen and held at –80°C for up to 14 d. Samples for LC/MS/HRMS analysis were treated as follows: 1) centrifugation at 17,000 × g for 14 min at 4°C to collect the protein-free supernatant to avoid contamination of the column in LC/MS/HRMS, 2) addition of 12 *M* KOH to the supernatant sufficient to adjust the pH range to between 4.0 and 8.0, 3) centrifugation at 3,000 × g for 10 min at 4°Cto separate the precipitate from the $KClO_4$ solution, 4) dilution of the obtained supernatant with 1 *M* Tris-HCl (pH 7.0) at 1:1 (vol/vol) to adjust the pH to the optimal value of 7.0, and 5) filtering with 0.45-μm regenerated cellulose filters (Labicom, Olomouc, Czech Republic).

Liquid Chromatography/Mass Spectrometry/High-Resolution Mass Spectrometry Quantification of Adenine Nucleotides and Creatine Phosphate

Adenine nucleotides of interest and CrP were quantified using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to an Accela 1250 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland), according to a method adapted from Jiang et al. (2012). A Hypercarb column (50 mm by 2.1 mm i.d. by 3-μm particles; Thermo Fisher Scientific) was used to separate target analytes. Mobile phases used for the separation consisted of ultrapure water (aqua-MAX-Ultrasystem; Younglin, Kyounggi-do, Korea) and acetonitrile (LiChrosolv Hypergrade; Merck, Darmstadt, Germany). Both were buffered with 2 m*M* ammonium acetate, and pH was adjusted to 10.0 using ammonia solution (measured off bottle with pH meter). The same amount of ammonia as for water was initially used for acetonitrile (Supplemental Table S1; see the online version of the article at http://journalofanimalscience.org).

Heated electrospray (HESI-II; ThermoFisher Scientific, San Jose, CA) in negative ion mode was used for the ionization of target compounds. Analysis was performed using product scan acquisition with the mass inclusion list, optimized collision energies, and expected retention times of the target analytes. The first quadrupole was operated at a 0.7-amu extraction window, and the Orbitrap spectrometer (ThermoFisher Scientific) was operated at 17,500 full width at half maximum. The m/z ratios for parent and product ions of target compound as well as their collision energy and retention times are presented in Supplemental Table S2 (see the online version of the article at http://journalofanimalscience.org).

Method performance was assessed in terms of its linearity, repeatability, recovery, limits of quantification, and matrix effect. For the quantification of target compounds, external calibration ranging from 1 to 500 ng/mL was used. Method performance parameters are presented in Supplemental Table S3 (see the online version of the article at http://journalofanimalscience.org). Method validation was performed using 10 replicates. Good linearity, repeatability, and limits of quantification values were obtained for all analytes. The matrix effect was evaluated by preparing calibration standard using sperm extract. Significant matrix effect (ion suppression) was observed only for CrP. To adjust calculated concentrations for low recovery rates, calculation coefficients were established (Supplemental Table S3; see the online version of the article at http://journalofanimalscience.org).

Statistical Analysis

Statistical analysis was conducted on ATP, ADP, and CrP, expressed as nanomoles per 10^9 spz, taking into account dilutions of the TS sample during in vitro maturation, activation, and preparation for LC/MS/HRMS.

Data distribution and homogeneity of dispersion were assessed with the Shapiro–Wilks and Levene's tests, respectively. Normally distributed data (velocity only) were analyzed by 1-way ANOVA followed by Fisher's LSD test. Due to a low number of observations ($n = 5$), a nonparametric Kruskal–Wallis ANOVA followed by multiple comparisons of mean ranks for all groups (post hoc procedure) was used for comparing spermatozoon motility rate and duration relative to ADP, ATP, and CrP content. Data were presented as median with percentiles (25%). Statistical significance was accepted at $P < 0.05$. Analyses and graphing were conducted using Statistica version 9.1 (Statsoft Inc., Tulsa, OK).

RESULTS

Motility Parameters

In the present study, spermatozoa concentration of TS ($28 \pm 9 \times 10^9$/mL) was significantly higher than that of WS ($0.5 \pm 0.4 \times 10^9$/mL; $n = 5$). Observed spermatozoon concentrations were typical of those for sturgeon (Dzyuba et al., 2012) with wide variation among individual fish, as was reported by Dzyuba et al. (2014).

Immediately after dilution with SF, testicular spermatozoa were immotile in AM. The percentage of motile spermatozoa at 10 s after activation in TS experimentally matured in AM was not significantly different in the initial motility period from that observed for WS (Table 1).

Motility duration of matured testicular spermatozoa was also not significantly different from that of WS (Table 1).

Macroergic Phosphates Content during In Vitro Maturation of Sterlet Spermatozoa

We found no significant differences in ATP and ADP content of matured and immature sturgeon spermatozoa before dilution in AM. A significant decrease was observed in CrP at 0.2 AU of incubation time compared with initial values (Fig. 1). Addition of 50 μ*M* CCCP diluted in DMSO to SF halted maturation and led to a significant decrease of CrP content whereas ADP content significantly increased and ATP content was unaffected (Fig. 2). Addition of 0.5% DMSO to SF as control did not affect the content of ATP, ADP, or CrP (Fig. 2).

Table 1. Motility parameters of matured testicular and Wolffian duct spermatozoa[1]

Sperm sample	Motility rate at 10 s after activation, %	Motility duration, s	Curvilinear velocity at 10 s after activation, μm/s
Testicular sperm (after maturation), $n = 5$	66 ± 11	126 ± 13	176 ± 24
Wolffian duct sperm, $n = 5$	86 ± 9	138 ± 11	182 ± 29

[1]For curvilinear velocity, no significant differences were found (Fisher's LSD test, $P = 0.44$) between testicular sperm and Wolffian duct sperm. No significant differences were found for motility rate and motility duration (Mann–Whitney U-test, $P = 0.32$ and $P = 0.47$, respectively)

Macroergic Phosphates Content in Matured and Immature Spermatozoa after Dilution with Activation Medium

Following sperm dilution in AM, total ATP content at 10 and 20 s after activation was significantly higher in immature sperm compared with matured spermatozoa (Fig. 3).

Although the overall level of CrP in immature sperm before activation was higher than in mature sperm, its content significantly decreased during the first 10 s after activation in both immature and matured spermatozoa (Fig. 4). An opposite effect was seen with ADP concentration, which significantly increased during the first 10 s after activation. Total ADP content was not significantly different in matured and immature spermatozoa before activation (Fig. 5).

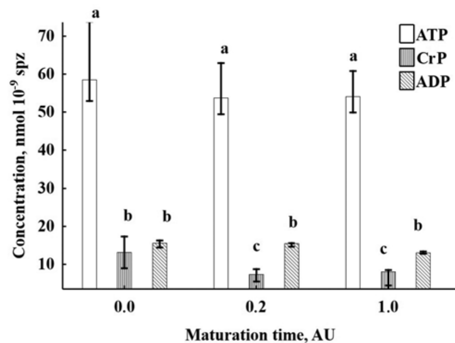

Figure 1. Adenosine triphosphate, creatine phosphate (CrP), and ADP content during the maturation of testicular sperm after dilution in seminal fluid ($n = 5$). [a–c]Columns with different letters differ significantly as determined by the nonparametric Kruskal–Wallis ANOVA test ($P < 0.05$). spz = spermatozoa; AU = arbitrary units.

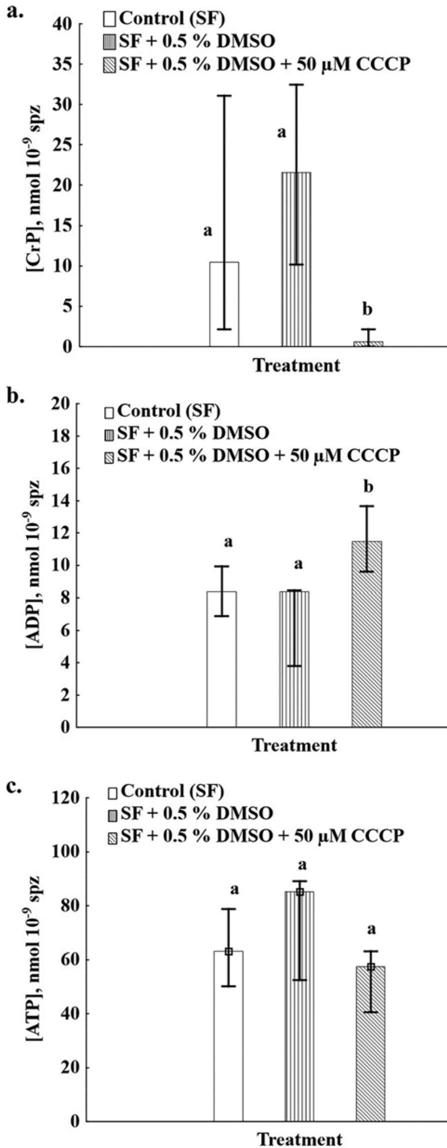

Figure 2. Creatine phosphate (CrP; a), ADP (b), and ATP (c) content during the maturation of testicular sperm after dilution in seminal fluid (SF) and treatment with dimethyl sulfoxide (DMSO) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; *n* = 5). [a,b]Columns with different letters differ significantly as determined by the nonparametric Kruskal–Wallis ANOVA test ($P < 0.05$). spz = spermatozoa.

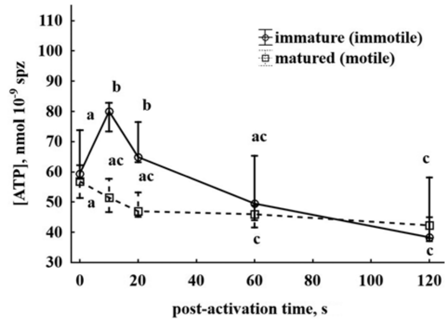

Figure 3. Adenosine triphosphate content of immature sperm (0 arbitrary units [AU] incubation time with seminal fluid [SF]) and matured sperm (1 AU incubation time with SF) after dilution with activation medium (*n* = 5). [a–c]Values with different letters differ significantly as determined by the nonparametric Kruskal–Wallis ANOVA test ($P < 0.05$). spz = spermatozoa.

DISCUSSION

Sperm contamination by urine is known as a factor influencing sperm motility parameters in teleost fish species (Rurangwa et al., 2004). Perchec Poupard et al. (1998) showed that contact with urine is deleterious to carp sperm and causes a significant decrease in motile cells and ATP content on motility activation compared with urine-free sperm. This illustrates the importance of avoiding contamination of milt with urine during collection and other manipulations. However, in sturgeon, incubation of TS with urine is a prerequisite for spermatozoon maturation. This process occurs in Wolffian ducts, where urine and sperm are naturally mixed. Such sperm and urine excretion is considered evolutionarily primitive among vertebrates, because more recent te-

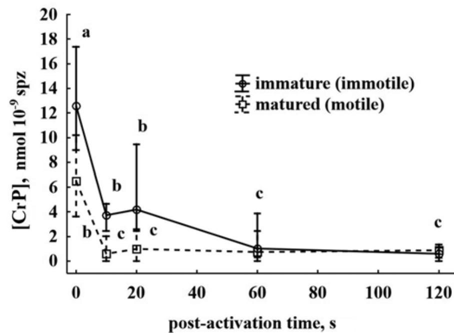

Figure 4. Creatine phosphate (CrP) content of immature sperm (0 arbitrary units [AU] incubation time with seminal fluid [SF]) and matured sperm (1 AU incubation time with SF) after dilution with activation medium (*n* = 5). [a–c]Values with different letters differ significantly as determined by the nonparametric Kruskal–Wallis ANOVA test ($P < 0.05$). spz = spermatozoa.

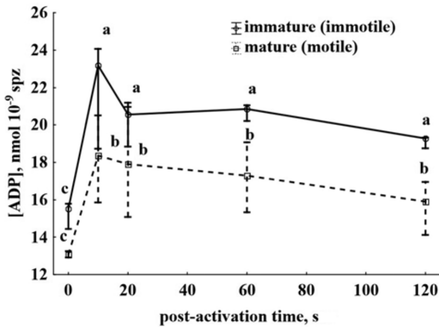

Figure 5. Adenosine diphosphate content of immature sperm (0 arbitrary units [AU] incubation time with seminal fluid [SF]) and matured sperm (1 AU incubation time with SF) after dilution with activation medium (n = 5). [a–c]Values with different letters differ significantly as determined by the nonparametric Kruskal–Wallis ANOVA test ($P < 0.05$). spz = spermatozoa.

leost taxa exhibit greater separation between urine and sperm ducts. This suggests that in the course of urogenital system evolution, the role of urine in spermatozoon maturation was dramatically altered. Although urine is a physiological participant in maturation of spermatozoa of the primitive sturgeon, it lost this role and became toxic to sperm of more evolved species.

The present study focused on alterations in ATP, ADP, and CrP content during sperm maturation that may result from overcoming osmotic shock when spermatozoa are released into hypotonic conditions. During in vitro maturation, osmotic shock appears at the transfer of TS from an osmotic environment of 220 to 230 mOsm/L into SF of about 50 mOsm/L (Dzyuba et al., 2014). Exactly this shock is leading to increased ATP consumption by spermatozoa ionic pumps activities, supporting cell homeostasis (Racker, 1976). Finally, this ATP consumption leads to ATP regeneration via CrP hydrolysis catalyzed by creatine kinase (Alavi and Cosson, 2006). It is known that motility rate and VCL reach maximum by the end of the in vitro TS maturation period (Dzyuba et al., 2014). Thereby, it could be supposed that this progression is associated with intracellular content of creatine and adenylate phosphates, both of which are involved in energy supply and regulation of spermatozoon maturation (Cosson, 2012). Physiological necessity of CrP and ADP participation in spermatozoa energy supply is determined by the fact that sites of ATP production (spermatozoa middle part) and its consumption (along entire flagellum) are spatially separated. Diffusion rates of ATP and CrP are essentially different and ATP can be regenerated from ADP via adenylate and creatine kinases (Cosson, 2012). As we found significant decrease in CrP during maturation (Fig. 1), we assume critical importance of ATP regeneration system (acting via creatine kinase) for the maturation process. The reasons for stable ADP content during maturation remain to be elucidated; however, minor involvement of adenylate kinase in maturation may be assumed. As information about sturgeon sperm bioenergetics at maturation is absent, it is only possible to compare our results with ones obtained in mature spermatozoa in another species.

In turbot sperm, the initial content of ATP, ADP, and CrP measured by nuclear magnetic resonance analysis before activation of motility was reported to be approximately 154, 99, and 727 nmol/10^9 spz, respectively (Dreanno et al., 2000), several-fold the values obtained for sterlet in the current study. High-performance liquid chromatography analysis for quantification of ATP and ADP in turbot spermatozoa (Dreanno et al., 1999b) showed ATP of approximately 240 nmol/10^9 spz before initiation of motility, several times the level observed in sterlet. For sea bass, reported level of ATP before activation was 150 nmol/10^9 spz, which is twice that obtained for sterlet in our study, with ADP at a similar concentration (15 nmol/10^9 spz; Dreanno et al., 1999a). The reasons for these differences are not clear but may be associated with species-specific energy metabolism strategies sustaining macroergic phosphates levels acquired during spermatozoa maturation and required for motility activation.

Taking into account increased blood circulation in sturgeon testes associated with gonad development before phase of spermiation, the respiration is assumed to have high importance for ATP synthesis during final stages of spermatogenesis and sperm maturation (Chebanov and Galich, 2009). Importantly, decrease of sperm concentration during passage through kidneys may lead to increase oxygen availability for spermatozoa. Our results on suppression of in vitro sperm maturation by CCCP, an uncoupler of mitochondrial respiration and oxidative phosphorylation, strongly indicate the importance of respiration for ATP synthesis during maturation. However, stable ATP content associated with decrease of CrP allows us to speculate that process of ATP synthesis by respiration during maturation does not predominate the processes ATP hydrolysis, although there is lack of information about bioenergetics in testicular spermatozoa of sterlet. Thereby, our results on ATP, ADP, and CrP dynamics involving respiration in matured and immature spermatozoa are pioneering in this area.

As immature spermatozoa are not able to initiate motility, comparative study of mature and immature spermatozoa is an essential experimental model for study of relationships between macroergic phosphates

content and motility associated with hypotonic conditions. The observed increase in ATP content in immature spermatozoa diluted with AM (Fig. 3) could be associated with stimulation of ATP synthesis by consumption of ionic pumps (Okada, 2004). As no motility occurs, ATP synthesis via respiration predominates the processes of ATP hydrolysis at initial postdilution time. However, this supposition should be further studied.

Dynein adenosine triphosphatase activity causes rapid intracellular ATP consumption (Morisawa and Okuno, 1982; Ingermann, 2008), as described in rainbow trout (*Oncorhynchus mykiss*; Christen and Gatti, 1987), common carp (*Cyprinus carpio*; Perchec and Jeulin, 1995), sea bass (*Dicentrarchus labrax*; Dreanno et al., 1999a), turbot (*Scophthalmus maximus*; Terner and Korsh, 1963; Dreanno et al., 2000; Spiropoulos et al., 2002), and Siberian sturgeon (*Acipenser baerii*; Billard et al., 1999). It has been demonstrated that ATP can be regenerated from ADP in trout sperm through intracellular adenylate kinase (Cosson 2004; Saudrais et al., 1998). Creatine phosphate has also been shown to support adequate ATP levels during motility via creatine phosphokinase activity in spermatozoa of chub (*Leuciscus cephalus*; Terner and Korsh, 1963; Lahnsteiner et al., 1992; Turman and Mathews, 1996) and rainbow trout (Saudrais et al., 1998).

Therefore, it is reasonable to assume that results of the present study confirm that the maturation of TS in sturgeon is an energy-dependent process involving mitochondrial respiration and ATP regeneration from CrP via creatine kinase reaction. These 2 processes are responsible for keeping ATP content at high level required for motility of mature spermatozoa.

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Table S1. LC gradient for the separation of target analytes

Time, min	A, %	B, %	Flow, µL min^{-1}
0.00	98.0	2.0	200.0
2.00	98.0	2.0	200.0
4.00	85.0	15.0	300.0
8.00	50.0	50.0	400.0
10.00	50.0	50.0	400.0
10.01	98.0	2.0	250.0
13.00	98.0	2.0	250.0

A = water + 2mmol L^{-1} ammonium acetate + ammonia

B = Acetonitrile + 2mmol L^{-1} ammonium acetate + ammonia

Table S2. MS/HRMS parameters

Analyte	Ion Mode	Parent ion (*m/z*)	Quantification transition (*m/z*)	Confirmation transition (*m/z*)	NCE[a] (%)	RT[b] (min)
CP	-	210	78.9591	96.9696	35	1.11
ADP	-	426	134.0472	328.0453	30	6.31
ATP	-	506	158.9253	408.0124	30	6.35

[a] Normalized collision energy

[b] Retention time

Table S3. Method performance parameters

Compound	Linearity (R^2)[a]	LOQ[b] (ng mL^{-1})	Absolute recovery (%)[c]	Repeatability (% RSD)[d]	Matrix effect (%)		CC[e]
					ion suppression	ion enchancement	
CP	1	5	53 (13)	6	54	-	1.9
ADP	0.9996	1	60 (4)	2	-	1	1.7
ATP	0.9975	1	73 (20)	2	-	4	1.4

[a] Linearity of the calibration curve in the range 1-500 ng mL^{-1}

[b] Limit of quantification

[c] RSD (%) of ten replicates are given in brackets

[d] ten replicates were analyzed

[e] Calculation coefficient

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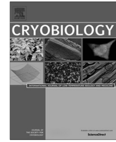

Brief Communication

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.

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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.

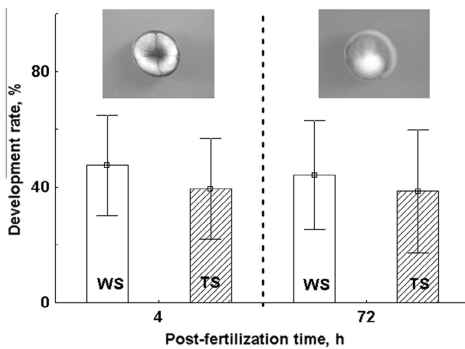

Fig. 1. Development rate of sterlet embryos obtained by frozen–thawed Wolffian duct sperm (WS) or testicular sperm after *in vitro* maturation (TS). The photos of developing embryo at 4 and 72 h post-fertilization are shown above the columns. No significant difference among groups was found ($P = 0.48$,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

CONTENT OF CREATINE- AND ADENYLATE PHOSPHATES IN EUROPEAN EEL SPERMATOZOA: INFLUENCE OF HORMONAL TREATMENT, *IN VITRO* STORAGE, AND MOTILITY

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My share on this work was about 70%.

CONTENT OF CREATINE- AND ADENYLATE PHOSPHATES IN EUROPEAN EEL SPERMATOZOA: INFLUENCE OF HORMONAL TREATMENT, *IN VITRO* STORAGE, AND MOTILITY

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ABSTRACT

It was shown in some fish species (e.g. sturgeon) that spermatozoa undergo maturation to get the ability to be activated and this process is associated with bioenergetic state of the cells. It is known that sperm of European eel also needs maturation for initiation of motility, and this can be triggered by hormonal treatment, but nothing is known about bioenergetic substrates content in these conditions. In addition, there are no data about effect of short-term storage conditions on bioenergetics of the sperm cells, which can be essential for eel aquaculture.

This study showed that European eel spermatozoa were virtually unable to be activated by dilution into seawater (SW) at the beginning of the artificially induced spermiation period (6th week of hormonal treatment with hCGrec). After 10 weeks of hormonal treatment, they acquired the ability of being activated into SW. This was associated with higher adenylate energy charge (AEC, the ratio of AMP, ADP, and ATP) on the 10th week of treatment in comparison to the 6th week. Hormonal treatment seems to induce a final maturation process, changing bioenergetic state, that in turn is important for acquiring motility by spermatozoa.

A significant increase of creatine phosphate (CP) and cyclic-AMP (cAMP) content in spermatozoa of the 10 weeks-treated males was observed prior to and post-activation if compared with cells from 6 weeks-treated fish. Current study represents the first successful quantification of cAMP in fish spermatozoa prior to and post activation using liquid chromatography coupled with high-resolution product scan (LC/HRPS).

Moreover, temperature and duration of short-term storage affected the content of CP, AMP, ADP, and ATP in European eel spermatozoa. Short-term storage (up to 7 days) at 4 °C resulted in higher macroergic phosphates content and higher motility traits compared to 20 °C.

Obtained results could be used for future improvement of spermiation induction technique and methods for short-term storage of European eel sperm.

Keywords: *Macroergic phosphates, sperm maturation, sperm bioenergetics, sperm storage*

Introduction

Sperm management by hormone treatment can become a vital tool for increasing of artificial reproduction success in aquaculture. Reproductive hormones are widely used to stimulate reproductive processes and induce spermiation and spawning. Among various reproductive hormones, pituitary gland (PG) extracts, human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone agonist (GnRHa) are commonly used for inducing or maintaining spermiation in many fish species (Miranda et al., 2005, Rzemieniecki et al., 2004). The pituitary gland produces and stores gonadotropin hormones, which play a decisive role in ovulation and spermiation. Human Chorionic Gonadotropin (hCG) is purified gonadotropin hormone used for induction of spawning and acts directly at the level of the gonad. Over recent years, hCG has been increasingly employed in spawning induction trials of many fish species. PG and hCG have been used to induce spermiation in Japanese eel (*Anguilla japonica*) (Kagawa et al., 2005), mullet (*Mugil cephalus*) (El-Greisy and Shaheen, 2007), bream (*Abramis brama*) (Kucharczyk et al., 1997), Pangasiid catfish (Legendre et al., 2000), and European eel (*Anguilla anguilla*) (Asturiano et al., 2005).

Several different hormones have been tested on European eels by now but the question of finding new, more optimal hormones still remains relevant (Asturiano et al., 2005). One of the specific features of the hormonal treatment of European eels is that it consists of consecutive weekly injections and usually lasts up to 12 weeks (Pérez et al., 2000). This applies to both males and females, and sometimes unsynchronized maturations can occur, which complicates fisheries practice. To overcome this obstruction, diluting media (extenders) were developed to avoid these problems and to improve the sperm handling. Usually, the diluents are used to maintain a high motility after short-term storage of spermatozoa (Penaranda et al., 2010), however the mechanism of action of these diluents is still not clear (Fauvel et al., 2012).

European eels are of high interest for aquaculture, thus better knowledge of their spermatology are valuable for artificial reproduction. Nowadays, different aspects of eel sperm biology become fairly well studied.

Cryopreservation of European eel sperm becomes currently popular and well-studied in different directions (Asturiano et al., 2016; Kasa et al., 2017; Penaranda et al., 2009; Szabo et al., 2005). Therefore, the morphology and viability of eel spermatozoa (Asturiano et al., 2007; Marco-Jimenez et al., 2006b) and effects of different agents on eel spermatozoa cryoresistance are also fairly well investigated (Garzon et al., 2008; Marco-Jimenez et al., 2006a). The initiation of eel sperm motility is also of high interest. Recent researches indicate the roles of calcium, sodium and potassium in the maintenance of spermatozoon volume, intracellular pH and the initiation of eel spermatozoa motility (Perez et al., 2016; Vilchez et al., 2016, 2017). In addition, the presence of different subpopulations of spermatozoa after the activation of eel sperm were found, which is additionally affected by hormonal treatment and the thermal regime (Gallego et al., 2015).

Just after release in water, fish spermatozoa require a source of energy to launch and sustain flagellar activity, in order to maintain sperm motility. The major role in the spermatozoa bioenergetics is played by the metabolism of adenylate-phosphates and creatine- / adenylate-phosphates. Generally, ATP is considered as an essential factor for initiation of force generation. ATP could be regenerated from ADP in the axoneme by the activity of adenylate kinase (Cosson, 2004, 2012; Saudrais et al., 1998) and as a complement, Creatine-Phosphate (CP) also supports the maintenance of ATP levels after the initiation of motility via creatine-phosphokinase reaction in spermatozoa of chub (*Leuciscus cephalus*) (Lahnsteiner et al., 1992; Terner and Korsh, 1963; Turman and Mathews, 1996), trout (Saudrais et al., 1998), carp and sterlet (Dzyuba et al., 2016). However, macroergic phosphates do not only sustain sperm

movement but also exert regulatory functions (Dzyuba et al., 2017). It is known that increased ADP concentration in contact with the axoneme exerts an inhibitory effect on flagellar beating (Okuno and Brokaw, 1979). Cyclic Adenosine Mono-Phosphate (cAMP) plays a regulatory role in the initiation of sperm motility. Intracellular concentration of cAMP increases in sperm cells of some, but not all fish species, by the contribution of adenylate cyclase activity that leads to set in motion the sliding of axonemal microtubules and flagella movement, which is supported by dynein ATPase activity. The later causes a rapid intracellular ATP consumption, as described in several species such as: rainbow trout (Oncorhynchus mykiss) (Christen et al., 1987), common carp (*Cyprinus carpio*) (Boryshpolets et al., 2009a; Perchec et al., 1995), sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*) (Dreanno et al., 2000; Spiropoulos et al., 2002; Terner and Korsh, 1963), eurasian perch (*Perca fluviatilis* L.) (Boryshpolets et al., 2009b), and sturgeon (Billard et al., 1999; Fedorov et al., 2015). Energy-supplying pathways in spermatozoa may be fundamentally different among different fish species. The duration of sperm motility among species is highly variable, which suggests that ATP can be stored and generated by different metabolic strategies present in fish spermatozoa (Cosson, 2012). Additionally, levels of ATP, ADP, and AMP of fish spermatozoa can be greatly affected by *in vitro* storage suggesting importance of these parameters for success of short-term storage (Zietara et al., 2004).

However, the bioenergetical side of European eel spermatozoa metabolism has been so far poorly investigated. Thereby, the quantification of creatine- and adenylate- phosphates during the motility period in European eel spermatozoa at different phases of hormonal treatment is currently of high interest.

The main objective of the study was to estimate the content of creatine- and adenylate phosphates after motility activation of European eel spermatozoa at the beginning and peak of spermiation induced by hCGrec hormonal treatment. Additionally, the influence of *in vitro* sperm storage on the content of creatine- and adenylate phosphates was studied.

Material and methods

Fish rearing conditions and hormonal treatment

Ten farmed European eel (*Anguilla Anguilla*) males (124.8 ± 20.7 g of body weight) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved into the facilities of the Institute for Animal Science and Technology (ICTA) at the Universidad Politecnica de Valencia. Fish were gradually acclimatized to seawater (salinity 37.0 ± 0.3 g l^{-1}; 20–22 °C) for 10 days. Fish were put in 90 l aquarium equipped with separated recirculation system (total recycling in 24 hours) and tagged with passive integrated transponders (P.I.T. tags) injected into the epaxial muscle for individual identification. Fish were fasted during the experiment. Males were hormonally induced to maturation and spermiation with weekly intraperitoneal injections of recombinant hCG (hCGrec) (1.5 IU g BW^{-1}) (Ovitrelle; Madrid, Spain), as described previously by Pérez et al. (Pérez et al., 2000).

During the experiment, the fish were starved and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

Sperm collection and sampling

Once a week during the spermiation period, from week 6–10 of hCGrec treatment, fish were sampled 24 h after the hormone administration. After cleaning the genital area with fresh water and thoroughly drying to avoid contamination of samples with feces, urine and seawater, total expressible milt was collected in a graduate tube after applying gentle abdominal pressure to anaesthetised males (benzocaine; 60 mg l^{-1}). Total expressible milt was expressed as ml 100 g fish^{-1} and samples were maintained at 4 °C. Determination of spermatozoa concentration was performed immediately after collection from all males and by the same technician to avoid bias in assessment. Concentration was estimated following the methodologies specified in Gallego et al. (Gallego et al., 2012).

Sperm motility recording and acquisition of motility parameters

All the motility analyses were performed following a method standardized by Gallego et al., 2013, by triplicate at 15 seconds after activation by the motility module of ISAS (PROiSER R + D, SL, Paterna, Spain) using an ISAS 782M camera recorder (60 fps; Hz). The chamber used in all experiments was a SpermTrack-10 (PROiSER) with × 10 negative phase contrast lens on a Nikon Eclipse (E-400) microscope.

Preparation of samples for content analysis of creatine, CP, AMP, ADP, ATP, and cAMP

Spermatozoa were diluted 1:100 in activation medium (AM) that consisted of sea water (SW). For the separate storage experiment, sperm was diluted 1:50 with P1 extender (Asturiano et al., 2004; Asturiano et al., 2003). After dilution with SW, 3 M perchloric acid (PCA) was added at 1:1 v/v (Lahnsteiner and Caberlotto, 2012) to activated sperm samples at 0, 60, and 120 s post-activation. After dilution with P1, 3 M PCA was added at 1:1 v/v to these diluted samples at 0, 1, 2, 3, and 7 days post-dilution. Samples were subsequently frozen in liquid nitrogen and held at -80 °C for 14 days. The samples were thawed at 4 °C and then stored on ice. The sample preparation for LC/HRPS analysis was carried out as follows: 1) centrifugation at 17 000 g for 14 min to collect the protein-free supernatant to avoid possible interference; 2) adjusting the pH range to 4.0–8.0 by addition of 12 M KOH to the supernatant; 3) centrifugation at 3000 g to separate the precipitate from the $KClO_4$ solution; 4) dilution of the supernatant with 1 M Tris-HCl (pH = 7.0) at 1:1 v/v to adjust the pH to 7.0; and 5) filtering through 0.45 µm regenerated cellulose filters (Labicom, Olomouc, Czech Republic) into autosampler vials.

LC/HRPS quantification of adenine nucleotides, CP, and creatine

Adenine nucleotides of interest and CP were detected using a hybrid quadrupole/orbital trap mass spectrometer (Q-Exactive mass spectrometer, Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Accela 1250 LC pump (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland). The LC method previously published by Jiang et al. (Jiang et al., 2012) was adapted to the LC/HRPS method for the analysis of target compounds. In brief, a Hypercarb column (50 mm × 2.1 mm ID × 3 µm particles; Thermo Fisher Scientific) was used to separate the target analytes. The mobile phases used for the separation consisted of ultrapure water (aqua-MAX-Ultrasystem, Younglin, Kyounggi-do, Korea) and acetonitrile (LiChrosolv Hypergrade, Merck, Darmstadt, Germany). More details about the method adaptation are in the previously published method (Fedorov et al., 2017).

The method's performance was assessed in terms of its linearity, repeatability, matrix effect, limit of quantification (LOQ), and trueness. For the quantification of target compounds, external calibration, ranging from 1 to 500 ng mL^{-1}, was used. The content of CP, AMP, ADP, ATP, and cAMP was expressed as nmol per 109 spermatozoa (spz), taking into account all dilutions of the sperm sample during activation and extraction.

Adenylate energy charge (AEC) proposed by Atkinson (1968) is defined as:

$$AEC = \frac{[ADP] + 2\,[ATP]}{[AMP] + [ADP] + [ATP]} \times \frac{1}{2} \times 100$$

Statistical analysis

The content of aforementioned creatine- and adenylate phosphates at 0 sec post-activation for each male was designated as 100% to reduce a high scatter in absolute values among males. All values of CP, AMP, ADP, ATP, and cAMP content were expressed as a relative (to 0 sec post-activation) content in percentage (%) and then statistical analysis was conducted. Due to a low number of observations ($n = 10$ for the conditions of motility activation and $n = 5$ for the storage of sperm in P1), a nonparametric Kruskal-Wallis ANOVA, followed by multiple comparisons of mean ranks for all groups, was used to compare the content of tested substances. Data were presented as medians with quartiles. Statistical significance was accepted at $P < 0.05$. Analyses and graphing were conducted using Statistica v. 13 (Statsoft Inc, Tulsa, OK, USA).

Results and discussion

The content of CP, AMP, ADP, ATP, and cAMP was measured in eel sperm after dilution with SW, which led to motility activation. For this purpose, we used the sperm from the beginning (sixth week after first injection) and the end (tenth week after the beginning of hormonal treatment) of the induced spermiation. We found a high male-to-male scatter in absolute levels of substances – up to 5 times from the lowest to highest, in all compounds but there are certain trends for each male. This dictates the necessity to normalize the absolute values of metabolites content to percentage changes with time to hide these big deviations.

Motility percentage, velocity curvilinear (VCL), and straight line velocity (VSL), estimated at the 15th sec post-activation of eel spermatozoa obtained at the peak of induced spermiation were 77.6% ± 7.7%, 146.5 ± 18.5 µm s^{-1}, 87.7 ± 16.0 µm s^{-1} respectively ($n = 10$) and were not significantly changed up to 120 sec post-activation. The values of obtained parameters corresponds fairly well with already studied using the same treatment (Gallego et al., 2013, 2012).

Spermatozoa from the beginning of spermiation did not initiate their motility in SW (motility < 5%), while spermatozoa from the end of spermiation period presented a motility percentage > 80% in average. We found no detectable cAMP in the spermatozoa collected at the end of spermiation period prior to motility activation; same occurred in spermatozoa from sixth week of hormonal treatment, prior and during whole post-activation period. However, the cAMP content significantly increased to a level of 0.5 nmol 10^{-9} spz in spermatozoa collected at the end of spermiation and activated in AM. There was, previously, an attempt to quantify cAMP content in the spermatozoa of sturgeon, and whitefish but with no success, even though the method itself is well elaborated for the quantification of cAMP in the fish sperm (Fedorov et al., 2017). Therefore, the current study is the first successful quantification of cAMP in fish spermatozoa using the LC/HRPS.

The presence of cAMP after the initiation of motility is probably the result of some regulatory physiological functions. Factors such as ATP or cAMP were shown to be responsible for the initiation and possibly maintenance of motility, as it was demonstrated by Morisawa and Okuno (1982) in salmonid sperm (Morisawa and Okuno, 1982). In contrast, we did not detect a significant amount of cAMP in eel spermatozoa after dilution in AM of the sperm collected at the begining of the spermiation period (sixth week of hormonal treatment), a situation where spermatozoa were unable to be activated. There was no cAMP detected within the whole post-activation period as well. These observations have two important implications: 1- the LC/HRPS method is successfully applicable for the measurement of cAMP content in fish sperm; 2- cAMP is an important factor related to maturation of eel sperm. It is remarkable that the cAMP concentration considerably increases within the 60 sec period following transfer into AM. A similar large and fast drop in the cAMP concentration was interpreted in salmonid sperm as being directly responsible of a protein phosphorylation leading to the motility-activating signal (Morisawa and Okuno, 1982). In case of eel sperm, this mechanism probably does not apply as it was shown by Gibbons et al. (1985) that cAMP is not needed to activate eel sperm motility.

We determined by LC/HRPS that the cAMP value ranges 0.5 nmol 10^{-9} spz spermatozoa in eel sperm where motility was activated since 60 seconds. This value compares to values obtained by Morisawa and Ishida (1987) for trout sperm immediately after motility activation (0.16 nmol 10^{-9} spz) and by Cosson et al. (1995) for trout sperm at end of the motility period (0.4 to 0.8 nmol 10^{-9} spz) using determination by a radioimmunoassay, in both cases (Morisawa and Ishida, 1982).

In case of the activated sperm collected during the peak of hormonally induced spermiation (potentially motile spermatozoa), we found a certain levels of all targeted compounds: CP, AMP, ADP, ATP, and cAMP. However, in the sperm collected during the beginning of the spermiation period and after diluted in AM (immotile spermatozoa), no CP was found. The level of CP in matured spermatozoa prior to and during post-activation was approximately 100 nmol 10^{-9} spz. AMP level remains in the same range (without significant changes of the content within the maturation period). ADP content was not statistically different in spermatozoa from start and end of the hormonal treatment during the whole post-activation period. ATP content prior to dilution of immotile sperm into AM was about 30 times lower.

According to literature, the relationship between ATP, ADP and flagella motility is not straightforward: Lindemann and Kanous (1989) showed that changes in the balance of ATP, Mg2+, and ADP are critical to establishment of the beat cycle of mammalian sperm flagella (Lindemann and Kanous, 1989). The relationship between ATP and ADP was explored in sea urchin spermatozoa by Yoshimura et al., who reported that, at neutral pH, ATP concentration of 1.0 mM inhibits motility (Yoshimura et al., 2007), and this inhibition can be counteracted by ADP (Kinoshita et al., 1995; Omoto et al., 1996), suggesting multiple roles of ATP in the dynein motile activity.

Generally, ATP is considered as a necessary factor for initiation of force generation, but in some conditions (such as at high concentrations) ATP can be an inhibitory factor reducing the velocity of movement (Inoue and Shingyoji, 2007). These authors hypothesized that ATP-induced inhibition and ADP-induced activation are probably acting through phosphorylation/dephosphorylation of the dynein outer arm associated protein(s). Whether a similar situation exists in fish spermatozoa remains to be better elucidated (Dzyuba et al., 2016). In some species such as trout, it was shown that the cAMP control of motility is itself regulated by the intracellular ATP/ADP levels (Cosson et al., 1995). On another hand, it was shown that in eel spermatozoa, cAMP is not playing a role in the initiation of flagella motility (Gibbons et al., 1985). Therefore, the change of cAMP sperm content observed in the present study

between the 6th and 10th week of hormonal induction cannot explain directly the change in the potentiality to be activated during the same period.

The decrease of adenylate nucleotides content and AEC values always appeared prior to the decline of motility parameters. However, in contrast with the results of Dreanno et al. (1999) obtained for turbot spermatozoa, no decrease of AEC during the 2 minutes post-activation was observed neither for spermatozoa from the 6th week of hormonal treatment nor for spermatozoa from the 10th week of hormonal treatment (Fig. 1). This suggests the existence of different metabolic pathways in spermatozoa of these two different fish species. Level of AEC in spermatozoa from the 10th week of hormonal treatment, which presented normal motility rate (77.6% ± 7.7%) was twice the AEC level in spermatozoa from the 6th week (fully immotile) during the whole post-activation period. This is in agreement with the idea developed for *E. coli* that viability of cells is maintained at EAC values between 80 and 50 (Chapman et al., 1971), while immotile eel spermatozoa from the beginning of hormonally induced spermiation represented AEC lower than 50. Moreover, AEC of motile eel spermatozoa in the present study corresponds well with the data previously reported for various organisms and tissues and supports the prediction, that the energy charge value is stabilized near 85 in intact metabolizing conditions for a wide variety of cell types (Chapman et al., 1971; Wiebe and Bancroft, 1975).

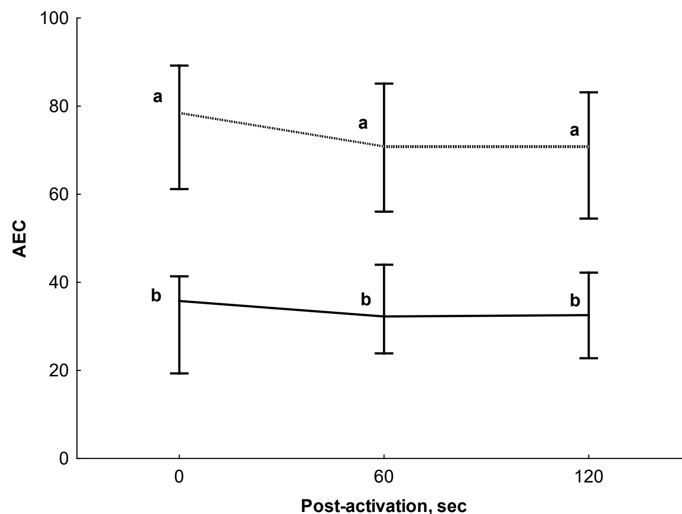

Figure 1. *Changes of adenylate energy charge (AEC) vs. time after dilution in activating medium (SW). (— spermatozoa from the 6th week of hormonal treatment; · · · · spermatozoa from the 10th week of hormonal treatment). Data presented as medians with quartiles. Values marked by different letters are significantly different (multiple comparisons of mean ranks for all groups, P < 0.05, n = 10).*

The next part of our experimental protocol aimed to measure CP, AMP, ADP, ATP, and cAMP during storage of European eel sperm in P1 without activation of motility by AM. For this purpose, sperm from the peak period of hormonally induced spermiation was used (10th week of treatment). Two conditions of storage were tested: at 4 °C and 20 °C for a period of 7 days.

The percentage of motile eel spermatozoa stored either at 4 °C or at 20 °C significantly decreased after the one day of storage (Fig. 2A) without significant difference in the percentage of motile cells between these two storage conditions. After a one day storage period, motility percentage of sperm stored at 4 °C did not significantly change until the seventh day of

storage, while motility percentage of sperm stored at 20 °C decreased to zero level by the end of the storage (Fig. 2A). Parameters such as VCL and VSL presented the tendency as motility percentage during the whole period of storage at 4 °C and 20 °C (Fig. 2B, C). These results about motility parameters are in good agreement with those already published on the short-term storage of European eel sperm (Asturiano et al., 2016; Penaranda et al., 2010).

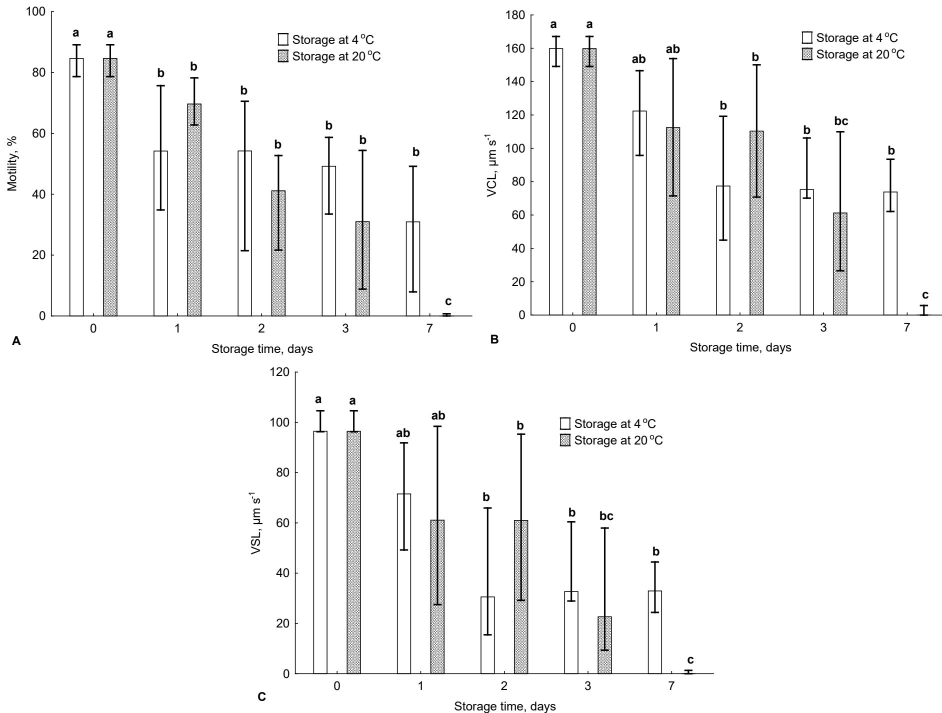

Figure 2. *The motility parameters of European eel sperm stored in P1 (at ratio 1:50) at 4 °C and 20 °C with agitation. Motility – A, velocity curvilinear (VCL) – B, velocity straight-line (VSL) – C. Data presented as medians with quartiles. Values marked by different letters (a–c) are significantly different (multiple comparisons of mean ranks for all groups, P < 0.05, n = 5).*

cAMP was not significantly detected in sperm cells from both conditions of storage within the whole period of investigation. In case of the storage at 20 °C, CP, ADP, and ATP decreased to a zero level until the end of the period of storage (Fig. 3A, C, D). During the storage at 4 °C, the decrease of CP, ADP, and ATP was also observed but to a 50% value as compared to the initial level at the beginning of the storage (Fig. 3A, C, D). Decrease of AMP did not present any significant change between the storage of eel sperm in P1 at 4 °C and 20 °C within the whole period of storage while the level of AMP decreased to approximately 10% of its initial level (Fig. 3B).

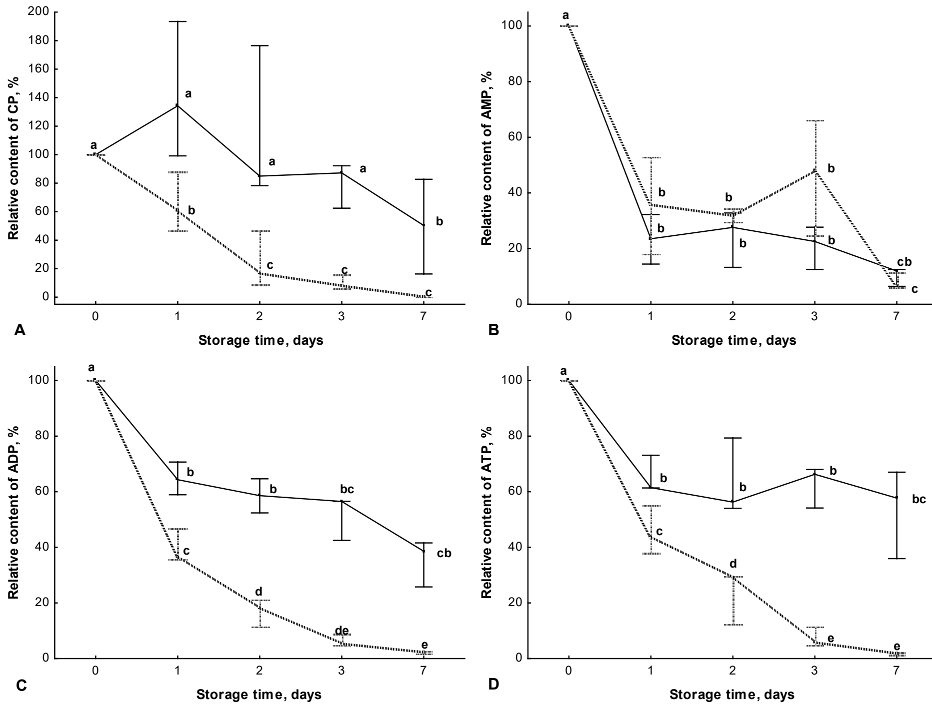

Figure 3. *Relative content of CP – creatine phosphate – A, AMP – B, ADP – C, and ATP – D in European eel sperm from the tenth week of hormonal treatment without dilution in seawater. Sperm was pre-diluted by P1 and was stored at 4 °C (—) and at 20 °C (· · · ·). Note: cAMP was not found at both conditions of storage. Data presented as medians with quartiles. Values marked by different letters (a–e) are significantly different (multiple comparisons of mean ranks for all groups, P < 0.05, n = 6).*

AEC dropped after 3 days of sperm storage at 20 °C, while it increased during the storage at 4 °C (Fig. 4). This suggests that the rate of ATP hydrolysis during the short-term storage at 20 °C is larger than the rate of ATP production by mitochondrial respiration. Similarly as for activated eel spermatozoa, AEC level during short-term storage of eel spermatozoa at 4 °C (Fig. 4) supports the prediction, that the energy charge of intact metabolizing cells is stabilized near 85 (Chapman et al., 1971; Wiebe and Bancroft, 1975).

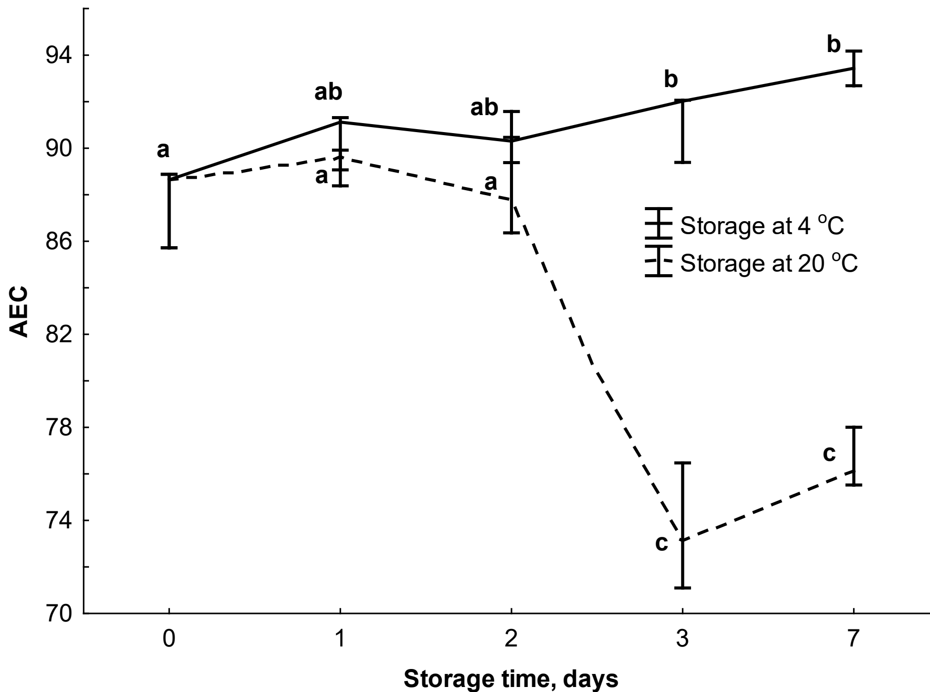

Figure 4. *Changes of adenylate energy charge (AEC) vs. time after dilution of European eel sperm in P1 extender (— storage at 4 °C; ···· storage at 20 °C). Data presented as medians with quartiles. Values marked by different letters (a-c) are significantly different (multiple comparisons of mean ranks for all groups, $P < 0.05$, $n = 6$).*

Conclusions

Spermatozoa at a sixth week of hormonal injections are not able to initiate their motility after their release into activating conditions. However, by the end of the hormonal treatment (tenth week), they acquire the ability to be activated after the dilution in AM. This is accompanied by the concomitant increase of CP and cAMP concentrations in spermatozoa. That momentous fact suggests the important role of the aforementioned substances in the process of eel sperm motility acquisition during the hormonally induced spermiation period.

Our results represent the first successful estimation of cAMP in fish spermatozoa during the motility period using the LC/HRPS, which will potentially improve the possibilities of description of the mechanism involved in the sperm motility activation in fish.

Short-term storage of eel sperm at 4 °C accompanied by higher macroergic phosphates content and higher AEC in comparison to the storage at 20 °C. This suggests that the rate of ATP hydrolysis during the short-term storage at 20 °C is larger than the rate of ATP production by mitochondrial respiration.

Obtained results could contribute to the development of new effective methods for improving of spermiation and short-term sperm storage in European eel aquaculture.

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CHAPTER 5

EFFECT OF ENVIRONMENT OSMOLALITY ON WHITEFISH SPERM SUBPOPULATIONS AND CONTENT OF CREATINE- AND ADENYLATE PHOSPHATES

Fedorov, P., Pastor, F.M., Fedorova, G., Grabic, R., Dzyuba, V., Rodina, M., Dzyuba, B. Effect of environment osmolality on whitefish sperm subpopulations and content of creatine- and adenylate phosphates. (manuscript)

My share on this work was about 70%.

EFFECT OF ENVIRONMENT OSMOLALITY ON WHITEFISH SPERM SUBPOPULATIONS AND CONTENT OF CREATINE- AND ADENYLATE PHOSPHATES

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ABSTRACT

At activation of fish sperm motility, flagellar dynein ATPase causes a rapid intracellular ATP consumption. ATP can be regenerated from ADP in axoneme by adenylate kinase activity and from creatine phosphate via creatine-phosphokinase. Motility duration and intracellular levels of macroergic phosphates (ATP, ADP, creatine phosphate) in fish spermatozoa are greatly affected by environmental factors such as osmolality and ionic composition. The aim of the present study was to investigate how the osmolality of the activating medium influences subpopulation distribution of motile spermatozoa, in relation with the contribution of creatine- and adenylate-phosphates changes observed at initiation and during the motility period. The levels of ATP, creatine phosphate and adenylate energy charge significantly decreased after dilution of sperm by activation media (AM) of 100, 200 or 300 mOsm kg^{-1} NaCl. Four sperm subpopulations with different motility characteristics were identified after cluster analysis of CASA data (Computer-Aided Sperm Analysis). The most abundant subpopulation at the initial stage of motility in AM was defined by spermatozoa with high velocity and linearity. AM 300 mOsm kg^{-1} supported longer motility and the prevalence of the subpopulation presenting longer period of straight-line and fast motility for a longer time. There was no relationship between content of creatine- and adenylate phosphates and cluster dynamics in spermatozoa activated in AM of different osmolality. Isotonic conditions favor the subpopulation of spermatozoa presenting longer period of straight-line and fast motility without increase in macroergic phosphates content.

Keywords: *sperm motility, Coregonus lavaretus maraena, clusters analysis, sperm bioenergetics*

Introduction

Spermatozoa of most fish species with external fertilization are maintained immotile in the seminal duct because motility is prevented by the osmolality and composition of the seminal fluid, but initiate motility immediately after dispersion into the water environment (Morisawa et al., 1983). Just after release in water, spermatozoa require a source of energy to launch and sustain flagellar activity, in order to maintain sperm motility.

The major role in the spermatozoa bioenergetics is played by the metabolism of creatine- and adenylate phosphates. These macroergic phosphates do not only sustain sperm movement and other functions, but also exert regulatory functions (Dzyuba et al., 2017). It is known that increased ADP concentration in contact with the axoneme exerts an inhibitory effect

on flagellar beating (Okuno and Brokaw, 1979). The relationship between ATP and ADP is not straightforward: Lindemann and Kanous (1989) showed that changes in the balance of ATP, Mg^{2+}, and ADP are critical to establishment of the beat cycle of mammalian spermatozoa (Lindemann and Kanous, 1989). Whether the same situation exists in fish spermatozoa remains to be better elucidated (Dzyuba et al., 2016). 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 (Yoshimura et al., 2007) suggesting multiple roles of ATP in the dynein motile activity (Kinoshita et al., 1995; Omoto et al., 1996). Generally, ATP is considered as an essential factor for initiation of force generation, and in some conditions (such as at high concentrations) ATP can be an inhibitory factor reducing the velocity of movement (Inoue and Shingyoji, 2007). These authors hypothesized that ATP-induced inhibition and ADP-induced activation are probably acting through phosphorylation/dephosphorylation of the dynein outer arm associated protein(s).

The sliding of axonemal microtubules and flagella movement is supported by dynein ATPase activity. The later causes a rapid intracellular ATP consumption, as described in several species such as: rainbow trout (*Oncorhynchus mykiss*) (Christen et al., 1987), common carp (*Cyprinus carpio*) (Boryshpolets et al., 2009a; Perchec et al., 1995), sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*) (Dreanno et al., 2000, Spiropoulos et al., 2002; Terner and Korsh, 1963), eurasian perch (*Perca fluviatilis* L.) (Boryshpolets et al., 2009b), and sturgeon (Billard et al., 1999; Fedorov et al., 2015). ATP can be regenerated from ADP in the axoneme by the activity of adenylate kinase (Cosson, 2004, 2012; Saudrais et al., 1998). Creatine-Phosphate (CP) also supports the maintenance of ATP levels after the initiation of motility via creatine-phosphokinase reaction, as demonstrated in spermatozoa from chub (*Leuciscus cephalus*) (Lahnsteiner et al., 1992; Terner and Korsh, 1963; Turman and Mathews, 1996) carp and sterlet (Dzyuba et al., 2016). The content estimation of all aforementioned adenylate phosphates gives a possibility to calculate the adenylate energy charge (AEC). This fundamental metabolic control parameter is used to measure the energy status of biological cells (Atkinson, 1968). It describes the ubiquitous regulatory signal in metabolism – the status of the adenylate system, which affects the rates of nearly all metabolic conversions and the partitioning of metabolically available substances between oxidation, synthesis of cell substance, and production of storage compounds (Atkinson, 1968; Chapman et al., 1971; Wiebe and Bancroft, 1975). This parameter was also successfully applied for the estimation of the energy status of fish spermatozoa as well (Dreanno et al., 1999b)

Energy-supplying pathways in spermatozoa may be different among different fish species. The duration of motility among species is different, which suggests that ATP can be stored and generated by different metabolic strategies in fish spermatozoa (Cosson, 2012). After activation, motility duration and levels of ATP, ADP, and CP in fish spermatozoa are greatly affected by environmental factors such as osmotic pressure and ionic composition (Cosson, 2004). Sensitivity of fish spermatozoa to osmolality and ion concentrations is one of the important factors controlling motility, which are related to ionic channel activities in the membrane and govern the motility mechanisms of axonemes (Cosson, 1999). In addition, a very important question is the amount of energy involved into the process of osmotic shock and ionic pumps activity. In a situation of low osmotic shock (when spermatozoa are unable to be activated), there is low energy consumption by ionic pumps and very low dynein ATPase activity. Oppositely, a high energy consumption is needed to sustain a normal physiological state of spermatozoa and high dynein ATPase activity following the high osmotic shock needed for rendering spermatozoa motile. Therefore, it is hypothesized that hypotonic conditions may lead to a higher energy consumption in comparison to isotonic conditions.

Contrarily to the initial idea that all spermatozoa from the same spermiation were activated similarly and would have a similar motility pattern (Cosson, 2007), recent researches have shown that different sperm subpopulations with different motility parameters could coexist within the sperm of the same male (Ferraz et al., 2014; Quintero-Moreno et al., 2003). Subpopulation analysis has been applied in a few studies in fish, including eels (genus Anguilla) (Gallego et al., 2015), the sole fish (*Solea senegalensis*) (Beirão et al., 2009; Martínez-Pastor et al., 2008), sea bream (*Sparus aurata*) (Beirão et al., 2011), and steelhead (*Oncorhynchus mykiss*) (Kanuga et al., 2012). In these studies, three to four subpopulations of spermatozoa were identified, one being defined as more desirable (containing fast and linearly motile cells) (Beirão et al., 2009; Martínez-Pastor et al., 2008). However, there are no studies about the relationship between sperm motility subpopulations and energy consumption or levels of macroergic phosphates. This topic is of high interest for the general description of sperm quality, combining sperm energy balance and the distribution of spermatozoa subpopulations.

Whitefish (*Coregonus lavaretus maraena*) was choosen as our experimental fish because its spermatozoa can be activated by both iso- and hypo-tonic conditions, and addition of 5 mM potassium chloride inhibits the motility in both conditions. Thereby, it is easy to manipulate motility activation and maintenance by adjusting the activation medium composition and its osmolality. In addition, whitefish spermatozoa have very interesting motility characteristics: during the motility period, there are clearly two populations of spermatozoa, with either straight or curved trajectories. This observation might confirm the presence of spermatozoa subpopulations defined by different motility parameters, which invites to apply cluster analysis, and consequently to establish the correspondence with the content of creatine- and adenylate phosphates.

We hypothesized that fish spermatozoa can change energy consumption and motility parameters in response to environment osmolality. The aim of this study was to check how osmolality of activating medium influences the content of creatine- and adenylate phosphates and motility parameters of whitefish spermatozoa from different subpopulations.

Material and methods

Ethical clearance

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 (RIFCH), Vodnany, Czech Republic. The FFPW USB and the RIFCH ran all experiments under the authorization for the use of experimental animals, reference number: 53100/2013-MZE-17214, and the authorization for breeding of experimental animals and delivery of experimental animals, reference number 53103/2013-MZE-17214, accredited by the Ministry of Agriculture of the Czech Republic. Scientists were trained to work with animals according to the Act No. 246/1992 on the protection of animals against cruelty. Technical workers were regularly trained according to the Public notice of the Ministry of Agriculture of the Czech Republic No. 419/2012.

Fish rearing conditions and sperm sampling

Whitefish, Coregonus lavaretus maraena, was selected as a model fish species. Sperm was obtained from 6 genotypically pure whitefish males (3–4 years, 0.6–1.0 kg body weight) at the Tisová hatchery (Rybářství Mariánské Lázně s.r.o.) during the natural spawning season (November) and collected in plastic syringes by gentle stripping, to avoid contamination by

urine, mucus, feces, or water that can lead to a decline in sperm quality. Semen was transported on ice to the laboratory within about 15 min from collection of first sample.

For the evaluation of sperm concentration, sperm was diluted in 0.9% NaCl solution at a ratio of 1:10000 (sperm/saline). Sperm concentration was calculated after counting the number of spermatozoa in 12 cells of a Burker hemocytometer (Meopta, Czech Republic) at ×200 magnification with an Olympus BX 50 phase-contrast microscope (Olympus, Japan).

Preparation of samples for ATP, ADP, AMP, creatine phosphate (CP), and creatine analysis

The obtained spermatozoa were diluted 1:100 in activation medium (AM) differing on osmolality (100, 200, 300 mOsm kg^{-1} NaCl; all media contained 10 mM Tris-HCl, pH 8.0) and non-activating media (NAM) of same osmolalities. Sperm motility in NAM was prevented by addition of 5 mM KCl. Motility duration was defined as the period of time to cessation of movement in 95% of spermatozoa visible in the microscopic field. After dilution with AM or NAM, 3 M perchloric acid (PCA) was added at 1:1 v/v to activated sperm samples (Lahnsteiner and Caberlotto, 2012) at 0, 20, 60, and 90 sec post-dilution. Samples were subsequently frozen in liquid nitrogen and held at -80 °C for 14 days. The samples were thawed at 4 °C and then stored on ice. The sample preparation for LC/HRPS analysis was carried out as follows: 1) centrifugation at 17 000 g for 14 min to collect the protein-free supernatant to avoid possible interference; 2) adjusting the pH range to 4.0-8.0 by addition of 12 M KOH to the supernatant; 3) centrifugation at 3000 g to separate the precipitate from the $KClO_4$ solution; 4) dilution of the supernatant with 1 M Tris-HCl (pH = 7.0) at 1:1 v/v to adjust the pH to 7.0; and 5) filtering through 0.45 µm regenerated cellulose filters (Labicom, Olomouc, Czech Republic) into autosampler vials.

LC/HRPS quantification of adenine nucleotides, CP, and creatine

Adenine nucleotides of interest, creatine, and CP were detected using a hybrid quadrupole/orbital trap mass spectrometer (Q-Exactive mass spectrometer, Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Accela 1250 LC pump (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland). The LC method, previously published by Jiang et al. (Jiang et al., 2012), was adapted to the LC/HRPS method for the analysis of target compounds. In brief, a Hypercarb column (50 mm × 2.1 mm ID × 3 µm particles; Thermo Fisher Scientific) was used to separate the target analytes. The mobile phases used for the separation consisted of ultrapure water (aqua-MAX-Ultrasystem, Younglin, Kyounggi-do, Korea) and acetonitrile (LiChrosolv Hypergrade, Merck, Darmstadt, Germany). More details about the method adaptation are in the recently published method (Fedorov et al., 2017).

The method's performance was assessed in terms of its linearity, repeatability, matrix effect, limit of quantification (LOQ), and trueness. For the quantification of target compounds, external calibration, ranging from 1 to 500 ng mL^{-1}, was used. The content of creatine, CP, AMP, ADP, and ATP was expressed as nmol per 109 spermatozoa (spz), taking into account all dilutions of the sperm sample during activation and extraction.

Adenylate energy charge (AEC) proposed by Atkinson (1968) is defined as:

$$AEC = \frac{[ADP] + 2\,[ATP]}{[AMP] + [ADP] + [ATP]} \times \frac{1}{2} \times 100$$

Sperm motility recording and acquisition of motility parameters

Sperm was diluted 1:2000 with AM and motility was immediately recorded until cessation, using a CCD video camera (SONY, SSCDC50AP) mounted on dark-field microscope (Olympus BX 41, 200x magnification; Olympus, Japan) combined with stroboscopic illumination (ExposureScope, Czech Republic). Sperm motility video records were analyzed using CASA-automated plugin (http://www.ucs.mun.ca/~cfpurchase/CASA_automated-files.zip) for ImageJ software (National Institutes of Health, USA). Obtained data were cleaned up and processed into a single dataset. Total motility and the median values of the kinematic variables were calculated for each individual sample (Purchase and Earle, 2012). Eleven parameters of whitefish spermatozoa motility were calculated: MOT – Percent motility, VCL – curvilinear Velocity, VAP – average–path Velocity, VSL – straight–line Velocity, LIN – Linearity, STR – Straightness, WOB – Wobble, ALH – amplitude of lateral head displacement, BCF – Beat cross frequency, DNC – Dance, DNCm – Dance mean. Parameters and ImageJ plugin are described by Wilson-Leedy and Ingermann (2007) (Wilson-Leedy and Ingermann, 2007).

Subpopulation and statistical analyses

Statistical analysis was conducted on ATP, ADP, AMP, creatine, and CP content. Due to a low number of observations (n = 6), a nonparametric Kruskal-Wallis ANOVA, followed by multiple comparisons of mean ranks for all groups, was used to compare ATP, ADP, AMP, CP, and creatine content. Data were presented as medians with quartiles. Statistical significance was accepted at $P < 0.05$. Analyses and graphing were conducted using Statistica v. 13 (Statsoft Inc, Tulsa, OK, USA).

Subpopulation and statistical analyses were carried out using the R statistical environment (R Core Team, 2013). The methodology was based on previous studies (Dominguez-Rebolledo et al., 2009; Gallego et al., 2015; Martinez-Pastor et al., 2005, 2011). In short, CASA data was cleaned up and processed into a single dataset. Total motility and the median values of the kinematic variables were calculated for each individual sample. Subpopulation analysis was carried out by carrying out a hierarchical clustering in each sample and then a second clustering on the median values of the first set of clusters.

Hypothesis testing on motility and clustering results were conducted by using linear mixed-effects models for data from motility and subpopulation analyses, with acquisition time and treatment (media) as fixed effects, and the sample as the grouping factor in the random part of the model. When needed, pairwise comparison between the levels of fixed factors was carried out by using Tukey's correction. Results are presented as mean ± SEM except if otherwise stated.

Results

Quantification of ATP, ADP, AMP, creatine, and CP content

The levels of ATP and CP significantly decreased after dilution of sperm by all AM solutions. However, no differences were observed between the content of the aforementioned compounds in both AM and NAM with different osmolalities (Fig. 1A, E). We did not find changes of target substances content in immotile spermatozoa after releasing into hypo- and iso-tonic conditions. No significant differences in ADP and creatine content between AM and NAM within whole post-activation period were found (Fig. 1B, D), their content remained stable after the dilution with AM and NAM. AMP content was not changed after dilution with

NAM for the whole post-activation period (Fig. 1C). However, when spermatozoa were diluted in AM, the content of AMP significantly increased at 60 sec post-activation, with a subsequent significant decrease to an initial level.

The AEC level was stable for inhibiting conditions (NAM) within post-dilution period (Fig. 2). Activating conditions (AM) represented a significant decrease of AEC after 20 sec post-activation (Fig. 2).

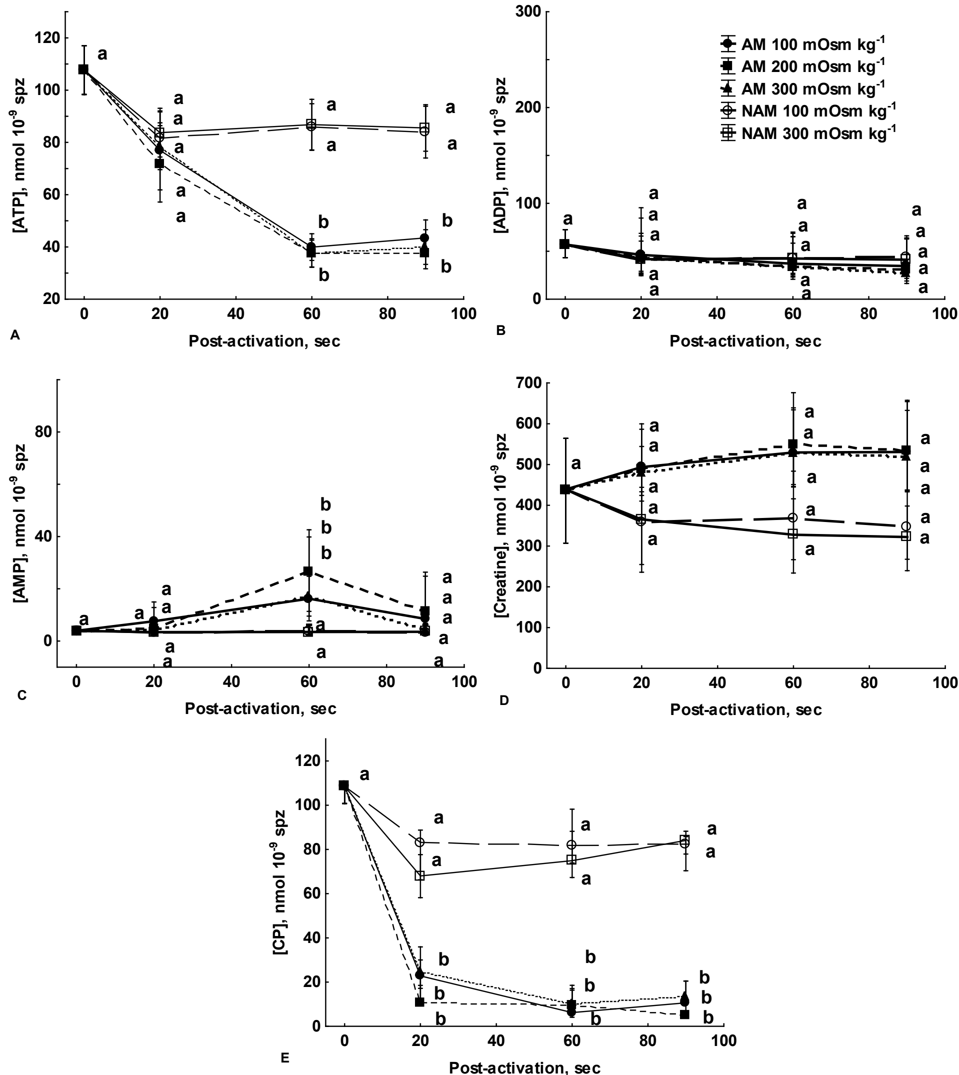


Figure 1. *Creatine- and adenylate-phosphates content of whitefish sperm after dilution with AM and NAM of different osmolalities. ATP (A), ADP (B), AMP (C), creatine (D), and CP (E). Activating conditions: activation media (AM) of osmolalities 100, 200, 300 mOsm kg*$^{-1}$ *NaCl and non-activating media (NAM) of osmolalities 100 and 300 mOsm kg*$^{-1}$ *NaCl. Motility in NAM was prevented by addition of 5 mM KCl. Data are presented as medians with quartiles. Values marked by different letters (a, b) are significantly different (multiple comparisons of mean ranks for all groups, P < 0.05, n = 6).*

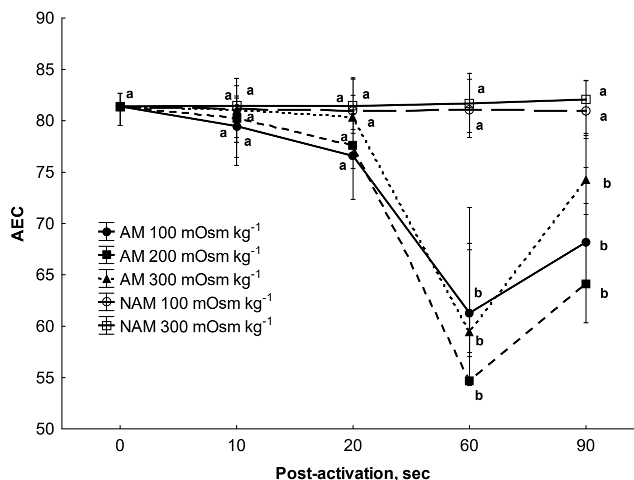
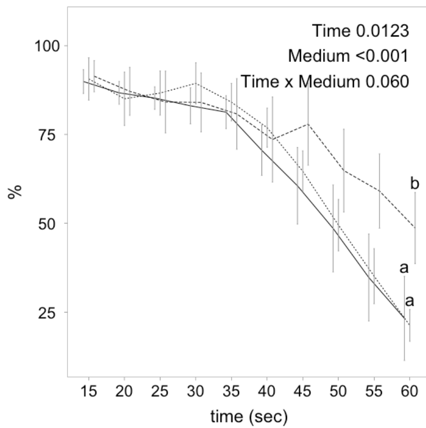


Figure. 2. *Changes of adenylate energy charge (AEC) vs. time after dilution of whitesfish sperm in different activating media (AM) of osmolalities 100, 200, 300 mOsm kg^{-1} NaCl and non-activating media (NAM) of osmolalities 100 and 300 mOsm kg^{-1} NaCl. Motility in NAM was prevented by addition of 5 mM KCl. Data presented as medians with quartiles. Values marked by different letters (a, b) are significantly different (multiple comparisons of mean ranks for all groups, $P < 0.05$, $n = 6$).*

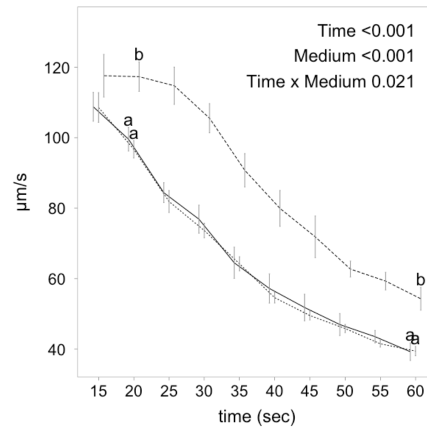
Motility and clusters analysis

Motility decreased with time as expected (Fig. 3). Total motility (Fig. 3a) decreased from 90.6% ± 2.5% at 15 sec post-activation to 31.1% ± 5.9% at 60 sec. The motility was significantly affected by time and treatment, with 300 mOsm kg^{-1} AM yielding slightly higher motility, especially by the end of the analysis time (48.7% ± 10.0% vs. 22.3% ± 5.4% pooled 100 and 200 mOsm kg^{-1} AM data). Sperm velocity was similarly affected, and it was clearly improved by activating with 300 mOsm kg^{-1} AM (Fig. 3b). Linearity variables were less dramatically affected, and the effect of time was not significant, but 300 mOsm kg^{-1} AM also significantly promoted more linear tracks (Fig. 3c). ALH (Fig. 3d) increased significantly with time and was also affected by the media. Activating with 300 mOsm kg^{-1} AM yielded higher ALH than 200 mOsm kg^{-1} AM ($P = 0.0174$), with 100 mOsm kg^{-1} AM being intermediate ($P > 0.05$). Considering pooled data 15-30 sec and 45-60 sec post-activation for comparing with the results for the creatine- and adenylate phosphates at 20 and 60 sec, there were not significant differences between media by 20 sec post-activation but by 60 sec 300 mOsm kg^{-1} AM showed the highest motility. However, velocity and linearity parameters showed the highest values at 300 mOsm kg^{-1} AM. For ALH, differences were significant only by 20 sec post-activation, with 300 mOsm kg^{-1} AM being the highest, with $P < 0.001$ with 200 mOsm kg^{-1} AM and $P = 0.156$ with 100 mOsm kg^{-1} AM.

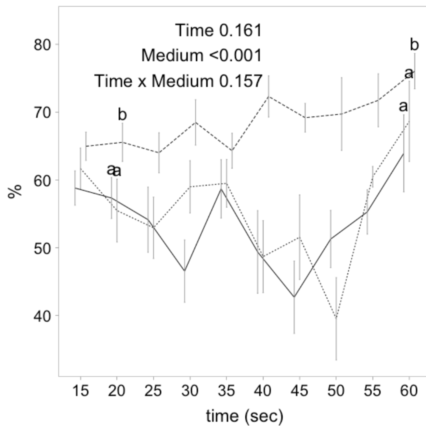
a) Total motility



b) VAP



c) STR



d) ALH

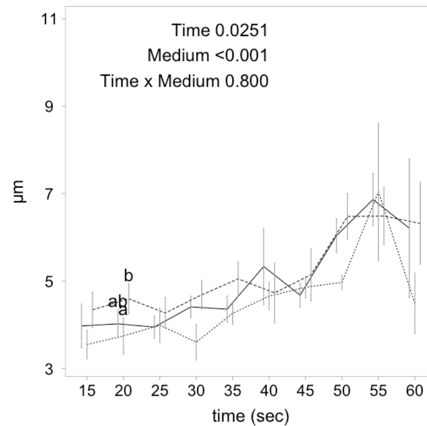


Figure 3. *Evolution of motility from 15 sec to 60 sec post-activation, CASA parameters. Inserts show the signification of the time, medium (— 100 mOsm kg^{-1}; · · · 200 mOsm kg^{-1}; --- 300 mOsm kg^{-1}) and interaction effects. Different letters indicate significant differences between media by 20 sec (15 to 30) or 60 sec (45 to 60), for comparison with results for macroergic phosphates (Fig. 1).*

VSL, VCL, and DNCm parameters were used for clustering analysis as the most influential variables. Four optimal subpopulations were found with different motility parameters: Subpopulation 1: High VCL, high LIN (VCL: 106.7 ± 32.1 µm s^{-1}, LIN 69.4% ± 14.9%), Subpopulation 2: High VCL, low LIN (VCL: 112.4 ± 23.8 µm s^{-1}, LIN 33.2% ± 15.1%), Subpopulation 3: Low VCL, high LIN (VCL: 57.1 ± 16.5 µm s^{-1}, LIN 72.3% ± 15.2%), Subpopulation 4: Low VCL, low LIN (VCL: 57.1 ± 19.4 µm s^{-1}, LIN 31.3% ± 21.9%).

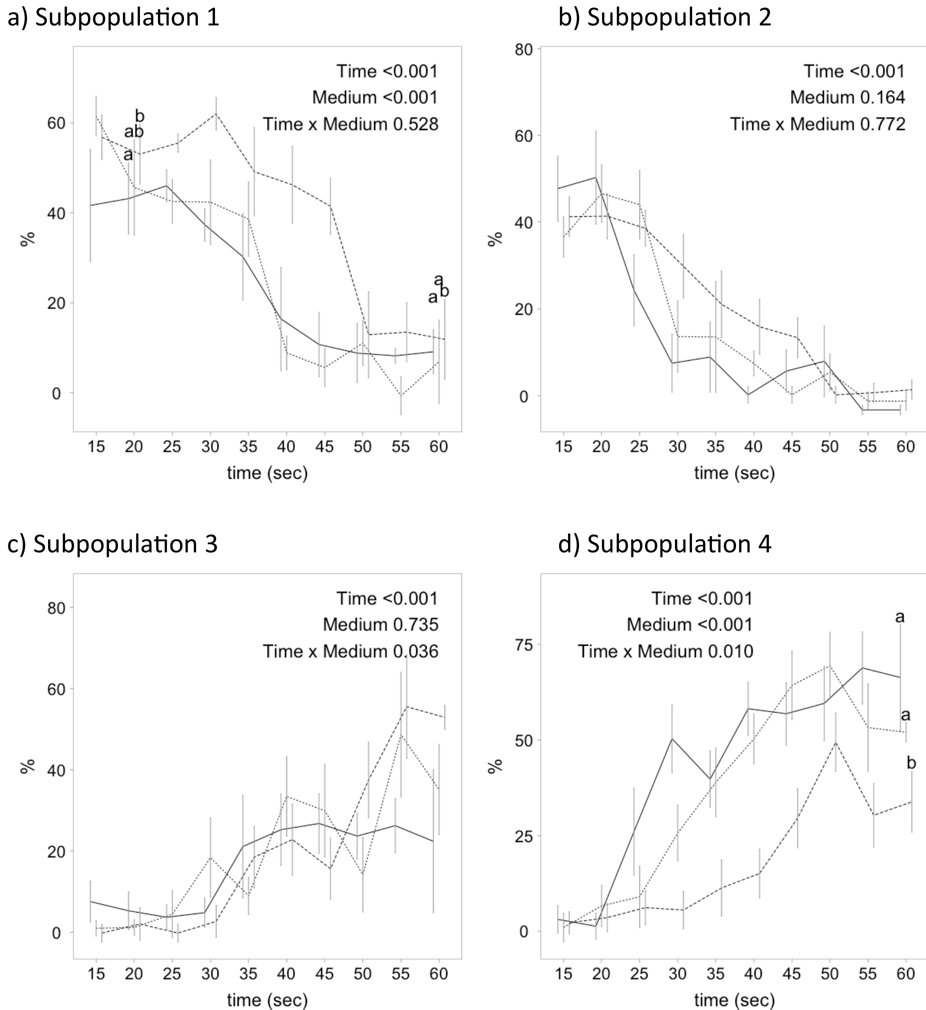


Figure 4. *Evolution of sperm subpopulations from 15 sec to 60 sec post-activation. Inserts show the signification of the time, medium (— 100 mOsm kg^{-1}; · · · 200 mOsm kg^{-1}; --- 300 mOsm kg^{-1}) and interaction effects. Different letters indicate significant differences between media by 20 sec (15 to 30) or 60 sec (45 to 60), for comparison with results for macroergic phosphates (Fig. 1).*

The predominant subpopulation at the initial stages of motility was cluster 1 (Fig. 4a). This subpopulation and cluster 2 (fast spermatozoa) decreased significantly with time, whereas cluster 3 and 4 (slow spermatozoa) increased (Fig. 4). The media significantly affected cluster 1 and 4 proportions, with 300 mOsm kg^{-1} showing the highest proportion of cluster 1 (Fig. 4a), and the lowest of cluster 4 (Fig. 4d). Cluster 2 and cluster 3 represented subpopulations of spermatozoa with intermediate motility characteristics, and there were not statistical differences in the proportion of these clusters between different AM (Fig. 4b and c). Time and medium significantly interacted for subpopulation 3 (Fig. 4c), with this subpopulation being less affected by time in 100 mOsm kg^{-1} AM.

Discussion

Our study shows that changes in sperm environment composition lead to different degrees of energy consumption. These results support previous research on sturgeon (Billard et al., 1999; Fedorov et al., 2015), carp (Perchec et al., 1995), trout (Christen et al., 1987) and many other fish species. Energy consumption significantly increased in motility activating condition, confirming that the flagellar dynein-ATPases are the main energy consumers but a basal level of energy consumption occurred in non-motile cells too, probably due to the need of fish sperm to control the ionic balance across its membrane in response to osmotic stress (Cosson, 2012), and possibly to basic dynein activity not producing movement. Nevertheless, the different media did not lead to a various degrees of energy consumption neither in activating nor in non-activating conditions, and irrespective of osmolality. We suppose that part of this consumption could be associated with the increased activity of ATP dependent ionic pumps, as proposed previously (Cosson, 2004; Cosson et al., 2008). However, this assumption requires further research to investigate it in more details.

ATP concentration was low at the end of the motility period but still high enough to sustain motility. Therefore, motility termination is probably not directly caused by ATP exhaustion because the Km (concentration of ATP for half maximal velocity) of dynein-ATPase for ATP is far below the minimal concentration measured when motility stops (Cosson et al., 1991, 1995, Dreanno et al., 2000). The decrease of ATP and CP after diluting the spermatozoa with AM suggests that both of them are involved in the energy supply for motility (Alavi and Cosson, 2006; Cosson, 2012). The physiological necessity of CP and ATP participation in the energy supply is supported by the fact that sites of ATP production (midpiece) and its consumption (axoneme mainly) are, in part, spatially separated. The diffusion rates of ATP and CP are different (Cosson, 2012), and therefore ATP must be regenerated along the axoneme from ADP and CP via adenylate- and creatine- kinases, in order to properly maintain a high ATP availability for dynein-ATPases (Saudrais et al., 1998).

In turbot sperm, the initial content of ATP, ADP, and CP measured before activation of motility was reported to be approximately 154, 99, and 727 nmol 10^{-9} spermatozoa, respectively (Dreanno et al., 2000). Whereas we obtained similar values for ATP, we get twice the concentration for APD and several-fold the values obtained for CP. Results of ATP and ADP quantification in turbot spermatozoa (Dreanno et al., 1999b), showed ATP content values of 240 nmol 10^{-9} spermatozoa before initiation of motility, twice the level observed by us in whitefish. For sea bass, the reported level of ATP before activation was 150 nmol 10^{-9} spermatozoa, similar to our results, whereas ADP levels were thrice those of whitefish (Dreanno et al., 1999ab). The reasons for these differences are not clear but may be associated with species-specific energy metabolism strategies sustaining macroergic phosphates levels required for motility activation.

The decrease of adenylate nucleotides content and AEC always appeared prior to the decline of motility parameters (Dreanno et al., 1999b). The same was also observed for whitefish sperm activated in all AM in the present study (Fig. 2). Spermatozoa diluted in NAM were immotile, however, they kept relatively high AEC level (slightly above 80) (Fig. 2), which corresponds with the data previously reported for various organisms and tissues and supports the prediction, that the energy charge is stabilized near 85 in intact metabolizing cells of a wide variety of types (Chapman et al., 1971; Wiebe and Bancroft, 1975). This suggests that even being immotile, spermatozoa can keep their energy status at a normal level, supposedly because ATP hydrolysis occurs at low rate. The decrease of AEC observed in all AM after 20 sec post-activation (Fig. 2) suggests that, in activated spermatozoa, the rate of ATP hydrolysis by dynein-ATPase is larger than the rate of ATP production by mitochondrial respiration. For

both activating and inhibiting conditions, the AEC level did not exceed the range between 85 and 50, which corresponds well with the idea investigated for *E. coli* that viability of cells is maintained at values between 85 and 50 (Chapman et al., 1971). As it was defined by Atkinson, the energy charge does not take into account an additional source of energy, i.e. CP. It is clear from our study as well as previous, such as in turbot (Dreanno et al., 1999b, 2000) that CP constitutes a main reservoir of chemical energy in fish spermatozoa (Cosson, 2012).

Another aim of this study was to relate the energy changes in whitefish sperm with the motility parameters and the sperm subpopulations at different post-activation times. The interest of using the cluster analysis in our study is that it allows determining groups of spermatozoa (subpopulations) with similar parameters at each post-activation time.

We have not found any publication using these two combined approaches (quantification of high-energy substances and subpopulation analysis of fish spermatozoa in one same conditions of experiment) so as to find if there is any relationship between energetics and spermatozoa subpopulation dynamics in fish sperm.

Our study suggests that four sperm subpopulations with distinct characteristics appear in whitefish sperm after activation of motility, successfully estimated using a statistical methodology described for other species (Gallego et al., 2015; Martínez-Pastor et al., 2008, 2011). This kind of analysis is promising, especially since many authors have suggested that sperm subpopulations might be defined by male genetics, originating during spermatogenesis (Martínez-Pastor et al., 2008). Nevertheless, these subpopulations can be modified by other events such as sperm maturation or even storage in the testes (Martínez-Pastor et al., 2008), or can be affected by environmental factors (extrinsic or intrinsic to the male). The subpopulations found in different studies present similar features (within the kinematics ranges of each species), generally presenting a fast-linear subpopulation, a fast and non-linear one and one or two slow (with more or less linear/wobbling movement). Thus, three subpopulations were reported in the semen of the gilthead sea bream (*S. aurata*) (Beirão et al., 2011) and four subpopulations in the semen of the three-spined stickleback (*G. aculeatus*) (Comber et al., 2004) and the Senegalese sole (*S. senegalensis*) (Martínez-Pastor et al., 2008).

This widespread consistency suggests that the presence of distinct subpopulations of motile sperm is either a component of the reproductive strategy of different species or an unavoidable consequence of sperm production or their microenvironment (Holt and Van Look, 2004).

Following activation of whitefish sperm motility in the present study, sperm cluster analysis allowed the detection of a gradual switch from straight-line to circular-track populations, probably related to a rise of internal Ca^{2+} concentration as it was shown for rainbow trout (Cosson et al., 1989). Despite different subpopulation/motility parameters profiles in different AM, we detected similar energy consumption. Therefore, this supports the idea that Ca^{2+} could indeed regulate motility between groups, while energy regulation might have a secondary role. For instance, osmotic shock at the onset of motility could have a major role in the subsequent motility duration and maintenance of certain motility patterns related to better sperm function (subpopulation 1, in this study). It was recently observed that intracellular concentrations of Ca^{2+} and K^{+} ions in European eel sperm increased after sperm activation in hyperosmotic conditions, with a progressive decrease in intracellular pH suggesting a flux of these ions through the spermatozoa membrane during sperm activation (Gallego et al., 2014). In addition, recent studies demonstrated the presence of a Na^{+}/Ca^{2+} exchanger involved in the sperm motility and that Ca^{2+} signaling can be a modulator of the sperm velocities and beat frequency of European eel spermatozoa (Perez et al., 2016).

The study of sperm subpopulations could be used to improve our knowledge about fish sperm motility, especially in species with short motility spans. Whereas we have used an unsupervised clustering method here (as it is common in the bibliography), supervised methods (based in machine-learning algorithms) could help to study subpopulations, once they have been defined by using unsupervised ones (Ramón et al., 2012). This approach allows using the same clustering criteria among different datasets, while making use of automated algorithms for that purpose.

Conclusions

We can conclude for whitefish spermatozoa that energy consumption and the balance of macroergic substrates are affected by environment composition. Energy consumption is significantly higher in motility activating conditions, regardless the osmolality conditions (at least, up to 300 mOsm kg^{-1}). We did not observe any relationship between energy consumption and motility parameters or subpopulation dynamics in spermatozoa activated in AM of different osmolality, which were affected by environmental variables. Isotonic conditions promote the subpopulation of spermatozoa presenting straight-line and fast motility and maintained motility for a longer period, however, without increase in energy consumption. This suggests that sperm energy management in whitefish is more efficient after activation in isotonic conditions.

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CHAPTER 6

GENERAL DISCUSSION
ENGLISH SUMMARY
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GENERAL DISCUSSION

Study of fish spermatology has attracted considerable interest over last several decades. Special attention is paid to energetical compounds as creatine- and adenylate phosphates. Major issues of concern are that these compounds are constantly used to support normal physiological state as well as motility of spermatozoa. Their simultaneous content in fish spermatozoa is mostly unknown. Therefore, development of new methods for the quantification studies of creatine- and adenylate phosphates is of great importance, as these data describes fish sperm physiology in more detail.

Development and application of LC/HRPS for quantification of adenine nucleotides, creatine phosphate, and creatine in sturgeon spermatozoa

In the framework of this thesis, a new method was developed for the simultaneous quantification of creatine- and adenylate phosphates in sturgeon spermatozoa (Fedorov et al., 2017). Different metabolites were examined including ATP, ADP, AMP, cAMP, CP and creatine using LC/HRPS method for measurements. Even with correction for the matrix effect (especially relevant for CP, where significant ion suppression was found) acceptable recoveries were not accomplished for all the analytes in the range given by the European Commission Decision 2002/657/EC on analytical methods performance (80–110%). To compensate for low recoveries of most of the target compounds, correction factors were established. Using these corrections for measured concentrations, calculations improved the quantification accuracy and compensated for probable analyte loss during sampling and sample handling. The study performed by Klawitter et al. (2007) with rat kidney tissue extracted in the same way reported acceptable recovery rates for ATP (76%) and ADP (80%), indicating that compound loss could be related to specific mechanisms in certain cells (Klawitter et al., 2007). However, the extraction efficiency of adenine nucleotides from sperm cells by PCA still needs estimation (even though it is the most frequently used approach). We believe that this is an important finding, as correct biological interpretation must be based on the "true" proportions of energy metabolites. The correction of data with this factor is crucial; otherwise, it can lead to misinterpretation of the real physiological state of the sperm. The dynamics of ATP content during the post-activation period of sterlet spermatozoa was similar to those obtained for Siberian sturgeon (*Acipenser baerii*) using a bioluminescence method (Billard et al., 1999). The median creatine content was 419 nmol 10^{-9} spz (95% range, 254–520 nmol 10^{-9} spz) without a statistically significant difference during the entire post-activation period; cAMP was not detected, possibly due to dilution during the collection and preparation of samples. To avoid the influence of these factors, a 15-fold pre-concentration was used, but no cAMP was found.

Regarding existing research, multi-compound analysis based on high-resolution nuclear magnetic resonance (NMR) has been applied to the sperm samples of several mammals (Lin et al., 2009; Smith et al., 1985). There was an attempt to measure ATP, ADP, CP, and other substances simultaneously in fish spermatozoa. Sperm cells of turbot (*Psetta maxima*) were tested with the help of NMR analysis but their content was obtained only prior to motility activation and there are no data reported during post-activation period (Dreanno et al., 2000).

There is no other report about simultaneous determination of ATP, ADP, AMP, CP and creatine in fish spermatozoa prior to, and during post-activation period, which emphasizes the advantages of proposed LC/HRPS method; this is much more available in laboratories than NMR. Application of this method was studied on sturgeon (Fedorov et al., 2015, 2017), whitefish, and European eel.

The study of sturgeon sperm maturation

The present study also focused on alterations in ATP, ADP, and CP content during sturgeon sperm maturation that may result from overcoming osmotic shock when spermatozoa are released into hypotonic conditions. During *in vitro* maturation, osmotic shock appears at the transfer of testicular sperm (TS) from an osmotic environment of 220–230 mOsm kg^{-1} into seminal fluid of about 50 mOsm kg^{-1} (Dzyuba et al., 2014). This shock leads to increased ATP consumption by spermatozoa ionic pumps activities, which supports cell homeostasis (Racker, 1976a, b). Finally, this ATP consumption leads to ATP regeneration via CP hydrolysis catalyzed by creatine kinase (Alavi and Cosson, 2006). It is known that motility rate and VCL reach a maximum by the end of the *in vitro* TS maturation period (Dzyuba et al., 2014). Therefore, it could be supposed that this progression is associated with intracellular content of creatine- and adenylate phosphates, both of which are involved in energy supply and regulation of spermatozoon maturation (Cosson, 2012). Physiological necessity of CP and ADP participation in spermatozoa energy supply is determined by the fact, that regions of ATP production (spermatozoa middle part) and its consumption (along entire flagellum) are spatially separated. Diffusion rates of ATP and CP are essentially different, and ATP can be regenerated from ADP via adenylate- and creatine kinases (Cosson, 2012). As we found significant decreases in CP during maturation, we assume critical importance of ATP regeneration system (acting via creatine kinase) for the maturation process. The reasons of stable ADP content during maturation remains to be determined, however, minor involvement of adenylate kinase in maturation may be supposed.

As immature spermatozoa are not able to initiate motility, comparative study of mature and immature spermatozoa is an essential experimental model for study of relationships between macroergic phosphates content, motility associated with hypotonic conditions. Observed increases of ATP content in immature spermatozoa diluted with activation medium (AM) could be associated with stimulation of ATP synthesis by consumption of ionic pumps (Okada, 2004). As no motility occurs, ATP synthesis via respiration dominates the processes of ATP hydrolysis at initial post-dilution time. However, this supposition should be further studied.

Content of creatine- and adenylate phosphates in European eel spermatozoa: influence of hormonal treatment, *in vitro* storage, and motility

We found all of the target compounds: CP, AMP, ADP, ATP, and cAMP in the activated eel spermatozoa from the peak of hormonally induced spermiation (spermatozoa, which represented 80% median motility). However, from the beginning of the spermiation we did not find any CP or cAMP in the sample diluted with activation medium (immotile spermatozoa). AMP in immotile spermatozoa was at the same level as in the matured spermatozoa (no changes of amount with time). ATP in immotile sperm ATP was about 30 times lower (5–15 nmol spz^{-9} without changes with time) than in motile sperm (150–450 nmol spz^{-9} with the significant decrease from 0 to 60 sec post-activation).

We found no cAMP in the European eel spermatozoa from the peak of induced spermiation before the activation of the motility, but the cAMP content significantly increased to a level of 0,5 nmol spz^{-9} after the initiation of motility (at 60 and 120 sec post-activation). There are no another reports of cAMP quantification in the fish sperm by the LC/HRPS. We attempted to quantify it in the spermatozoa of sturgeon, carp, and whitefish without any success, taking into account that the method itself is well elaborated by our group for the quantification of cAMP in the fish sperm (Fedorov et al., 2017). Current study represent a first successful estimation of cAMP in fish spermatozoa during the motility period using the LC/HRPS.

To conclude, spermatozoa at a sixth week of hormonal injections (being immotile) are characterized by the absence CP and cAMP. However, by the peak of the hormonal treatment (tenth week), they acquire the ability to be activated after the dilution with SW. This is accompanied by a significant increase of CP and cAMP content in spermatozoa.

The presence of cAMP after the initiation of motility can probably be the result of some regulatory physiological function. Factors such as ATP or cAMP can be responsible for the initiation and possibly maintenance of motility. This was confirmed by Morisawa and Okuno (1982) (Morisawa and Okuno, 1982). In contrast, we found no cAMP during whole post-activation period in eel spermatozoa from the beginning of the spermiation period, which were immotile after the dilution of the sperm with the activation medium.

We estimated that the content of CP, AMP, ADP, and ATP during the short-term storage of European eel sperm was affected by the temperature. It follows by the significant decrease of CP, ADP, and ATP content and AEC during the whole period of storage at 20 °C in comparison to 4 °C, while the motility parameters do not represent statistical difference until seventh day of storage.

In the current study of eel sperm, no antibiotics were used to prevent the bacteria propagation during short-term storage in P1 extender. This extender was shown as an appropriate extender for the successful short-term storage of eel sperm (Asturiano et al., 2004, 2003). This study was a preliminary study of the bioenergetics of eel sperm; and currently it is hard to conclude, whether such results may be interconnected with possible bacteria propagation. This question definitely needs to be studied in the future experiments.

Effect of environment osmolality on whitefish sperm subpopulations and content of creatine- and adenylate phosphates

Another important study was on whitefish sperm response to the environmental osmolality. Our study suggests that four sperm subpopulations with distinct motility characteristics exist in whitefish sperm. Subpopulations can be successfully identified by using a multivariate clustering analysis. This kind of analysis is promising, especially since many authors have suggested that sperm subpopulations are primarily defined by male genetics, originating during spermatogenesis (Abaigar et al., 1999; Beirão et al., 2009; Dominguez-Rebolledo et al., 2009; Kanuga et al., 2012; Martínez-Pastor et al., 2008). Nevertheless, these subpopulations could be modified by other events such as sperm maturation or even sperm storage in the testes (Martínez-Pastor et al., 2008), or could be affected by environmental factors (extrinsic or intrinsic to the male). The subpopulations found in different studies present similar features (within the kinematics ranges of each species), generally presenting a fast-linear subpopulation, a fast and non-linear one and one or two slow (with more or less linear/ wobbling movement). Likewise, three subpopulations were reported in the semen of the gilthead sea bream (*S. aurata*) (Beirão et al., 2011) and four subpopulations in the semen of the three-spined stickleback (G. aculeatus) (Comber et al., 2004) and the Senegalese sole (*S. senegalensis*) (Martínez-Pastor et al., 2008). The current results are also consistent with the presence of subpopulations of motile sperm in other vertebrates, such as boar, gazelle, Iberian red deer, human, turkey (Abaigar et al., 1999; Chantler et al., 2004; King et al., 2000; Martinez-Pastor et al., 2005), as well as invertebrates, such as insects (Parker, 1970). This widespread consistency suggests that the presence of distinct subpopulations of motile sperm is either a component of the reproductive strategy of different species or an unavoidable consequence of sperm production or their microenvironment (Holt and Van Look, 2004).

We found that there was no relationship between content of creatine- and adenylate phosphates and cluster dynamics in spermatozoa activated in AM of different osmolality. Since energy consumption is the same in all AM, and the concentration of molecules involved in energy balance regulation (ATP/ADP, AMP) is similar, the resulting hypothesis is that motility modulation under the conditions tested in this study may depend on factors other than simply the energy balance (Cosson, 2004; Dzyuba et al., 2017; Ingermann, 2008; Lahnsteiner et al., 1999; Lahnsteiner and Caberlotto, 2012). For instance, osmotic shock at the onset of motility could have a major role in the subsequent motility duration and maintenance of certain motility patterns related to better sperm function (subpopulation 1 in this study, which possess spermatozoa with the highest levels of linearity and curvilinear velocity). There are different dynamics of cluster proportion in different AM. We have not found articles using two approaches (quantification of energetical substances and subpopulation analysis of fish spermatozoa in one experiment) to find if there is any relationship between energetics and spermatozoa subpopulation dynamics in fish sperm.

The study of sperm subpopulations could be used to enhance our knowledge about fish sperm motility, especially in species with short motility spans. Whereas we have used an unsupervised clustering method here (as it is common in the bibliography), supervised methods (based in machine-learning algorithms) could help to study subpopulations, once they have been defined by using unsupervised ones. This approach allows the use the same clustering criteria among different datasets, while making use of automated algorithms for that purpose.

Conclusions and future perspectives

This study presents new analytical methods for the simultaneous measurement of creatine- and adenylate phosphates, which was applied for the sturgeon and whitefish sperm. This method has significant advantages over the existing ones: it allows measuring several macroergic metabolites in one analytical run and their consequent quantification. Further research is needed to overcome the limitations and to expand the number of metabolites of high interest.

Results of the present study confirm that the maturation of TS in sturgeon is an energy-dependent process involving mitochondrial respiration and ATP regeneration from CP via creatine kinase reaction. These two processes are responsible for ATP synthesis required for motility of mature spermatozoa.

European eel spermatozoa at a sixth week of hormonal injections were not able to initiate motility after releasing into activating conditions. However, by the peak of the hormonal treatment (tenth week), they acquired the ability to be activated after dilution with activation medium. This was accompanied by a concomitant appearance of CP and cAMP levels in spermatozoa. It suggests the importance of aforementioned substances in the process of spermatozoan motility acquisition during the induced spermiation period. cAMP was found in the fish sperm for the first time using the LC/HRPS, which is the next step of the using of this method for the description of mechanism activation in fish spermatozoa. The content of CP, AMP, ADP, and ATP during the short-term storage of European eel sperm can be affected by the temperature. Short-term storage of eel sperm at 4 °C was accompanied by higher marcoergic phosphate content and higher AEC in comparison to the storage at 20 °C. This suggests that the rate of ATP hydrolysis during the short-term storage at 20 °C is greater than the rate of ATP production by mitochondrial respiration. Results potentially can help in developing of effective method for improving spermiation and increasing sperm production in European eel.

Whitefish spermatozoa have various degrees of energy consumption in response to environment composition. Energy consumption is significantly higher in motility activating conditions without any relation to osmolality. It was found that there was no relationship between energy consumption and cluster dynamics in spermatozoa activated in AM of different osmolality. Isotonic conditions favor the subpopulation of spermatozoa presenting longer period of straight-line and fast motility without increase in energy consumption.

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ENGLISH SUMMARY

Fish spermatozoa metabolites content in various physiological conditions

Investigation of creatine- and adenylate phosphates involvement in fish spermatozoa metabolism is of high interest for fish spermatology. These compounds are necessary to support a normal physiological state and motility of spermatozoa. The simultaneous change in content of creatine- and adenylate phosphates in fish spermatozoa prior to, and during their motility are quite unclear. Therefore, development of new methods for the quantification of creatine- and adenylate phosphates in spermatozoa of different fish species under such physiological conditions as maturation and *in vitro* manipulation are of high importance.

One of the study outputs was the developed LC/HRPS (liquid chromatography coupled with high-resolution product scan) method for the analysis of creatine- and adenylate phosphates content in fish spermatozoa (Chapter 2). Its main advantage is the possibility to detect and quantify several compounds (creatine, creatine phosphate (CP), AMP, ADP, ATP, and cAMP) simultaneously so as to obtain maximum information with minimum analytical effort. The method was validated taking into account such key parameters as limit of quantification, selectivity, recovery and repeatability. It represented an excellent performance allowing determination of target compounds in highly diluted fish sperm samples.

Consequently, the method was applied for the quantification of aforementioned substances during sterlet (*Acipenser ruthenus*) spermatozoa maturation and *in vitro* manipulation with sperm of whitefish (*Coregonus lavaretus maraena*) and European eel (*Anguilla Anguilla*).

The present study showed that immature sterlet spermatozoa are not able to initiate motility. Significant decrease of CP and stable levels of ATP and ADP content during their maturation were found. The critical importance of ATP regeneration system and oxidative phosphorylation for the maturation process of sterlet sperm as a prerequisite for successful fertilization was assumed (Chapter 3).

Further experiments revealed that European eel spermatozoa were not able to initiate motility by activation medium (AM) at the start of the induced spermiation. However, they acquired the ability to be activated after the dilution with AM at the end of hormonal treatment. This was accompanied by an increase of CP and cAMP levels in spermatozoa after activation. That allowed us to assume the involvement of ATP regenerating system and cAMP-dependent regulatory pathways in the process of spermiation caused by the hormonal treatment (Chapter 4). Current study represents a first successful estimation of cAMP in fish spermatozoa during the motility period using the LC/HRPS.

Important issues concerning the short-term storage of European eel sperm were posed. The content of CP, AMP, ADP, and ATP during the short-term storage of European eel sperm was affected by the temperature. Short-term storage of eel sperm at 4 °C was accompanied by higher marcoergic phosphates content and higher motility in comparison to the storage at 20 °C. It suggests the involvement of metabolic processes during the short-term storage, which corresponds with the macroergic phosphates metabolism. (Chapter 4). Results might contribute to the development of new effective methods for improving of spermiation and short-term sperm storage in European eel aquaculture.

Various degrees of energy consumption in response to environment composition were found in whitefish spermatozoa. Energy consumption was significantly higher in motility activating conditions. No effect of osmolality was found on this process. The content of CP and ATP was significantly higher when cells were in motility-inhibiting medium compared to activation medium. No relationship between content of CP, ADP, and ATP and spermatozoa

motility parameters in AM of different osmolality was found. Isotonic conditions favor the spermatozoa with longer motility period, higher linearity, and fast velocity without increase in ATP content (Chapter 5). This suggests that the energy management of whitefish sperm is more efficient after activation in isotonic conditions. Results are of high interest for elaboration of new sperm motility activating media for fisheries practice.

CZECH SUMMARY

Obsah metabolitů ve spermiích ryb za různých fyziologických podmínek

Výzkum zapojení kreatinfosfátu (CP) a adenosyl fosfátů do metabolizmu rybích spermií je pro spermatologii ryb zásadní. Tyto sloučeniny jsou nezbytné k navození normálního fyziologického stavu spermií a pro jejich pohyblivost. Souběžně probíhající změny obsahu kreatinu a adenylovaných fosfátů v rybích spermiích před a během jejich pohybu nejsou zcela objasněny. Na studium a vývoj nových metod kvantifikace kreatinu a adenylovaných fosfátů ve spermiích různých druhů ryb za fyziologických podmínek, jako je zrání a manipulace in vitro, je proto kladen velký důraz.

Jedním z výstupů této dizertační práce je vyvinutí metody pro analýzu obsahu kreatinu a adenylovaných fosfátů v rybních spermiích s využitím LC/HRPS (kapalinová chromatografie pojená se skenováním produktů ve vysokém rozlišení, kapitola 2). Hlavní výhodou této metody je možnost současné detekce a kvantifikace několika sloučenin (kreatin, CP, AMP, ADP, ATP a cAMP) s cílem získání maximálního množství informací s vynaložením minimálního úsilí. Metoda byla ověřena při zohlednění klíčových parametrů, jako jsou mez kvantifikace, selektivita, výtěžnost a opakovatelnost. Představuje vynikající nástroj ke stanovení cílových sloučenin ve vysoce ředěných vzorcích spermatu ryb.

Následně byla tato metoda použita pro kvantifikaci výše uvedených látek během procesu zrání spermií jesetera malého (*Acipenser ruthenus*) a při in vitro manipulaci se spermatem síha severního marény (*Coregonus lavaretus maraena*) a úhoře říčního (*Anguilla anguilla*).

První ze studií ukázala, že nezralé spermie jesetera malého nejsou schopné pohybu. Během jejich dozrávání bylo zaznamenáno průkazné snížení CP a stabilní hladiny ATP a ADP. Proto je předpokládán klíčový význam systémů regenerace ATP a oxidativní fosforylace při procesu dozrávání spermií jesetera malého a pro úspěšné oplodnění jiker (kapitola 3).

Další experimenty ukázaly, že spermie úhoře říčního po přidání aktivačního média (AM) na počátku hormonálně indukované spermiace ryb nebyly schopné pohybu. Tuto schopnost však získaly ke konci hormonální stimulace. Celý proces byl doprovázen zvýšením hladin CP a cAMP ve spermiích po jejich aktivaci. Na základě výsledků předpokládáme, že při procesu spermiace vyvolané hormonální stimulací se uplatňuje regenerační systém ATP a na cAMP závislých regulačních cest (kapitola 4). Tato studie je první, kde se podařilo stanovit obsah cAMP ve spermiích ryb během jejich pohybu pomocí LC/HRPS.

Při výzkumu vyvstaly důležité otázky týkající se krátkodobého uchování spermatu úhoře říčního. Obsah kreatinu, CP, AMP, ADP a ATP během krátkodobého uchování spermatu byl ovlivněn teplotou. Při krátkodobém uchování spermií při 4 °C byl pozorován vyšší obsah makroergických fosfátů i vyšší pohyblivost spermií ve srovnání s uchováním při 20 °C. Při krátkodobém uchovávání spermií je předpokládáno zapojení metabolických procesů souvisejících s makroergickými fosfáty (kapitola 4). Získané výsledky by mohly přispět k rozvoji nových účinných metod pro zlepšení hormonálně indukované spermiace a krátkodobého uchování spermií v akvakulturním chovu úhoře říčního.

V souvislosti s okolním prostředím byly zjištěny u spermií síha severního marény různé úrovně spotřeby energie. Spotřeba energie byla výrazně vyšší v médiu aktivujícím pohyblivost spermií. Vliv osmolality na tento proces nebyl prokázán. Obsah CP a ATP byl významně vyšší v médiu inhibujícím pohyblivost oproti aktivačnímu médiu. Nebyl zjištěn žádný vztah mezi obsahem CP, ADP a ATP a parametry pohyblivosti spermií v AM s různou osmolalitou. Izotonické podmínky napomáhají k delší době pohyblivosti spermií, vyšší linearitě jejich dráhy a k vyšší rychlosti spermií bez zvýšení obsahu ATP (kapitola 5). To naznačuje, že využití energie spermiemi u síha severního marény je účinnější po aktivaci v izotonických podmínkách. Získané výsledky

jsou velmi důležité pro rybářskou praxi při přípravě nových médií potřebných pro aktivaci pohyblivosti spermií.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Dzyuba, B., Dzyuba, V., Boryshpolets, S., **Fedorov, P.**, Bondarenko, O., Rodina, M., Linhart, O., Shelton, W.L., Cosson, J., 2017. Sperm maturation in sturgeon (*Actinopterygii*, Acipenseriformes): A review. Theriogenology 97, 134–138 (IF 2016 = 1.986)

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Methodologies

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Dzyuba, B., Cosson, J., Dzyuba, V., Bondarenko, O., Boryshpolets, S., **Fedorov, P.**, Rodina, M., Linhart, O., 2015. Specific sperm maturation determined by urogenital system anatomy in sturgeon. In: 5th International Workshop on the Biology of Fish Gametes, Abstract Book, Ancona, September 7–11, 2015, pp. 33–34.

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Fedorov P., Dzyuba B., Fedorova G., Grabic R., Martínez-Pastor F., Cosson J., Dzyuba V., Rodina M., 2015. Macroergic phosphates content and cluster analysis of motility in spermatozoa of whitefish *Coregonus lavaretus maraena* in response to environment osmolality. In: 5th International Workshop on the Biology of Fish Gametes, Abstract Book. September 7–11, 2015, Ancona, Italy, p. 35–36. (*Oral presentation*).	2015

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