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Fish sperm respiration: species specificity and effect of environmental temperature

Deepali Rahi

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. General overview

Fish spermatozoa synthesize energy in the form of ATP when they reside at quiescent state in seminal plasma, before coming in contact with the external environment. During this stage, energy is synthesized and stored as a pre-accumulated stock. The energy compound, ATP, can be produced in the cytoplasm and/or mitochondria (located in the middle piece) via various energy supplying pathways such as glycolysis, Krebs cycle, fatty acid oxidation, oxidative phosphorylation (OXPHOS). However, OXPHOS is the most ATP yielding pathway among all, and it is known to be the most prevalent in fish spermatozoa (Kholodnyy et al., 2020; Perchec et al., 1995). The stored ATP in the middle piece is then transported to flagellum, where it is hydrolyzed by dynein ATPases leading to the motility of the entire spermatozoon body. This transport is supported by phosphocreatine-creatine (PCr-Cr) shuttle system (Tombes and Shapiro, 1985). Once the energy reaches the axoneme of flagellum, it is readily available and can be rapidly consumed for sperm motility activation (Ziętara et al., 2009).

After the spermatozoon is activated, the energy gets drained quickly since the mitochondrial respiration capacity cannot fulfill the demand of highly activated flagella and it leads to very short motility duration (Perchec et al., 1995). However, the motility duration of sperm varies from species to species and follows no general trend, based on taxa specificity or thermal habitat history (Dzyuba and Cosson, 2014). On the basis of difference in sperm motility parameters among fish species, the existence of different metabolic strategies for storage and generation of ATP are suggested (Ingermann, 2008). Nevertheless, the role of metabolic pathways for ATP generation/regeneration and distribution along flagellum may be species specific, vary at motile or immotile state, and is not fully understood.

Furthermore, in spermatozoa of few fish species – carp (*Cyprinus carpio*) and trout (*Oncorhynchus mykiss*) possibility of a second activation after completion of the first motility period has been observed (Benau and Terner, 1980; Linhart et al., 2008). For this second activation, ATP exhausted spermatozoon has ability to revive its ATP and regain fertilizing capacity (Cosson, 2004). Though, any further information regarding its species specificity, mode of motility reinitiation, or energy supplying pathways during reactivation state are entirely unknown.

Overall, the current work was designed to determine the relative contribution of different energy supplying pathways, focusing on sperm mitochondrial respiration at motile and immotile states of spermatozoa of fish species spawning at a wide range of temperature and possessing differences in their sperm motility duration. Additionally, the possibility of sterlet sperm reactivation, most important bioenergetic pathways involved in the energy supply during reactivated state, and capacity of reactivated sperm to fertilize the egg cells were studied.

1.2. Sperm motility duration

During motility, spermatozoon of externally fertilizing fish is majorly dependent on ATP pre-store (produced at quiescent state) and entirely on endogenous nutrients (Ingermann, 2008). Once the fish spermatozoa come in the contact of an aqueous environment and attain motility, the event occurs for a very short lapse and ultimately stops. The time duration for spermatozoa being motile varies among different fish species. Generally, longer motility duration is reported for spermatozoa of fish species that possess internal fertilization than for externally fertilizing fish (Dzyuba and Cosson, 2014). Also, sperm motility duration of freshwater fish is generalized to be shorter than in marine fish species (Rurangwa et al., 2004). However, in either case, the sperm motility duration is much less than that in mammalian sperm (Cosson, 2012).

Spermatozoa of numerous freshwater fish species exhibiting external fertilization and marine species with internal fertilization support the above-mentioned trend. Among freshwater fish, carp, trout, burbot (*Lota lota*) (sperm motility duration is ≤ one min) and among marine fish; turbot (*Scophthalmus maximus*), cod (*Gadus morhua*), rabbitfish (*Siganus rivulatus*), or internally fertilizing fish; medaka (*Oryzias latipes*) (having sperm motility duration up to 10–60 min) are few examples (Chauvaud et al., 1995; Cosson et al., 1985; Lahnsteiner and Patzner, 1999; Perchec et al., 1995; Trippel and Morgan, 1994; Yang and Tiersch, 2009). However, minority of fish groups are exceptional and hence rule out the idea of generalization. For example, among freshwater species, spermatozoa of species belonging to Acipenseridae family can remain motile up to 30 min (Toth et al., 1997). Among marine species, sperm motility duration of coral reef fish, the bluehead wrasse (*Thalassoma bifasciatu*) is reported to be only 15–30 s (Petersen et al., 1992).

Also, it is important to note that there is no direct relation or interdependency between higher ATP content and longer motility duration. ATP content at quiescent state in the spermatozoa of fish species with the short motility duration (carp and trout) and the long motility duration (sturgeons) lies between the range of 18–110 and 60–80 nmol $(10^9$ spermatozoa$)^{-1}$ respectively (Fedorov et al., 2015; Ingermann, 2008; Perchec et al., 1995). However, higher ATP content was found in the spermatozoa of marine fish – turbot or sea bass (*Dicentrarchus labrax*) – 110–240 nmol $(10^9$ spz$)^{-1}$ (Dreanno et al., 1999a,b). The similar data exhibiting uneven relation between motility duration and level of macroergic compounds among different fish species has also been reported (Dzyuba et al., 2017).

The above-mentioned diversity in data on sperm motility duration among different fish species suggests that bioenergetics of fish sperm does not come into a common umbrella based on taxa specificity or mode of reproduction. Various metabolic pathways with different strategies to utilize the energy efficiently for sperm motility must be adapted by species in the evolutionary time. Additionally, sperm motility duration is also associated with numerous other factors such as ionic composition and osmolality of activation medium (AM), osmotic damages of the cellular structures, mode of reproduction (external or internal), oxygen tension, temperature of activation media (Alavi and Cosson, 2005, 2006; Dadras et al., 2017). This makes the idea of generalization even more complicated. Technical differences for taking motility observations can also create differences in motility duration estimation (Cosson et al., 2008)

1.3. Energy supplying pathways

A positive relation between sperm motility, ATP content, concentration of substrates required for energetic pathways, flagellum beat frequency, and fertilization success has been found in numerous studies (Ingermann et al., 2003; Lahnsteiner et al., 1996, 1998, 1999; Zilli et al., 2004). Based on decades of research on fish sperm bioenergetics, a standard and conventional rule is accepted for the spermatozoa of most of the teleosts. According to that, synthesis of ATP in spermatozoa occurs at quiescent stage when spermatozoa are residing in seminal plasma, and energy is stored as a pre-accumulated stock of macroergic phosphates in the form of ATP, ADP, and phosphocreatine (PCr) (Cosson, 2012). Later, when spermatozoon is activated in an aqueous environment, the pre-stored energy is rapidly utilized to sustain the motility. However, it is important to note that albeit the species specificity for length of motility period or availability of various macroergic phosphates in fish spermatozoa, ATP hydrolysis is the only energy source that can be utilized by spermatozoa for motility. Therefore, ADP and PCr must be converted into ATP for energy utilization (Dzyuba et al., 2017).

The readily available energy source – ATP is produced by metabolizing various organic molecules through a series of enzymatically controlled reactions that are differentiated into

various metabolic pathways – glycolysis, Krebs cycle, OXPHOS, fatty acid oxidation. However, the choice of organic molecule to be metabolized and ultimately relative importance of bioenergetic pathways for energy synthesis is species specific and may vary at motile or immotile state of spermatozoa. The dominant contribution of a certain pathway for energy production in spermatozoa is a topic of debate not only for different fish species but also for higher animals (Ruiz-Pesini et al., 2007; Storey, 2004). Glycolysis and Krebs cycle activities are potentially much higher in carp spermatozoa than in the African catfish (*Clarias gariepinus*) (Ziętara et al., 2009). Studies on enzymatic activities and content of metabolites revealed that cyprinid spermatozoa are much relied on carbohydrate (specifically monosaccharides) metabolism rather than lipid metabolism (Lahnsteiner et al., 1992, 1994, 1996, 1999). Lipid catabolism and β-oxidation of fatty acids played an essential role during sperm motility of the African catfish (Mansour et al., 2003) in contrast to some representatives of cyprinids, whose spermatozoa were unable to catabolize lipids and lack lipase enzyme (Lahnsteiner et al., 1996, Lahnsteiner et al., 1992). Salmonid spermatozoa can metabolize lipids and possess lipase, phospholipase, and fatty acid synthase (Lahnsteiner et al., 1993). Lipids are available as extra- (seminal plasma) and intracellular material in salmonid spermatozoa and the level of lipids positively correlates with fertilization success in salmonid spermatozoa (Lahnsteiner et al., 1998, 2009).

Additionally, the role of mentioned pathways for energy production can also vary at motile and immotile state of spermatozoa. Mansour et al. (2003) found that lipid catabolism, β-oxidation of fatty acids, Krebs cycle and oxidative phosphorylation are primarily important energy-delivering pathways for immotile spermatozoa whereas glycolysis, lipid catabolism and β-oxidation of fatty acids are major energy providing pathways for motile spermatozoa in African catfish spermatozoa. The OXPHOS, Krebs cycle, and glycolysis played a central role for energy synthesis during motility whereas fatty acid level remained constant and amino acid catabolism had no role in spermatozoa of Danube bleak (*Chalcalburnus chalcoides*) (Lahnsteiner et al., 1999). In rainbow trout spermatozoa, glycolysis and triglyceride catabolism occur simultaneously during the immotile state. During motility, glycolysis occurs in the initial 30 s, but as the ATP level cannot continue to be maintained by glycolysis, a shift to triglyceride metabolism allows regeneration of ATP (Lahnsteiner et al., 1993).

1.4. Role of mitochondrial respiration

Fish spermatozoa have been demonstrated to be capable for glycolysis, lipid metabolism, and OXPHOS. Nevertheless, the major contribution to energy production required for sperm motility has been from OXPHOS (Ziętara et al., 2009). Several studies performed on the spermatozoa of freshwater and marine fishes have demonstrated the diminishing effect of respiratory inhibitors on oxygen consumption rate (OCR), motility in AM, motility activation after incubation in non-activation media (NAM), or ATP content (Dreanno et al., 1999b; Ingermann et al., 2011; Lahnsteiner et al., 1999; Mansour et al., 2003; Perchec et al., 1995). This suggests a central role of OXPHOS for energy production during active and non-active state of fish spermatozoa. Additionally, a similar pattern of oxygen or ATP consumption after motility activation was observed (a sudden fall in respiration and ATP content immediately after motility initiation followed by maintaining a constant low level) whether spermatozoa of fish species possess short or long sperm motility duration (Billard et al., 1999; Dreanno et al., 1999b; Perchec et al., 1995). It indicates that the level of OXPHOS for ATP production has remained insufficient to fulfil high energy demand during motility. Therefore, majority of energy is deriving from a pre-stored stock of ATP. While this idea could fit well for spermatozoa of species with short motility duration, it cannot be said with certainty that it works well for another group of fish with longer sperm motility duration such as sturgeons or marine fish.

A direct method to determine the input of OXPHOS to energy supply before/after motility activation is by measuring oxygen consumption rate (OCR) and comparing it before and after attaining motility. In spermatozoa of some species, an enhanced OXPHOS after motility activation was observed. However, the level of enhancement may be a species-specific phenomenon. After motility activation, in spermatozoa of carp, African catfish, and turbot, OXPHOS intensity was enhanced 2–4, 20–25, and 2–4 times, respectively, in comparison with that at a quiescent state (Ingermann, 2008). On the other hand, no enhancement of OXPHOS after motility activation was observed in the spermatozoa of coldwater fish species – trout (Ingermann et al., 2003). It is also important to note that the OCR of trout spermatozoa lies in the lowest range while comparing among most of the studied teleosts (Ingermann et al., 2003). For reference, the range of OCR at quiescent and motile state of fish spermatozoa is broad and lies between 0.6–280 nmol O_2 min^{-1} $(10^9 spz)^{-1}$ (Ingermann, 2008). So far, very few studies have been done on sperm respiration of coldwater fish; trout being the coldest species studied till now that spawn at 10 °C. To test the capacity to maximize sperm OCR at motile or quiescent state of spermatozoa, there is a tool often used in laboratories – uncouplers of OXPHOS. Uncouplers such as FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) or CCCP (carbonyl cyanide m-chlorophenylhydrazone) break the coupling between substrate oxidation and ATP synthesis and, as a result, OCR increases without generation of ATP.

1.5. Phosphocreatine-creatine shuttle system

Even though mitochondrial OXPHOS is the most energy yielding pathway, the energy generation through it is not efficient enough to satisfy the high energy demand during motility. One possible reason of this inefficiency could be the fact that the site of ATP production (middle piece, containing mitochondria) and the site of its consumption (axoneme, containing dynein ATPase) are distantly located. Due to which ATP molecules have to diffuse from the site of production to the site of utilization. As diffusion coefficient of ATP molecules is low (Takao and Kamimura, 2008), an alternative/additional mechanism linking the sites of ATP production and consumption and thus maintaining a homogenous concentration of ATP all along the flagellar compartments is present in sperm cells (Cosson, 2012). This mechanism, called phosphocreatine-creatine (PCr-Cr) shuttle system was previously well documented in cardiac muscles, and was described in sea urchin sperm during late 1900s (Tombes et al., 1987; Tombes and Shapiro, 1985; Williamson, 1979).

Phosphocreatine-creatine shuttle system operates via series of spatially separated reactions. Creatine is transphosphorylated to PCr (macroergic phosphate, having high diffusion coefficient) by ATP hydrolysis in spermatozoon middle piece, which diffuses along flagellum. Due to ATP hydrolysis by dynein ATPases, flagella have high level of ADP, which favours resynthesis of ATP using PCr. The mechanism works with the help of distinctly localized creatine kinase (CK) isoenzymes catalysing the formation of ATP from ADP and PCr in flagellum and the formation of PCr from Cr and ATP in middle piece (Saudrais et al., 1998).

Spermatozoa of salmonids and cyprinids possess CK and PCr. The level of PCr was decreased with onset of motility (Kamp et al., 1996; Lahnsteiner et al., 1996; Robitaille et al., 1987) representing the importance of mentioned pathway in cyprinid and salmonid spermatozoa. Later, the involvement of shuttle system at/after initiation of spermatozoon motility for transporting high energy phosphate was also confirmed in the spermatozoa of zebrafish (*Danio rerio*) and marine fish turbot (Dreanno et al., 1999b; Ingermann et al., 2011; Saudrais et al., 1998; Tombes and Shapiro, 1985). Fluorodintrobenzene (FDNB) – an inhibitor of CK is often used to assess the role of PCr-Cr in fish spermatozoa. In fish spermatozoa, there is another way of ATP regeneration, that is accomplished with participation of adenylate kinase

(AK). It allows the conversion of two molecules of ADP into ATP and AMP. Recently, the activity of CK and AK was studied in demembranated spermatozoa of sterlet (*Acipenser ruthenus*) and the data on enzyme activities were compared to ones in carp spermatozoa. The essential role of PCr-Cr shuttle system for ATP regeneration was confirmed in both the species (Dzyuba et al., 2016). Despite difference in spermatozoon structure, motility duration and mode of motility activation (ionic in sterlet and osmotic in carp), similar energy distribution system was found in both taxonomically different fish species. However, the functioning of AK and CK was less efficient for energy maintenance in demembranated sterlet spermatozoa.

1.6. Temperature dependency in fish sperm energetics

As teleosts are ectotherms and possess external fertilization, sperm motility is directly affected by the temperature of spawning environment (Dadras et al., 2017). Spermatozoon motility and activity of various enzymes that are involved in bioenergetic pathways are suggested to be changed with change in temperature (Alavi and Cosson, 2005; Lahnsteiner and Mansour, 2012).

Generally, the relation between enzymatic activity and temperature is a classic "bell-shaped curve". In addition, the relation between sperm motility and temperature has been thoroughly studied. With rise in temperature there are enhanced sperm velocity, motility rate, flagellum beat frequency, and ATP consumption rate which lead to shorter motility duration (Dadras et al., 2017). Nevertheless, a handful of studies have been demonstrating the results otherwise (Lahnsteiner, 2011; Lahnsteiner and Mansour, 2012; Vladiĉ and Jätrvi, 1997). Therefore, it is difficult to draw a "general rule" for the effect of temperature on enzymatic activities involved in energy supply during sperm motility. Considering the role of taxa specificity offered by the wide range of spawning temperature among various fish species makes the interpretation of "general rule" even more complicated.

Till now, it is clear that the energy for fish sperm motility is significantly contributed by OXPHOS that occurs at quiescent state (Ingermann, 2008). However, the effect of variation in temperature due to seasonal/climatic change or migration of fish on the energy budgeting strategy in fish spermatozoa is unclear. To maintain enough ATP for motile spermatozoa that are capable of successful fertilization at variable (for example, low) temperature, two possible strategies can exist. Either OXPHOS is occurring at low rate but for longer period or the enzymes involved in OXPHOS are least affected by the temperature (or able to tolerate the change in temperature) leading the rate of OXPHOS at low temperature similar to the rate at higher temperature. The latter strategy is due to some degree of independence of metabolism from change in temperature and is well known in some ectotherms as "thermal compensation" (Johnston et al., 1994). However, the extent of thermal compensation and strategy behind it are affected by multiple factors and therefore are difficult to be predicted for a species (Guderley, 2004). No such study till now has been performed on fish sperm, thus leaving a huge knowledge gap.

1.7. Sperm reactivation

The motility terminated spermatozoon is energetically exhausted, possesses deformed ultrastructure and loss of capability to fertilize the egg (Billard et al., 1995; Linhart et al., 2008). The studies on the spermatozoa of cyprinids and salmonids have demonstrated that the termination of sperm motility is linked with huge but not complete depletion of ATP (Christen et al., 1987). Therefore, it was suggested that osmotic damage or morphological alterations are not the only reason behind termination of motility, insufficient availability of

energy to sustain motility is one of the major reasons. Thus, once activated spermatozoon is metabolically active even at the end of motility period (Cosson, 2010). Spermatozoon can be reactivated if it is allowed to have a resting period to restore the initial energy level, internal ionic composition and osmotically compromised structure.

In several studies, reactivation was achieved either after sperm incubation in non-activation solution or when previous dilution was made in saline/ovarian fluid/Ringer's medium or if spermatozoa were reactivated in presence of phosphodiesterase inhibitors such as 3-isobutyl-l-methylxanthin or theophylline (Benau and Terner, 1980; Cosson, 2004; Ginzburg, 1968). The re-attainment of sperm motility in these studies was also associated with reversal of morphological alterations, regaining of fertilization capacity, that is due to the reloading of ATP (Billard et al., 1995; Cosson, 2004).

The mentioned phenomenon is known as sperm reactivation or second activation. It was first time described in 1938 in trout spermatozoa (Cosson, 2004). The reactivation of trout spermatozoa was achieved by incubation of once activated spermatozoa in K^+ rich solution (with osmolality similar to one of seminal fluid) for 30 min. This was followed by activation of incubated sperm in K^+ solution with osmolality lower than in seminal plasma that allowed the induction of motility. Few years later, similar studies were reported in other salmonid species (Billard et al., 1974; Ginzburg, 1968; Yamamoto, 1962). Apart from salmonids, the phenomenon of reactivation has also been shown for spermatozoa of channel catfish (*Ictalurus punctatus*) and carp (Guest et al., 1976; Linhart et al., 2008; Sneed and Clemens, 1956). However, the possibility to initiate the second period of sperm motility could be species specific and could also be regulated by multiple factors. Till today the insightful information on sperm reactivation in fish is limited. In-depth studies of energy budgeting (involved pathways, level of OCR, content of different metabolites, etc.) during reactivation state will be novel and forthcoming steps in fish spermatology. If the mentioned information could be available in more diverse fish groups, this will help to enhance our fundamental knowledge in fish spermatology field. At applied level to achieve a better fertilization success, poorly motile spermatozoa could be revived after incubation in reactivation medium. This approach also holds a great potential, as the sperm of poor quality but from valuable individuals (especially in case of endangered or vulnerable fish species) can be used for conservation purposes.

1.8. Objectives of the thesis

The recognition of spermatozoa biology in bioenergetics are of important interest from the practical point of view. Better knowledge regarding these topics could be fruitful in terms of artificial reproduction improvement. Noticing the above-mentioned knowledge and knowledge gaps in the field of fish sperm bioenergetics, the research was based on the hypothesis that various energy supplying pathways occurring at motile and immotile state of the spermatozoa may vary from species to species, depending on their sperm motility duration or spawning temperature. Therefore, the present study was designed as a comprehensive investigation of the bioenergetic pathways of spermatozoa in fish species spawning at different temperatures and possessing wide range of motility duration. To accomplish this, following objectives were pursued:

1. To study the relative contribution of different bioenergetic pathways involved in energy supply in spermatozoa of Siberian sturgeon (*Acipenser baerii*) at motile and immotile state.
2. To study the energy supplying pathways in an under-ice spawning fish burbot and adaptation of sperm mitochondrial activity to the maximum critical temperature.

3. To study the possibility of sterlet sperm reactivation in terms of regaining motility and re-achieving fertilizing ability.
4. To evaluate the functioning of different bioenergetic pathways in quiescent, motile, and reactivated sterlet spermatozoa.

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CHAPTER 2

ENERGY PATHWAYS ASSOCIATED WITH SUSTAINED SPERMATOZOON MOTILITY IN THE ENDANGERED SIBERIAN STURGEON *ACIPENSER BAERII*

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Energy pathways associated with sustained spermatozoon motility in the endangered Siberian sturgeon *Acipenser baerii*

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Abstract

Sturgeon spermatozoa are unique for their sustained motility. We investigated the relative importance of bioenergetic pathways in the energy supply of Siberian sturgeon *Acipenser baerii* spermatozoa during motile and immotile states. Spermatozoon motility and oxygen consumption rate (OCR) were analysed following exposure to inhibitors of oxidative phosphorylation (sodium azide, NaN_3), glycolysis (2-deoxy-D-glucose, DOG) and β-oxidation of fatty acids (sodium fluoride, NaF), and to an uncoupler of oxidative phosphorylation (carbonyl cyanide *m*-chlorophenyl hydrazine, CCCP). No significant difference in curvilinear velocity was observed after addition of these reagents to activation medium (AM) or nonactivation medium (NAM) for incubation. Incubation of spermatozoa in NAM containing CCCP or NaN_3 resulted in significantly decreased motility duration compared to controls. The OCR of sturgeon spermatozoa in AM (11.9 ± 1.4 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$) was significantly higher than in NAM (8.2 ± 1.5 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$). The OCR significantly declined with addition of NaN_3 to AM and NAM. No significant difference in motility parameters or OCR was observed with NaF or DOG. These results suggest active oxidative phosphorylation in both immotile and motile spermatozoa. Nevertheless, mitochondrial respiration occurring during motility is not sufficient to meet the high energy demands, and the energy required for sustained motility of Siberian sturgeon spermatozoa is derived from adenosine triphosphate accumulated during the quiescent state.

KEYWORDS
fatty acid oxidation, fish, glycolysis, oxidative phosphorylation, spermatozoon motility

1 | INTRODUCTION

Spermatozoon motility and its duration are crucial to successful fertilization. Adenosine triphosphate (ATP) is the only energy source for motility that can be generated and stored in sperm cells (Dzyuba *et al*., 2017). Studies have shown a positive correlation among spermatozoon motility, ATP content, concentration of substrates required for energy supplying pathways and fertilization success (Ingermann *et al*., 2003; Lahnsteiner *et al*., 1996, 1998). Differences in spermatozoon motility variables among fish species suggest variation in metabolic strategies for storage and generation of ATP (Ingermann, 2008). For example, glycolysis and Krebs cycle activity are presumed to be higher in common bleak *Alburnus alburnus* spermatozoa than in African catfish *Clarias gariepinus* (Zietara *et al*., 2009), while lipid catabolism and β-oxidation of fatty acids play important roles in motility of African catfish spermatozoa (Mansour *et al*., 2003). The relative importance of an energy supplying pathway can vary with sperm motile state. In rainbow trout *Oncorhynchus mykiss* spermatozoa, glycolysis and triglyceride catabolism occur simultaneously during the immotile state. During motility, glycolysis occurs in the initial 30 s, but as the ATP level cannot continue to be maintained by glycolysis, a shift to triglyceride metabolism allows regeneration of ATP (Lahnsteiner *et al*., 1993).

Spermatozoa of some fish species exhibit enhanced capacity for oxidative metabolism while activated. Activation of motility in African

catfish spermatozoa was accompanied by a 23-fold increase in respiration rate (Mansour *et al.*, 2003). The spermatozoon of rainbow trout possesses low capacity to increase the rate of oxidative phosphorylation, and thus respiration rate, at motility activation (Christen *et al.*, 1987; Ingermann *et al.*, 2003). To estimate the capacity for enhanced oxygen consumption, uncouplers of oxidative phosphorylation, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, are commonly used.

Spermatozoa of externally fertilizing fish exhibit two primary strategies for energy regulation. First is the production and storage of ATP during the immotile state for use during motility. The rate of ATP consumption exhausts its supply after a brief motile period, usually less than 1 min (Boryshpolets *et al.*, 2009b; Christen *et al.*, 1987). A second and rarer strategy is present in fish with long-lasting motility, such as sturgeon. The data on motility duration of sturgeon spermatozoa is contradictory, with reports ranging from 4 to 30 min (Toth *et al.*, 1997) depending on the composition of activation medium (AM) and its dilution factor (Alavi *et al.*, 2004a,b). Little information is available on spermatozoon metabolism in sturgeon, and the physiology underlying the extended motility duration is unclear.

Billard *et al.* (1999), in an initial study of sturgeon sperm energetics, observed the ATP content of immotile Siberian sturgeon *Acipenser baerii* spermatozoa to be 5.7–9.4 nmol $(10^8 \text{ spz})^{-1}$. The ATP content declined significantly to 2.7-5.6 nmol $(10^8 \text{ spz})^{-1}$ within 5 s after motility activation and then remained stable. A similar range of ATP concentration at quiescent state (6.0–8.1 nmol $(10^8 \text{ spz})^{-1}$) and a similar trend in its sudden decline after motility activation followed by a constant concentration of ATP (2.5–3.8 nmol $(10^8 \text{ spz})^{-1}$) were also observed in sterlet *Acipenser ruthenus* spermatozoa (Fedorov *et al.*, 2017). In addition, the ATP concentration in specified sturgeon species is <50% of that reported for rainbow trout and common carp *Cyprinus carpio* spermatozoa (Christen *et al.*, 1987; Perchec *et al.*, 1995). These points strongly suggest that motility in sturgeon spermatozoa is supported not only by accumulated ATP, as in rainbow trout and common carp, but also by ATP produced during the motile period.

The goal of this study was to determine the relative contribution of primary pathway(s) of energy production in immotile and motile states of Siberian sturgeon spermatozoa by comparing the spermatozoon motility and oxygen consumption rate when exposed to inhibitors and uncouplers of major energy pathways to those of spermatozoa of fish species with short motility duration. To the best of our knowledge, this is the first report on energy-supplying pathways in Siberian sturgeon spermatozoa. Results can aid in understanding sustained motility and in the improvement of *in vitro* gamete manipulation in the world's most endangered group of species (Ruban & Bin, 2010).

2 | MATERIALS AND METHODS

2.1 | Broodstock, spermiation and sperm collection

Experiments were conducted in compliance with the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice. Siberian sturgeon males (>7 kg) were kept at the genetic fisheries centre at the Faculty of Fisheries and Protection of Waters. Prior to the experiment, during the March–April spawning season, fivemature Siberian sturgeon males were transferred from an outdoor pond to a closed water recirculation system at the hatchery of the South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic. The water temperature was increased from 3 to 15°C with a 1°C rise per day. Fish were fed commercial pellets (Alltech Coppens Premium Select 20 mm: crude protein 34%, crude fat 15%; Nettetal, Germany). Spermiation was induced by intramuscular injection of carp pituitary extract dissolved in a 0.9% (w/v) NaCl solution at 4 mg kg^{-1} body weight 24 h before sperm collection. A 4 mm plastic catheter was inserted into the urogenital duct and semen was collected in a 250 ml tube and stored on ice. As a quality control, only samples with >80% motile spermatozoa were used.

2.2 | Activation and nonactivation media

Buffered spermatozoon motility activating medium (AM) consisted of 10 mM Tris–HCl and 0.25% pluronic acid (Dzyuba *et al.*, 2014a), and the buffered spermatozoon motility inhibiting (nonactivating) medium (NAM) comprised 16 mM NaCl, 3 mM KCl, 0.19 mM $CaCl_2$ and 10 mM Tris. The pH was adjusted to 8.0, and osmolality was 10–15 and 50–55 mOsmol kg^{-1} for AM and NAM, respectively.

2.3 | Inhibitors and uncoupler

The widely used inhibitors 2-deoxy-D-glucose (DOG), sodium fluoride (NaF) and sodium azide (NaN_3) were used for inhibition of glycolysis, β-oxidation of fatty acids and oxidative phosphorylation, respectively. Carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) was used as an uncoupler of oxidative phosphorylation. The concentrations of inhibitors and the uncoupler were 1 mM and 50 μM, respectively, as selected by preliminary experiments. The pH of AM and NAM was not altered by addition of inhibitors or the uncoupler.

2.4 | Sperm incubation and motility analysis

Motility parameters were analysed to determine the contribution of energy pathways in Siberian sturgeon sperm in its motile and immotile states. To identify primary metabolic pathways in motile sperm, sperm were exposed to AM with and without inhibitors or uncoupler. To quantify the input of these pathways in the quiescent state, sperm were incubated on ice for 60 min in NAM with and without inhibitors or uncoupler, followed by activation in AM containing the same reagents.

For sperm activation, 40 μl of AM was mixed with 0.5 μl of sperm on a slide to obtain the desired cell concentration. Motility recordings were made at 15°C.

Video recordings were made at ×10 magnification, using a negative phase-contrast condenser with an Imaging Development System (IDS) digital camera equipped with uEye Cockpit software, set at 25 frames per second. Recordings were saved in AVI format and analysed using an integrated system for semen analysis (ISAS software; Proiser, Paterna, Spain) at 10, 60 and 180 s post activation. The selected motility parameters included curvilinear velocity (VCL, μm s^{-1}), straight-line velocity (VSL, μm s^{-1}), average-path velocity (VAP, μm s^{-1}), straightness of track (STR, %) and linearity of track (LIN, %). Spermatozoa with VCL <10 μm s^{-1} were considered nonmotile. Spermatozoon motility duration was defined as time from activation to cessation of movement in 95% of spermatozoa (spz).

2.5 | Determination of spermatozoon concentration and oxygen consumption rate

Spermatozoon concentration was evaluated using a Burker cell haemocytometer (Marienfeld, Germany) and Olympus BX 50 phase contrast microscope (×200 magnification; Olympus, Japan).

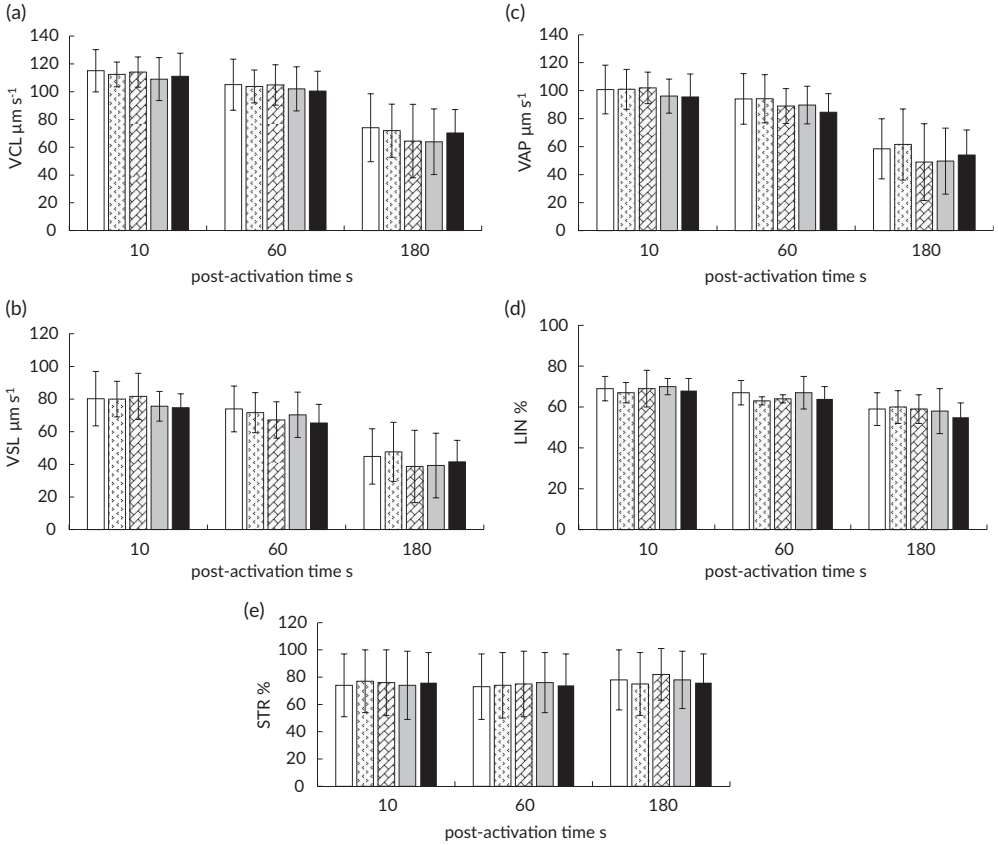

FIGURE 1 Effect of inhibitors and uncoupler on spermatozoon motility at 10, 60 and 180 s post activation in AM. (a) Curvilinear velocity (VCL): (□), control; (⁙), CCCP; (▨), DOG; (▥), NaF; (■), NaN_3. (b) Straight-line velocity (VSL): (□), control; (⁙), CCCP; (▨), DOG; (▥), NaF; (■), NaN_3. (c) Average-path velocity (VAP): (□), control; (⁙), CCCP; (▨), DOG; (▥), NaF; (■), NaN_3. (d) Linearity of track (LIN): (□), control; (⁙), CCCP; (▨), DOG; (▥), NaF; (■), NaN_3. (e) Straightness of track (STR): (□), control; (⁙), CCCP; (▨), DOG; (▥), NaF; (■), NaN_3. Mean ± S.D. presented. Control, motility activation in basal AM; CCCP, motility activation with 50 μM of carbonyl cyanide *m*-chlorophenyl hydrazine (uncoupling agent of oxidative phosphorylation); DOG, motility activation with 1 mM of 2-deoxy-D-glucose (inhibitor of glycolysis); NaF, motility activation with 1 mM of sodium fluoride (inhibitor of β-oxidation of fatty acids); NaN_3, motility activation with 1 mM of sodium azide (inhibitor of oxidative phosphorylation). There were no significant differences between groups at the same post-activation time ($P > 0.05$, one-way ANOVA followed by Tukey's HSD test)

TABLE 1 Effect of inhibitors DOG, NaF and NaN_3, and uncoupler CCCP on motility duration of Siberian sturgeon spermatozoa with and without preliminary incubation in nonactivating medium

Exposure	Motility duration in AM (min)	Motility duration after preliminary incubation (min)
Control	5.5 ± 0.4[a]	5.1 ± 0.9[a]
CCCP	4.6 ± 0.5[a]	3.3 ± 0.5[b]
DOG	4.8 ± 0.6[a]	4.0 ± 0.6[a]
NaF	5.1 ± 0.6[a]	4.2 ± 1.2[a]
NaN_3	5.2 ± 0.7[a]	3.2 ± 0.5[b]

Values with different letters are significantly different ($P < 0.05$, One-way ANOVA followed by Tukey's HSD test).
Note. AM, activating medium; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; Control, no reagent; DOG, 2-deoxy-D-glucose; NaF, sodium fluoride; NaN_3, sodium azide.

A Clark-type polarographic oxygen probe (YSI 5300A Biological Oxygen Monitor; Ohio, USA) immersed in a chamber with a water jacket and magnetic stirrer (IKA magnetic stirrer; Staufen, Germany) was used to determine the oxygen consumption rate (OCR). Since the concentration of Siberian sturgeon sperm was insufficient (0.3--1.5 × 10^9 spz ml^{-1}) to measure changes in oxygen content in the polarographic system, the sperm samples were centrifuged at 500 × g for 10 min at 4°C to obtain 3.5–9.7 × 10^9 spz ml^{-1}. The motility rate of concentrated samples was determined and for each observation 50 µl of the concentrated sample was inserted into the insertion hole of the chamber with a Hamilton syringe.

After addition of sperm samples, the oxygen content in the chamber was recorded in real time at 15°C with stirring at 800 rpm. The OCR of spermatozoa in AM and NAM with or without inhibitors/uncoupler was calculated taking into account the changes in oxygen content and cell concentration, and was expressed as nmolO_2 min^{-1} (10^9 spz)$^{-1}$. The calculated OCR values at 3 min post activation were compared in the presence/absence of studied reagents. Inhibitor and uncoupler of oxidative phosphorylation (NaN_3 and CCCP) were used as for motility analysis, while DOG and NaF were used in combination at a final concentration of 1 mM. Additionally, to observe the trend of OCR in AM and NAM in absence of reagents, the OCR values were calculated at each minute for 5 min.

2.6 | Statistical analyses

Statistical analyses of the group-coded dataset (groups = activation medium, nonactivation medium, inhibitor, uncoupler, control, post-activation time points) were conducted in STATISTICA v. 12 (Statsoft Inc., USA) and R (R Development Core Team, 2015; Vienna, Austria). All experimental values were obtained from mean values of five individual fish, each with two repetitions. The mean values of five fish combined were then calculated and subjected to statistical analysis. The data distribution characteristics and homogeneity of dispersion were evaluated using Shapiro–Wilk and Levene's test, respectively. The normally distributed data with similar dispersion values (VCL, VSL, VAP, STR, motility duration, OCR in AM and NAM, and LIN in AM) were analysed by one-way ANOVA followed by Tukey's honest significant difference test. Nonhomogenous data (LIN after incubation in NAM in the presence and absence of reagents) were analysed by a nonparametric Kruskal–Wallis ANOVA followed by multiple comparison of mean ranks for all groups. Statistical significance was accepted at $P < 0.05$. Data were presented as mean ± S.D. A locally estimated scatterplot smoothing (LOESS) graph generated with the 'ggplot2' package in R (Cleveland *et al*., 1993; Wickham, 2016) was used to illustrate the trend of spermatozoon respiration rate in activation and nonactivation medium for up to 5 min.

3 | RESULTS

3.1 | Motility parameters

Spermatozoon velocity (VCL, VSL, VAP), LIN and STR in the presence of any studied reagent did not differ significantly from controls ($P > 0.05$) at any post-activation time point (Figure 1a–e). Motility duration in AM was 5.5 ± 0.4 min and did not significantly differ ($P > 0.05$) with addition of DOG, NaF, NaN_3 or CCCP (Table 1).

After 60 min of incubation of sperm samples in NAM in the presence of inhibitors/uncoupler, no significant differences were observed in VCL, VSL, VAP, LIN and STR compared to controls at any time post activation (Figure 2a–e). Spermatozoon motility duration after 60 min of incubation in NAM and activation in AM without reagents was 5.1 ± 0.9 min and reduced significantly with CCCP and NaN_3 application (Table 1).

3.2 | Oxygen consumption rate

In the absence of inhibitors and uncoupler, OCR of sturgeon spermatozoa in AM at 3 min post activation was 11.9 ± 1.0 nmolO_2 min^{-1} (10^9 spz)$^{-1}$, significantly higher than in NAM (8.2 ± 1.0 nmolO_2 min^{-1} (10^9 spz)$^{-1}$). At 3 min post activation, presence of CCCP was associated with significantly higher OCR (12.0 ± 1.3 nmolO_2 min^{-1} (10^9 spz)$^{-1}$) in NAM as compared to control but did not result in significant change from controls in AM. With sodium azide, OCR was significantly lower than controls in both AM (7.5 ± 1.0 nmolO_2 min^{-1} (10^9 spz)$^{-1}$) and NAM (4.2 ± 1.3 nmolO_2 min^{-1} (10^9 spz)$^{-1}$). A combination of DOG and NaF reduced OCR in both AM and NAM (10.5 ± 3.4 nmolO_2 min^{-1} (10^9 spz)$^{-1}$ and 6.0 ± 0.5 nmolO_2 min^{-1} (10^9 spz)$^{-1}$, respectively) compared to controls but did not reach significance (Figure 3). From 1 to 5 min, OCR decreased from 13.2 ± 1.0 to 11.2 ± 1.0 nmolO_2 min^{-1} (10^9 spz)$^{-1}$ in AM and from 9.0 ± 0.4 to 8.5 ± 2.1 nmolO_2 min^{-1} (10^9 spz)$^{-1}$ in NAM (Figure 4).

4 | DISCUSSION

For decades the investigation of bioenergetics in fish spermatozoa has employed methods such as ^{14}C-labelled glucose (Gardiner, 1978),

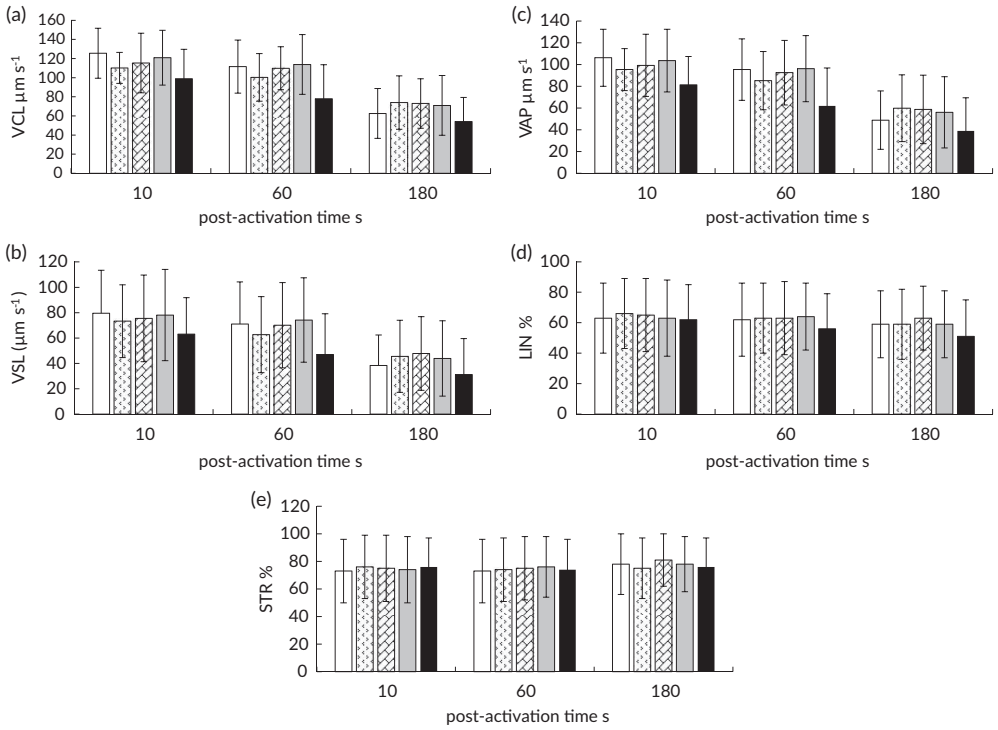

FIGURE 2 Effect of inhibitors and uncoupler on spermatozoon motility at 10, 60 and 180 s post activation after 60 min of incubation in NAM followed by activation in AM containing inhibitors/uncouplers. (a) Curvilinear velocity (VCL): (□), control; (⁘), CCCP; (▨), DOG; (▤), NaF; (■), NaN_3. (b) Straight-line velocity (VSL): (□), control; (⁘), CCCP; (▨), DOG; (▤), NaF; (■), NaN_3. (c) Average-path velocity (VAP): (□), control; (⁘), CCCP; (▨), DOG; (▤), NaF; (■), NaN_3. (d) Linearity of track (LIN): (□), control; (⁘), CCCP; (▨), DOG; (▤), NaF; (■), NaN_3. (e) Straightness of track (STR): (□), control; (⁘), CCCP; (▨), DOG; (▤), NaF; (■), NaN_3. Data are presented as mean ± S.D. Control, no reagent; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone (uncoupling agent of oxidative phosphorylation); DOG, 2-deoxy-D-glucose (inhibitor of glycolysis); NaF, sodium fluoride (inhibitor of β-oxidation of fatty acids); NaN_3, sodium azide (inhibitor of oxidative phosphorylation). Uncoupler and inhibitors were added at 50 µM and 1 mM, respectively, to both AM and NAM. There were no significant differences between groups at the same post-activation time ($P > 0.05$, one-way ANOVA followed by Tukey's HSD test)

nuclear magnetic resonance (Dreanno *et al.*, 2000; Robitaille *et al.*, 1987), liquid chromatography/mass spectrometry/high-resolution mass spectrometry (Fedorov *et al.*, 2015), extraction of enzymes important for bioenergetic pathways (Burness *et al.*, 2005; Lahnsteiner & Mansour, 2012), analysis of spermatozoon motility after application of inhibitors, metabolites, substrates and co-enzymes of pathways involved in energy production (Dreanno *et al.*, 1999; Ingermann *et al.*, 2011; Lahnsteiner *et al.*, 1999; Zietara *et al.*, 2004), and Clark-type polarography (Lahnsteiner *et al.*, 1999; Ingermann *et al.*, 2003; Mansour *et al.*, 2003; Boryshpolets *et al.*, 2009a).

The value for spermatozoon VCL at 10 s post activation without reagents was in accordance with Dadras *et al.* (2019), but low compared to other published data on sturgeon (Dzyuba *et al.*, 2013,b; Xin *et al.*, 2018). This difference may be attributed to factors previously reported to influence the motility of fish spermatozoa: composition of AM, dilution rate, ions, pH and osmolality (Alavi *et al.*, 2004a, 2004b; Alavi & Cosson, 2006). In contrast to the presented results, swimming velocity of African catfish spermatozoa in AM was significantly reduced by the addition of inhibitors of the tricarboxylic acid cycle (malonate), oxidative phosphorylation (atractyloside, 2,4-dinitrophenol and potassium cyanide) and lipid metabolism (hydroxybutyrate and acetoacetate) with and without storage (Mansour *et al.*, 2003). This suggests that in African catfish spermatozoa, in contrast to those of sturgeon, lipid catabolism, β-oxidation of fatty acids, the tricarboxylic acid cycle and oxidative phosphorylation play an important role during the immotile state, while glycolysis, lipid catabolism and β-oxidation of fatty acids along with mitochondrial respiration are important during motility (Mansour *et al.*, 2003). Additionally, in sterlet spermatozoa

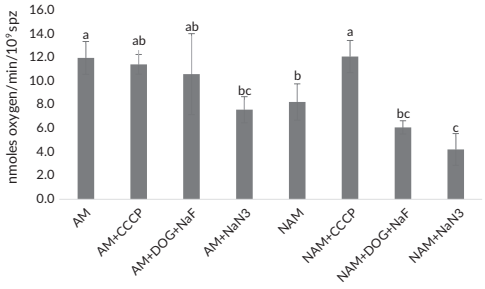

FIGURE 3 Effect of inhibitors and uncoupler on oxygen consumption rate of sturgeon spermatozoa in motility AM and NAM. Data are presented as mean ± S.D. AM + CCCP, activating medium containing carbonyl cyanide *m*-chlorophenylhydrazone; AM + DOG + NaF, activating medium containing a mixture of 2-deoxy-D-glucose and sodium fluoride; AM + NaN_3, activating medium containing sodium azide; NAM + CCCP, nonactivating medium containing carbonyl cyanide *m*-chlorophenylhydrazone; NAM + DOG + NaF, non-activating medium containing amixture of 2-deoxy-D-glucose and sodium fluoride; NAM + NaN_3, nonactivating medium containing sodium azide. Values with different letters are significantly different ($P < 0.05$, one-way ANOVA followed by Tukey's HSD test)

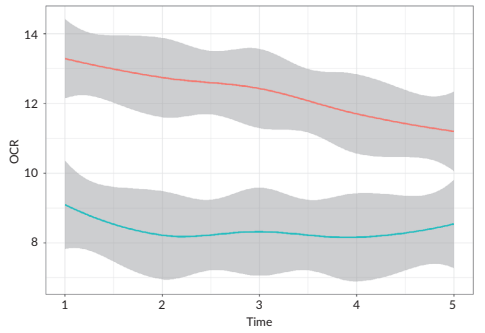

FIGURE 4 Oxygen consumption rate (OCR) in $nmolO_2\ min^{-1}\ (10^9\ spz)^{-1}$) of *A. baerii* (Siberian sturgeon) for 5 min in motility activating medium (AM) and nonactivating medium (NAM). LOESS plots were made with the 'ggplot2' package in R. Graph represents mean ± S.E. (coloured line ± shaded area).(▤), AM; (▤), NAM

few studies have indicated the contribution of the creatine-phosphocreatine shuttle system for the regeneration of ATP, an alternative for energy transport from middle piece to flagella (Dzyuba *et al.*, 2016; Fedorov *et al.*, 2017). A similar mechanism has also been approved in cyprinids and salmonids spermatozoa (Lahnsteiner *et al.*, 1996; Robitaille *et al.*, 1987; Saudrais *et al.*, 1998).

The duration of Siberian sturgeon spermatozoon motility with and without preliminary incubation in NAM was comparable to that reported for other sturgeon species (Lahnsteiner *et al.*, 2004). Motility duration was not significantly reduced in AM containing the inhibitors and uncoupler without preliminary incubation in NAM (Table 1). A significant decrease in motility duration was observed when either CCCP or NaN_3 were present in both incubation and activation medium (Table 1), suggesting involvement of mitochondrial oxidation in generation of ATP stores. Negative effects of respiratory inhibitors (potassium cyanide, 2,4-dinitrophenol, atractyloside) on motility duration, swimming velocity, motility percentage and sperm viability have been observed in Danube bleak *Chalcalburnus chalcoides* (Lahnsteiner *et al.*, 1999). The effect was similar to the present study, suggesting oxidative phosphorylation to be the central energy-supplying pathway. In the present study, the effect of inhibitors and uncoupler were further validated by applying them to spermatozoa to measure oxygen consumption rate.

The mitochondrial respiration rate of Siberian sturgeon spermatozoa in both AM and NAM was significantly reduced by the addition of the respiratory inhibitor NaN_3 (Figure 3). The effect of NaN_3 on both motility duration and OCR confirms that Siberian sturgeon spermatozoa possess the capacity for oxidative phosphorylation during both immotile and motile states. The negative effect of mitochondrial inhibitors has been observed in spermatozoa of numerous marine and freshwater species with motility duration ranging from 30 s to 10 min, including common carp, rainbow trout, turbot *Psetta maxima* and gilthead seabream *Sparus aurata* (Christen *et al.*, 1987; Dreanno *et al.*, 1999; Lahnsteiner & Caberlotto, 2012; Perchec *et al.*, 1995). This indicates active oxidative phosphorylation during motility, like our results. However, in the present study, the lack of effect of NaN_3 on VCL in AM suggests that most of the energy supply required for motility is generated by oxidative phosphorylation in the quiescent state.

The DOG and NaF, inhibitors of glycolysis and β-oxidation of fatty acids, had no significant effect on spermatozoon velocity, LIN, STR (Figures 1 and 2) or respiration rate (Figure 3). This agrees with observations of the effect of DOG and NaF on spermatozoon motility, linearity, nonlinearity, circular swimming motion and viability in spermatozoa of Danube bleak and rainbow trout (Lahnsteiner *et al.*, 1999). The contribution of triglyceride catabolism and glycolysis in rainbow trout and chub *Leuciscus cephalus* spermatozoon in energy production has been verified (Lahnsteiner *et al.*, 1992, 1993; Terner & Korsh, 1963a).

We found the rate of oxygen consumption at 3 min post activation to be higher in AM (11.9 ± 1.4 $nmolO_2\ min^{-1}\ (10^9\ spz)^{-1}$) than in NAM (8.2 ± 1.5 $nmolO_2\ min^{-1}\ (10^9\ spz)^{-1}$) (Figure 3). This pattern was also shown for spermatozoa of the freshwater African catfish, Danube bleak and the turbot (Dreanno *et al.*, 1999; Lahnsteiner *et al.*, 1999; Mansour *et al.*, 2003). In contrast to Siberian sturgeon, spermatozoa of some fish species show a similar level of oxygen consumption before and after motility activation (Terner & Korsh, 1963b; Christen *et al.*, 1987; Inoda *et al.*, 1988; Ingermann *et al.*, 2003).

No significant alteration in OCR of Siberian sturgeon spermatozoa from 1 to 5 min post activation was observed in AM (13.2 ± 1.0–11.2 ± 1.8 $nmolO_2\ min^{-1}\ (10^9\ spz)^{-1}$) or NAM (9.0 ± 0.4–8.5 ± 2.1 $nmolO_2\ min^{-1}\ (10^9\ spz)^{-1}$) (Figure 4). In contrast, Dreanno *et al.* (1999), in a study of activated spermatozoa of turbot (which shows spermatozoon

motility duration comparable to that of sturgeons at 8–10 min), reported a sharp decrease in respiration rate in the first minutes after motility initiation followed by gradual decrease until cessation of movement. Oxygen consumption rate of turbot spermatozoa in NAM was constant, consistent with our results in Siberian sturgeon. The trend observed in the present study indicates active participation of mitochondrial respiration not only in the quiescent state but also throughout the motility period, suggesting enough available substrates for respiration during motility. Our observation on nonsignificant changes in OCR during the active state of Siberian sturgeon spermatozoa can also be related to the reported trend of almost-stabilized ATP content across the motile state of Siberian sturgeon and sterlet spermatozoa (Billard *et al.*, 1999; Fedorov *et al.*, 2017).

The capacity to enhance oxidative phosphorylation on spermatozoon motility activation is a species-specific characteristic. In the present study, the oxidative phosphorylation uncoupling agent CCCP enhanced respiration rate in NAM while no significant difference from controls was observed in AM (Figure 3). The addition of CCCP to sturgeon spermatozoa in NAM resulted in the increase of OCR to the value shown for activated spermatozoa, indicating that, after activation, oxidative phosphorylation functions at its maximum rate. Similar findings have been reported in immotile spermatozoa of turbot and common carp (Boryshpolets *et al.*, 2009a; Dreanno *et al.*, 1999). Little or no effect of an uncoupler was reported in rainbow trout spermatozoa (Christen *et al.*, 1987; Inoda *et al.*, 1988; Ingermann *et al.*, 2003).

In general, there is a trade-off between the energy stock of ATP reserves and motility duration. The present study found no evidence of a contribution of glycolysis and β-oxidation of fatty acids to energy production in motile and immotile sperm. This is similar to spermatozoa of chub and Danube bleak, in which no lipase and phospholipase enzymes were found, and no β-oxidation of fatty acids was detected (Lahnsteiner *et al.*, 1992, 1999). Glycolysis played an important role during the motility of spermatozoa of common bleak (Lahnsteiner *et al.*, 1996). Rainbow trout spermatozoa, in comparison, showed enzyme capacity for both glycolysis and phospholipid catabolism (Lahnsteiner *et al.*, 1993). In the African catfish spermatozoa, glycolysis and β-oxidation of fatty acids occurred simultaneously during motility, while no important role of glycolysis during the quiescent state was recorded (Mansour *et al.*, 2003).

Our results suggest that, in sturgeon spermatozoa, oxidative phosphorylation plays a central role in energy production not only in the quiescent state but throughout the motility period. Nevertheless, taking into account well described previously the dynamic of ATP content during motility period (Billard *et al.*, 1999; Fedorov *et al.*, 2017), the continuing energy production *via* mitochondrial respiration during motility is not sufficient to cope with the high energy demands. Therefore, the energy store accumulated in the quiescent state represents the major contribution to, but is not the sole source of, energy for long-lasting motility of sturgeon spermatozoa. This is similar to turbot and zebrafish *Danio rerio* spermatozoa, in which it has been demonstrated that the primary source of energy for motility is stored ATP, while prolonged motility can rely on continuing ATP generation through mitochondrial oxidative phosphorylation (Dreanno *et al.*, 1999; Ingermann *et al.*, 2011). In contrast, low capacity for mitochondrial oxidation during the motility phase is a crucial reason for the short motility duration of common carp and rainbow trout spermatozoa (Christen *et al.*, 1987; Perchec *et al.*, 1995). Altogether, the elucidation of metabolic pathways involved in ATP generation before motility activation and during the motility phase in sturgeon spermatozoa requires future study to understand the bioenergetics of long-lasting sperm motility in sturgeons.

Unlike teleost fishes, the eggs of sturgeons have several micropylar canals and hence there is a risk of abnormal polyspermic fertilization if several spermatozoa enter the egg simultaneously. In conditions of natural spawning, polyspermy is not common since the sperm are discharged in rapid water currents and thus a low number of spermatozoa can reach the eggs simultaneously because of sperm dilution (Ginzburg, 1972). We may presume that in these conditions longer motility duration is an advantage for successful fertilization and may be regarded as a species adaptation.

5 | CONCLUSIONS

The results suggest mitochondrial oxidative phosphorylation to be the primary pathway of energy production in Siberian sturgeon spermatozoa, with oxidative phosphorylation taking place in both immotile and motile states. Nevertheless, mitochondrial respiration is insufficient to meet high energy demands during motility. Hence, the major source of energy required for the sustained motility of sturgeon spermatozoa is ATP generated and stored during the quiescent state. Considering our results as a step in uncovering the energetic background of long-lasting sperm motility in sturgeons, we presume that further study of the level of endogenous metabolites and enzymatic activities involved in the energy supply of sturgeon sperm is required.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

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CHAPTER 3

BIOENERGETIC PATHWAYS IN THE SPERM OF AN UNDER-ICE SPAWNING FISH, BURBOT (*LOTA LOTA*): ROLE OF MITOCHONDRIAL RESPIRATION IN A VARYING THERMAL ENVIRONMENT

Rahi, D., Dzyuba, B., Policar, T., Malinovskyi, O., Rodina, M., Dzyuba, V., 2021. Bioenergetic pathways in spermatozoa of an under-ice spawning fish burbot (*Lota lota*): role of mitochondrial respiration in varying thermal environment. Biology 10, 739.

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Article

Bioenergetic Pathways in the Sperm of an Under-Ice Spawning Fish, Burbot (*Lota lota*): The Role of Mitochondrial Respiration in a Varying Thermal Environment

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Simple Summary: The burbot (*Lota lota*) is the only endangered or threatened freshwater gadoid that usually spawns in icy waters (<6 °C), and whereas the sperm bioenergetics of many fish species have been studied in the context of adaptation to warmer environments, the sperm of cold-water fish are the least explored. Therefore, this study was undertaken to determine both the roles of the most important energy-supplying pathway(s) in the burbot before and after sperm become motile at the spawning temperature, and the mitochondrial adaptation at the maximum temperature that is tolerable. The results reveal that burbot sperm have a naturally low oxygen consumption rate (respiration) and a limited capacity for enhancement under exposure to an uncoupler. Oxidative phosphorylation is more realized near the critical thermal tolerance limit. However, similar to the sperm of most other freshwater species, this pathway, which occurs during motility, is insufficient to fulfill the large energy demands of the motile sperm. Therefore, be it at the spawning temperature or at a higher temperature, the majority of the energy required for motility is derived from pre-stored ATP reserves produced during a quiescent state.

Abstract: Regarding the sperm of cold-water fish, the contributions of different bioenergetic pathways, including mitochondrial respiration, to energy production at the spawning temperature and its adaptation at the maximum critical temperature (CTmax) are unclear. The roles of glycolysis, fatty acid oxidation, oxidative phosphorylation (OXPHOS) at 4 °C, and OXPHOS at 15 °C for energy production in burbot (*Lota lota*) spermatozoa were studied by motility and the oxygen consumption rate (OCR) (with and without pathway inhibitors and the OXPHOS uncoupler). At both temperatures, the effects of the inhibitors and the uncoupler on the motility duration, curvilinear velocity, and track linearity were insignificant; in addition, the OCRs in activation and non-activation media differed insignificantly and were not enhanced after uncoupler treatment. After inhibitor treatment in both media, OXPHOS was insignificantly different at the 2, 30, and 60 s time points at 4 °C but was reduced significantly at the 30 and 60 s time points after treatment with sodium azide at 15 °C. In conclusion, for burbot sperm at both the spawning temperature and the CTmax, the energy synthesized via OXPHOS during motility was insufficient. Therefore, the majority of the energy required to sustain motility was derived from pre-accumulated energy produced and stored during the quiescent state of the spermatozoa.

Keywords: oxidative phosphorylation; glycolysis; fatty acid oxidation; sperm motility; spawning temperature; maximum critical temperature; cold-water fish

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1. Introduction

The burbot (*Lota lota*) is a holarctic, cold-water stenotherm that undertakes long migrations during the spawning season (mid-winter) and reproduces externally [1], meaning

the temperature affects its life cycle and fertilization. The optimum feeding temperature, optimum spawning temperature, and maximum critical temperature (CTmax) are 12 °C, 1–4 °C (often under ice), and 12–14 °C, respectively (the fish avoids temperatures > 14 °C) [2]. Therefore, the physiological functions of this cold-water fish are realized in a narrow temperature range: 1–15 ± 1 °C.

In an externally fertilizing fish, sperm are released into the water and remain motile for only a moment. Motility, a prerequisite for successful fertilization, is regulated by enzymes, including those involved in energy-supplying pathways. Generally, the relation between enzymatic activity and temperature is a classic bell curve, but unlike the "classic rule," widely accepted by fish spermologists, a different observation was recorded for burbot sperm. For example, after the temperature changed from 4 to 12 and 20 °C, the key energy-supplying enzymes (ATPase, adenylate kinase, pyruvate kinase, and malate dehydrogenase) showed reduced activity [3]. Since the burbot is a species that spawns in icy conditions, it offers a rare opportunity to study the energetic pathways of fish sperm at extremely low temperatures and their adaptations to enhanced temperature.

It is commonly accepted that most of the energy required to maintain adequate ATP for motility in fish is generated through mitochondrial respiration; however, mitochondrial respiration's contribution to energy production might vary with the temperature. The general rule implies that as the temperature rises, the prevalence of sperm motility and sperm velocity increase because of an enhanced respiration rate due to higher enzymatic activity. Contrary to the general rule though, these enhancements are sometimes caused even by lower temperatures [4–6]. This phenomenon, well known in some ectotherms, is referred to as "thermal compensation" [7] and has been studied mostly in somatic tissues. The present study, however, focuses on the gamete (sperm).

In the sperm of fish that fertilize externally, it is commonly recognized that the energy for motility activation and prolongation is produced in the form of ATP during a quiescent but bioenergetically active state, stored, and then expended after activation. Various energy-supplying pathways (glycolysis, phospholipid catabolism, triglyceride metabolism, the Krebs cycle, and oxidative phosphorylation (OXPHOS)) are recruited to produce energy for motility. Nevertheless, the dominant contribution of any of these at a particular motile or immotile state is debatable, species-specific, and follows no general trend [8]; moreover, different spawning temperatures make the generalization even more complicated.

To the best of our knowledge, no study has been conducted on the sperm mitochondrial respiration role (before and during sperm motility) for energy production in fish that spawn in extremely cold water and have special emphasis on mitochondrial activity that adapts to temperature increases. Whether such adaptations are species-specific or can be generalized or extrapolated from somatic tissue studies cannot be distinguished with any certainty.

Furthermore, when the coupling of substrate oxidation and ATP synthesis is incomplete, a mitochondrial anomaly called "proton leak" occurs, and the respiration rate rises but does not generate energy (ATP). In various externally fertilizing fish, such as the cold-water trout (*Oncorhynchus mykiss*) and the warm-water carp (*Cyprinus carpio*) and turbot (*Psetta maxima*), studies have evaluated the enhancements in respiration during active and inactive states [8]. It has been revealed that the spermatozoa of some fish enhance oxidative metabolism after motility activation or after being exposed to an uncoupling agent [8–10]. Despite this documented knowledge, no prediction has yet been made for the enhanced respiration in a particular fish species based on its thermal habitat or taxon.

Although the sperm mitochondrial function has been explained for several fish species from varying thermal regimes, the only extreme cold water data, thus far, concern the trout, which spawns at 10 °C [11]. Therefore, this study was designed to reveal the following for burbot sperm: (a) the relative contribution of specific energy-supplying pathways (glycolysis, fatty acid oxidation, and OXPHOS) in activated and non-activated states at a spawning temperature of 4 °C; (b) changes in the role of the most important energy-supplying pathway when the environmental temperature approaches the critical limit

(CTmax 15 °C); and (c) any significant enhancements in oxidative metabolism (in the presence of an uncoupler or after motility activation) at the spawning temperature or CTmax. The results were then compared with the known trends of various fish with distinct thermal preferences (cold water vs. warm water).

2. Materials and Methods

2.1. Broodstock, Spermiation, and Sperm Collection

Experiments were conducted in compliance with the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice. Three-year-old adult male broodstock (spawning season: January–February; average body weight ~250 g) were cultured in a circular holding tank (40–60 individuals per 500 L tank) at the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Vodnany, Czech Republic. The tank's temperature was kept below 5 °C, and the photoperiod mimicked the preferred natural spawning habitat: 9 h light, 15 h dark. Sperm were stripped by abdominal massage, collected into a 3 mL syringe, kept on ice, and then transferred to a laboratory. As a quality control, only sperm samples having motility >80% were accepted.

2.2. Basal Solutions for Activation and Non-Activation Media

The buffered sperm motility activation medium (AM) used for the burbot consisted of 50 mM NaCl, 10 mM Tris-HCl, and 1 mM $CaCl_2$. The buffered sperm non-activation medium (NAM) comprised 100 mM NaCl, 40 mM KCl, 10 mM Tris, and 1 mM $CaCl_2$. The pH was adjusted to 8.5. Osmolality was 110 mOsmol/kg for AM, and 290 mOsmol/kg for NAM.

2.3. Reagents Used in the Experiment—Inhibitors and an Uncoupler

The inhibitors 2-deoxy-d-glucose (DOG), sodium fluoride (NaF), and sodium azide (NaN_3) were used to inhibit the key enzyme of glycolysis (glucose-6-phosphatase [12]), fatty acid oxidation (fatty acid oxidase, enolase [13]), and OXPHOS (cytochrome oxidase [14]), respectively. Additionally, to assess the enhanced respiration rate in active and non-active burbot sperm, the oxygen consumption rate (OCR) was studied by exposure of sperm to an OXPHOS uncoupling agent, carbonyl cyanide m-chlorophenyl hydrazine (CCCP) [15]. The concentration of every inhibitor (DOG, NaF, and NaN_3) was 1 mM, and that of the uncoupler (CCCP) was 1 μM. The concentrations of the reagents were standardized after conducting a preliminary experiment. The pH and osmolality of both AM- and NAM-containing reagents were confirmed to be unchanged prior to the experiment.

2.4. Motility Assessment

A dominant energy contribution of any of the energetic pathways during the motile state was determined by analyzing motility parameters in AM without (control) and with each inhibitor (DOG, NaF, and NaN_3) and the uncoupler (CCCP). For this, a 40 μL drop of AM (with or without reagents) containing 0.25% pluronic acid was placed onto a microscope slide, and sperm were added using the tip of an insulin syringe needle and thoroughly mixed to a dilution of ~1:10,000 [16] to obtain the appropriate concentration for analysis. All motility records were taken at 4 ± 1 °C. Additionally, to evaluate the OXPHOS activity at a higher temperature, motility with and without OXPHOS inhibitor and uncouplers (NaN_3 and CCCP) was studied at 15 ± 1 °C.

A thermoblock (HLC BO50/15, Landsberger, Berlin, Germany) was used to adjust the temperatures of the solutions. The microscope was equipped with a cooling stage (Olympus IX83, Southend-on-Sea, United Kingdom). The adjusted temperature was monitored via a copper-constantan thermocouple (Omega, L-044T, Taipei, Taiwan) using a data logger thermometer (Omega, HH127, Taipei, Taiwan). Video records were created microscopically under a 10× lens and a negative phase-contrast condenser with an Imaging Development Systems (IDS) digital camera equipped with uEye Cockpit software set at 25 frames·s^{-1}.

Video records were saved in avi format, and analysis was conducted with an Integrated System for Semen Analysis (ISAS software; PROISER, C/ Catedrático Agustín Escardino, Paterna, Spain) at 10, 20, 30, 40, 50, and 60 s, and at 10, 20, 30, and 40 s post-activation at 4 and 15 °C, respectively. Sperm curvilinear velocity (VCL), linearity of track (LIN), and motility duration were selected as the studied parameters for both temperature groups. Sperm having a velocity less than 10 $\mu m \cdot s^{-1}$ were considered to be non-motile. Motility duration was recorded from each video from the beginning of motility until 95% of the spermatozoon stopped moving. The stepwise methodology used to record motility and further analysis are provided in Supplementary Figure S1.

2.5. Sperm Concentration and Measurement of Oxygen Consumption Rate

Sperm concentration was evaluated by using a Burker cell hemocytometer (Meopta, Kabelikova 1, Prerov, Czech Republic) and an Olympus BX 50 phase-contrast microscope (200× magnification; Olympus, Shinjuku, Tokyo, Japan) [17].

The OCR of burbot sperm was measured with a Clark-type polarographic oxygen probe (YSI 5300A Biological Oxygen Monitor; Brannum Lane Yellow Springs, OH, USA) immersed in a chamber with a water jacket (regulating the temperature to 4 or 15 °C) kept on a magnetic stirrer (frequency of rotation 800 rpm). For assessing the influences of inhibitors of energy-supplying pathways (DOG, NaF, and NaN_3) on the sperm OCR, the chamber was filled (1000 µL) with media (AM or NAM) with or without (control) inhibitors and then closed with an oxygen probe. A 50 µL sperm sample was injected through the insertion hole a few seconds after obtaining a stable signal. For the assessment of the influence of the CCCP uncoupler on the OCR, a sperm sample was injected in a closed chamber containing media (AM or NAM without an uncoupler), and a reagent was then injected 120 s after the injection of sperm. Additionally, to study the role of OXPHOS at a higher temperature, the OCR was measured in the presence and absence of an inhibitor and uncoupler (NaN_3 and CCCP) of OXPHOS in AM and NAM at 15 °C.

With a polarographic system, oxygen saturation data (%) at each 2 s interval were obtained in real time. Taking into account the cell concentration for each male and the solubility of oxygen at different temperatures, the final OCR was calculated from the oxygen content (%) for each group (males, media combinations, temperature, and time). For this, OCR was calculated every 2 s (from 0 to 72 s) while keeping a 10 s tracking interval (0–10, 2–12, 4–14, etc., up to 62–72 s). Finally, to demonstrate the OCR changes occurring in the presence and absence of inhibitors at 0–70 s, average values from five males and two replicates for each group were calculated. The dots obtained from the averaged values were connected and plotted on a line graph.

Additionally, to analyze the effects of inhibitors on the OCR of burbot sperm, statistical analysis was performed (elaborated in Section 2.6) at the beginning, middle, and end of motility (2, 30, and 60 s, respectively). The effect of the uncoupler at each temperature (4 and 15 °C), in each medium (AM and NAM), was determined by calculating the OCRs at the 3rd min (120–180 s) in the presence and absence of CCCP at 1 µM concentration. All the calculated values are expressed in nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$. The general experimental design and workflow are presented in Supplementary Figure S2.

2.6. Statistical Analyses

Statistical analyses for each group (temperature, media combinations (AM/NAM with and without reagents), and time points were conducted in STATISTICA v12 (Statsoft Inc., Hillview Avenue Palo Alto, CA, USA). Each experimental value was obtained by averaging the results of five male fish with two replicates each. The mean values of the males were calculated and used for statistical analyses. Interactions were considered statistically significant at $p \leq 0.05$.

The data distribution characteristics and homogeneity of dispersion were evaluated by using the Shapiro–Wilk test and Levene's test, respectively. A parametric test, one-way ANOVA followed by Tukey's honest significant difference (HSD), was applied for the

normally distributed data with similar dispersion values. For the abnormally distributed data or data with an absence of homogeneity (OCR values at 15 °C), a non-parametric test (Mann–Whitney test) was applied to analyze the effect of the inhibitor in the presence of the respective media (AM with AM + NaN_3 and NAM with NAM + NaN_3).

3. Results

3.1. Sperm Motility Parameters

At 4 and 15 °C, the reduction in the burbot sperm VCL after treatment with each reagent (inhibitors and uncoupler) at any post-activation time point was insignificant (Figure 1a,b). Similar results were obtained for the LIN (Figure 2a,b).

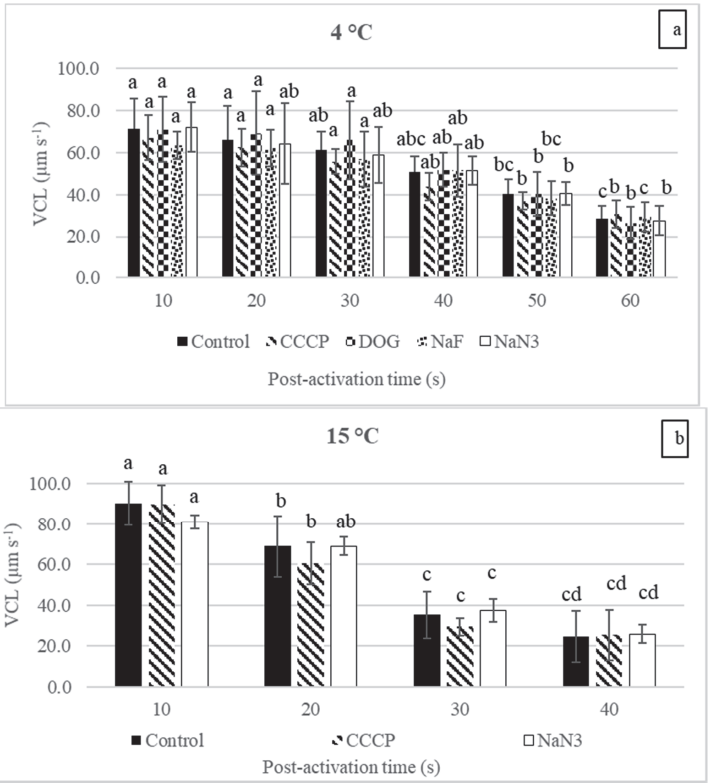

Figure 1. The influences of inhibitors and an uncoupler on the curvilinear velocity (VCL) of burbot (*L. lota*) sperm at different post-activation time points in AM at (**a**) 4 °C; (**b**) 15 °C. Control, no reagent; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; NaF, sodium fluoride; DOG, 2-deoxy-D-glucose; NaN_3, sodium azide. Inhibitors and the uncoupler were used at 1 mM and 1 μM concentrations, respectively. Values with different letters at different post-activation times are significantly different ($p < 0.05$, Tukey's HSD test). Data are presented as mean ± S.D.

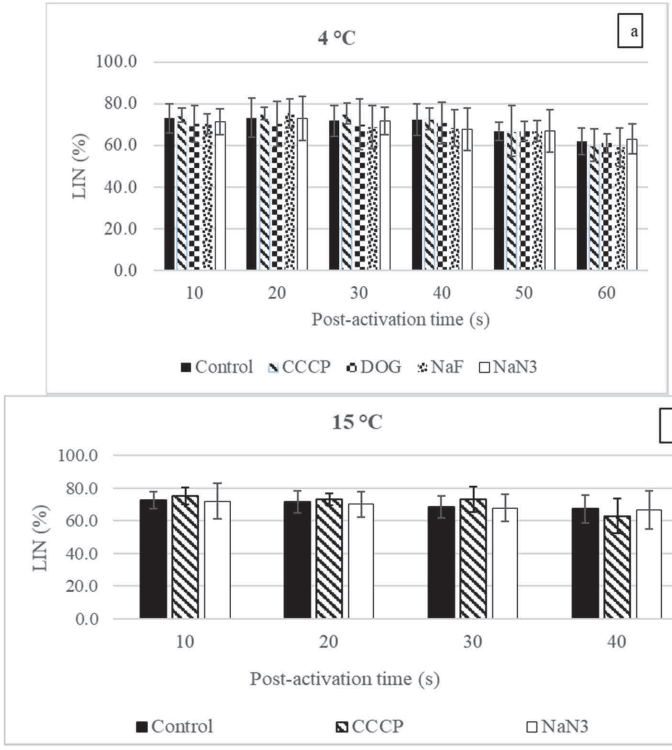

Figure 2. The influences of inhibitors and an uncoupler on the linearity of track (LIN) of burbot (*L. lota*) sperm at different post-activation time points in AM at (**a**) 4 °C; (**b**) 15 °C. Control, no reagent; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; NaF, sodium fluoride; DOG, 2-deoxy-D-glucose; NaN_3, sodium azide. Inhibitors and the uncoupler were used at 1 mM and 1 μM concentrations, respectively. At 4 and 15 °C, no significant changes were observed either after treatment with any reagents or past the post-activation time ($p > 0.05$, Tukey's HSD test). Data are presented as mean ± S.D.

In the control, the reduction in the VCL post-activation was slow at 4 °C and decreased significantly only at 50 s compared with 10 s (Figure 1a). At 15 °C, the reduction in the VCL over the post-activation time was faster than at 4 °C (the VCL was significantly reduced at 20, 30, and 40 s compared with 10 s (Figure 1b)). No significant changes were observed in the LIN at any time point at 4 and 15 °C (Figure 2a,b). Additionally, after raising the temperature from 4 to 15 °C, the VCL at 10 s post-activation was significantly increased in the control and CCCP groups (1.27 and 1.33 times, respectively) ($p < 0.05$, one-way ANOVA), but no significant enhancement was observed in the NaN_3 group ($p > 0.05$, one-way ANOVA).

At 4 and 15 °C, the motility duration in the control group was insignificantly changed after treatment with any studied reagent ($p > 0.05$, one-way ANOVA). At 15 °C, the motility duration was significantly reduced compared to 4 °C in all groups: control, NaN_3, and CCCP—1.40, 1.38, and 1.45 times, respectively ($p < 0.05$, one-way ANOVA) (Table 1).

Table 1. Effects of inhibitors (at 1 mM) and an uncoupler (at 1 μM concentration) on the motility duration of burbot (*L. lota*) sperm in an activation medium at 4 °C and 15 °C.

Exposures	4 °C (s)	15 °C (s)
Control	65.8 ± 5.1 a	46.7 ± 1.8 b
CCCP	63.0 ± 5.4 a	45.6 ± 2.6 b
NaF	67.1 ± 3.1	-
DOG	68.7 ± 4.2	-
NaN_3	68.2 ± 5.3 a	47.0 ± 5.9 b

Control, no reagent; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; NaF, sodium fluoride; DOG, 2-deoxy-D-glucose; NaN_3, sodium azide. Values with different letters within rows are significantly different ($p < 0.05$, Tukey's HSD test). Data are presented as mean ± S.D.

3.2. Oxygen Consumption Rate (OCR)

At 4 °C, as with the situation at 15 °C, the OCRs in AM and NAM at all time points (2, 30, and 60 s) were insignificantly different from each other (Figure 3a–c). In addition, at 4 °C, no significant change in the OCR compared to the control was found after treatment with any inhibitor at 2, 30, or 60 s in either medium (AM or NAM) (Figure 3a,b).

At 15 °C, in each medium, no significant effect of NaN_3 at 2 s was observed compared to the control ($p > 0.05$, Mann–Whitney U test). At 30 and 60 s, a significant reduction in the OCR after exposure to NaN_3 was observed in AM and NAM ($p < 0.05$, Mann–Whitney U test) (Figure 3c, Table 2). The OCR significantly increased at 15 °C compared to 4 °C in AM and NAM at 2 (1.71 and 1.90 times), 30 (1.77 and 1.75 times), and 60 s (1.89 and 1.63 times) ($p < 0.05$, one-way ANOVA). No significant changes were observed after exposure to an uncoupling agent (CCCP) in AM or NAM at 4 or 15 °C.

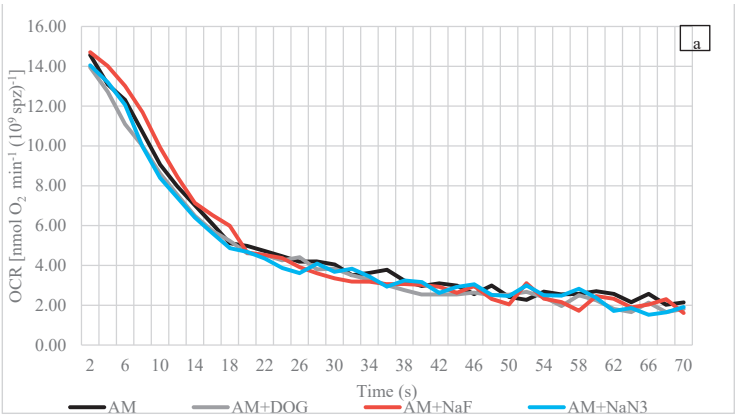

Figure 3. *Cont.*

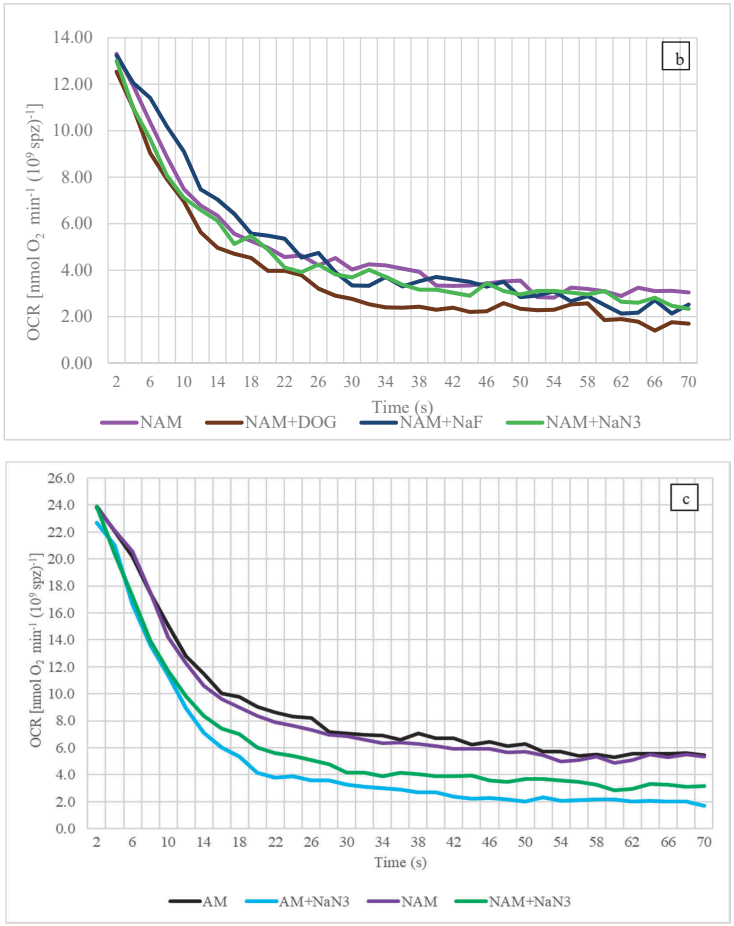

Figure 3. Oxygen consumption rate (OCR, nmol O_2 min^{-1} $(10^9\ spz)^{-1}$) of burbot (*L. lota*) sperm at (**a**) 4 °C in AM; (**b**) 4 °C in NAM; (**c**) 15 °C in AM and NAM. Line plots were created with and without inhibitors at 0–70 s. Each line was obtained by connecting the dots of average values for combinations of male/medium composition/time. AM, activation medium; NAM, non-activation medium; AM + NaF, activation medium containing sodium fluoride; AM + DOG, activation medium containing 2-deoxy-D-glucose; AM + NaN_3, activation medium containing sodium azide; NAM + NaF, non-activation medium containing sodium fluoride; NAM + DOG, non-activation medium containing 2-deoxy-D-glucose; NAM + NaN_3, non-activation medium containing sodium azide. Values of OCR at 2, 30, and 60 s are presented in Table 2.

Table 2. Effects of inhibitors (at 1 mM concentration) on oxygen consumption rate (nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$) of burbot (*L. lota*) sperm at 2, 30, and 60 s in AM and NAM.

Exposures	2 s	30 s	60 s
4 °C			
AM	14.6 ± 3.3 a	4.0 ± 0.8 a	2.7 ± 1.0 a
AM + NaF	14.7 ± 2.7 a	3.3 ± 1.3 a	2.6 ± 0.9 a
AM + DOG	13.8 ± 2.3 a	3.9 ± 1.9 a	2.3 ± 0.6 a
AM + NaN_3	14.0 ± 4.1 a	3.7 ± 1.5 a	2.4 ± 1.8 a
4 °C			
NAM	13.3 ± 2.6 a	4.0 ± 1.0 a	3.0 ± 0.8 a
NAM + NaF	13.2 ± 4.1 a	3.3 ± 1.5 a	2.5 ± 1.2 a
NAM + DOG	12.5 ± 3.0 a	2.8 ± 0.7 a	1.9 ± 0.4 a
NAM + NaN_3	13.0 ± 2.9 a	3.7 ± 1.6 a	3.1 ± 1.2 a
15 °C			
AM	23.9 ± 5.2 a	7.1 ± 1.4 a	5.3 ± 0.8 a
AM + NaN_3	22.7 ± 5.1 a	3.2 ± 0.7 b	2.1 ± 0.6 b
15 °C			
NAM	23.5 ± 8.8 a	6.5 ± 2.2 a	4.9 ± 1.9 a
NAM + NaN_3	23.9 ± 8.1 a	4.2 ± 2.0 b	2.9 ± 1.6 b

AM, activation medium; NAM, non-activation medium; AM + NaF, activation medium containing sodium fluoride; AM + DOG, activation medium containing 2-deoxy-D-glucose; AM + NaN_3, activation medium containing sodium azide; NAM + NaF, non-activation medium containing sodium fluoride; NAM + DOG, non-activation medium containing 2-deoxy-D-glucose; NAM + NaN_3, non-activation medium containing sodium azide. In columns, no significant changes are observed in AM and NAM after treatment with any reagent at 4 °C ($p > 0.05$, Tukey's HSD test). In each column, values with different letters represent differences at 15 °C ($p \leq 0.05$, Mann–Whitney U test). Data are presented as mean ± S.D.

4. Discussion

The burbot is the only freshwater gadoid that is endangered or threatened in North America and Eurasia [18,19]. Increased temperatures due to climate change are one reason among many, such as pollution, urbanization, and invasive non-native species, behind the declining population of the burbot. Several studies have been conducted to mitigate the population decline directly or indirectly. Most of them have focused on understanding its life cycle, enhancing reproduction in hatcheries, or understanding and improving sperm physiology [3,16,20–24]. Nevertheless, sperm energy budgeting (production, accumulation, and usage) and its adaptation to raised temperatures were still unexplored before this study commenced. This article is the first report on the determination of the dominant energy-supplying pathway (glycolysis, fatty acid oxidation, or mitochondrial respiration) that contributes to the energy production required for sperm motility at its spawning temperature. Additionally, the modulation in the role of OXPHOS (the most important bioenergetic pathway in most freshwater species) was studied at CTmax (15 °C). Furthermore, the obtained results at the spawning temperature and CTmax were compared to published data on various freshwater species that spawn in different thermal regimes.

Since it is the only freshwater species belonging to the order Gadiformes, the burbot has retained many characteristics of ancestral marine and cold stenothermal fish, such as high fecundity and a pelagic larval stage [21]. Nevertheless, the spermatozoon motility duration (which is known to be generally shorter for freshwater species and longer for marine fish) at 4 °C was typical of other freshwater species such as the Arctic char (*Salvelinus alpinus*) and the pikeperch (*Sander lucioperca*) in temperatures of 2–5 °C [25,26]. The VCL was well within the range of previous values found for the burbot, but lower than the VCL of trout sperm [20,27,28].

4.1. Influences of the Inhibitors and Uncoupler on Sperm Motility at Spawning Temperature

The relative contributions of energy-supplying pathways have been debated for decades, not only for fish spermatozoa but also for mammals [29,30]. An additional question often asked among fish spermologists is whether fish sperm generate and use energy during motility or produce energy (ATP) before motility and then use only pre-stored energy. Most studies on fish found that energy generation during sperm motility was the least contributing factor [31–33]. Thus, the majority of energy required was derived from a pre-accumulated source produced while in a quiescent state. However, a handful of studies on trout presented contradictory results that carried the debate forward [34].

In sperm bioenergetics, determining the most important energy-supplying pathways by studying the influences of inhibitors and uncouplers on sperm motility or the OCR has been going on for decades. The reagents used in this study (NaN_3, CCCP, DOG, and NaF) were previously used widely on animals belonging to different taxa [35–38]. The results reveal that at 4 °C, the sperm VCL and LIN were insignificantly changed after exposure to any inhibitor at any post-activation time (Figures 1a and 2a). Similar results were observed for the motility duration (Table 1). In line with the present study, the insignificant effect of the OXPHOS inhibitor (potassium cyanide, KCN) was observed on the sperm swimming velocity of the common carp [33]. In the zebrafish (*Danio rerio*), OXPHOS inhibitor (KCN) and an uncoupler (2,4 DNP) did not inhibit the sperm motility percentage until 90 s, which was more than its motility duration [32]. In the Danube bleak (*Chalcalburnus chalcoides*), insignificant changes in sperm motility, linearity, nonlinearity, circular swimming motion, and viability were observed after treatment with DOG or NaF [35]. These data indicate that burbot sperm are metabolically depressed and have no major contribution from any studied pathway during motility at the spawning temperature (icy conditions). The results also suggest that the burbot adopts the energy budgeting strategy adopted by most fish because no energy-supplying pathway provides enough energy to maintain motility during the active state. Thus, most of the energy was derived from pre-accumulated energy produced during a quiescent but metabolically active state.

An altogether different approach is adopted by the spermatozoa of another warm-water species, the African catfish (*Clarias gariepinus*), which uses glycolysis, lipid catabolism, and fatty acid oxidation for energy production during motility [36]. The catfish is rare among other teleosts in that these pathways contribute to energy production during sperm motility. While teleost species (cyprinids and salmonids, having been intensively studied) have the metabolic capacity for glycolysis, oxidative phosphorylation, and lipid metabolism [35,39–41], the contributions of these pathways for energy supply during motility are the least known [42].

The formation of reactive oxygen species (ROS), mainly during biological processes such as respiration, is a common cellular phenomenon. Metabolic byproducts such as superoxide radicals ($O_2\bullet-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($\bullet OH$), and singlet oxygen ($^1O_2$) are common ROS species that are formed during phosphorylation reactions and oxygen metabolism. They eventually manifest into oxidative stress [43]. At higher temperatures, when the OCR increases substantially in either motile or quiescent sperm, there is a concomitant increase in ROS production [44]. As lipids are perhaps the most prone to oxidation, even at low temperatures, they are the largest contributor of ROS [45]. Fortunately, cellular systems can detoxify themselves [43]. Burbots have a ω6/ω3 fatty acid (FA) ratio, which is more skewed towards ω6 than ω3: 1.6:1 to ~3:1 (re-calculated from [20]). Such a proportionately high level of ω6 FAs is known to slow down lipid oxidation and ROS formation [46,47]. Therefore, burbot sperm may be naturally predisposed to protect themselves from ROS attacks. This study did not characterize ROS formation at the high temperatures where the OCR increases, but future efforts may be directed towards understanding this mechanism.

4.2. The Influence of Temperature on Sperm Motility

There is a conventional opinion that the level of enzymatic activity increases with temperature to a certain point. Any further increases in temperature reduce activity. Thus, we have a bell curve. Additionally, among fish spermologists, it is well known that when the temperature rises, the sperm velocity, motility rate, flagellum beat frequency, and ATP consumption rate are enhanced, leading to a shorter motility duration [48]. Nevertheless, some fish species behave in the opposite way [3,49,50]. The burbot spermatozoon VCL at 10 s post-activation and motility duration increased and decreased significantly, respectively, with a rise in the temperature. The results of the motility parameters are aligned with those of previous studies conducted on numerous species, including the burbot, which showed a higher motility percentage when the temperature ranged from 5 to 20 °C [23,51]. The results suggest that the motility parameters (VCL and motility duration) for the burbot follow the general rule.

An increase in the velocity with an increase in the temperature was not observed when NaN_3 inhibited mitochondrial respiration (Figure 1a,b). Additionally, in the control group, the reduction in the VCL over the post-activation time at 15 °C was faster than at 4 °C. The above-mentioned results clearly indicate predominant ATP production (via OXPHOS) and faster consumption at a higher temperature. These findings are in agreement with a study conducted on carp, a warm-water fish, that showed that the decrease in the ATP content during motility is faster at 20 °C than at 2 °C [33].

4.3. Oxygen Consumption Rate

Recently, to measure the OCRs in biological samples, several novel methodologies have been devised, among which are the MitoXpress fluorescent assay [52], electron paramagnetic resonance oximetry [53], the Seahorse metabolic assay [54], the scanning electrochemical microscopy method [55], and the respiratory detection system [56]. Nevertheless, most mitochondrial respiration studies in fish spermatozoa, including this one, were performed using the conventional Clark-type polarographic method [10,31,35,36,57].

To gain a better understanding of the bioenergetic processes during the most important phase of fish spermatozoa, i.e., motility, ATP content, or the OCR of the spermatozoa of several fish, species have been investigated from the first moments of motility (0–60 s) [33,52,58,59]. In this study, at 4 °C, burbot sperm OCRs in AM and NAM at 2 s were 14.6 ± 3.3 and 13.3 ± 2.6 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$, respectively. Within 30 s, the AM and NAM values dropped dramatically by 3.60 and 3.33 times, respectively, and then remained similar until the final point of study at 60 s (Figure 3a,b; Table 2). The relatively low (compared to 2 s) but unaltered values of the OCR in AM and NAM during motility (30 to 60 s) were comparable to those of another cold-water species, the trout [11,60]. The burbot OCRs we measured, accompanied by the trout values, lie in the lower range of data for teleosts (1 to 280 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$) [8]. In contrast to these cold-water species, a very high OCR, followed by a sharp fall within 2 min, was observed for the turbot. This trend was observed only in AM. In NAM, a constant OCR was observed from 0 to 12 min [31]. The results of this study suggest that OXPHOS in motile burbot sperm at the spawning temperature (ice conditions) occurs at a very low level and does not account for any major contribution to energy production during motility.

Fish sperm, in general, are metabolically depressed, which is common for cold-water species. The OCR values at all studied times (2, 30, and 60 s) did not vary significantly between AM and NAM (Figure 3a,b; Table 2). This observation is supported by the majority of the data for the trout [11,57,59]. In contrast, the trend of an enhanced OCR after motility activation is common in temperate, warm-water, or marine species such as the carp, African catfish, Siberian sturgeon (*Acipenser baerii*), and turbot [9,10,31,36]. To the best of our knowledge, the burbot in the presented study and the trout mentioned above are the only cold-water species in which spermatozoon metabolic pathways have been studied by means of the OCR. Therefore, based on limited data, the conclusion that the

mitochondrial OCR of a cold-water species has no capacity for enhancement after motility begins is not strong enough, and numerous intensive cold-water fish studies are needed.

4.4. Influences of Reagents (Inhibitors and Uncoupler) on OCR at Spawning Temperature

At 2, 30, and 60 s, no significant inhibitor influence on the burbot sperm OCR was observed at 4 °C in AM or NAM (Figure 3a,b; Table 2). These results support the findings for motility (VCL, LIN, and duration) (Figures 1a and 2a). In contrast, the negative effects of mitochondrial inhibitors on the sperm OCR or its outcome, ATP, have been observed in numerous marine and freshwater species, including the common carp, the rainbow trout (*Oncorhynchus mykiss*), the turbot, and the gilthead seabream (*Sparus aurata*), suggesting the existence of ATP production via mitochondrial respiration during motility. However, due to the low capacity of OXPHOS during motility, the major energy source is pre-stored ATP [31,33,57,59,61]. Other cold water-spawning species, such as the rainbow trout, also metabolize lipids when in a non-active state. Those lipids are available as intra- and extra-cellular materials which could aid in sperm motility [62,63], but we did not observe lipid oxidation to be a statistically significant energy-supplying pathway in an active or non-active state, even though lipids have the highest calorific value (9 kcal·g^{-1}).

Existing data on the lipid composition of burbot sperm indicate that the ratio of polyunsaturated fatty acids (PUFAs)/saturated or mono-unsaturated fatty acids (SFAs + MUFAs) is ~1:1 [20]. Although mitochondrial β-oxidation in fish happens more freely for SFAs and MUFAs, β-oxidation of PUFAs can be quite variable or complicated [64]. One of the reasons that burbot sperm mitochondrial β-oxidation was not significant is that the lipid composition was not completely skewed towards "β-oxidation labile" SFAs + MFAs but was in equilibrium with PUFAs (more complicated for β-oxidation).

In this study, the OCRs in AM and NAM at 4 and 15 °C were not enhanced after uncoupler treatment. The results are similar to the data for the trout at 10 °C [11,57]. While warm-water species such as the carp, sturgeon, and turbot spawn at 15–20 °C, enhanced OCRs after uncoupling in a non-active state have been observed [9,10,31]. The results suggest a homologous pattern for an uncoupling effect in the spermatozoa of cold-water (no enhanced capacity) and warm-water fish (enhanced capacity). However, the lack of background studies on cold-water fish species prevents us drawing the conclusion that cold-water fish have no capacity for enhanced mitochondrial oxidative metabolism.

4.5. The Influence of Temperature on OCR

At 15 °C, the increases in the sperm OCR in AM and NAM at each time point (2, 30, and 60 s) were significant. However, as with the results for the VCL, the decrease from 2 to 60 s was much faster at 15 °C (23.9 to 5.3 nmol O_2 min^{-1} (10^9 spz)$^{-1}$ in AM, and 23.5 to 4.9 nmol O_2 min^{-1} (10^9 spz)$^{-1}$ in NAM) than at 4 °C (14.6 to 2.7 nmol O_2 min^{-1} (10^9 spz)$^{-1}$ in AM, and 13.3 to 3.1 nmol O_2 min^{-1} (10^9 spz)$^{-1}$ in NAM). In line with the results of this study, more than double the OCR in African catfish sperm was observed in AM and NAM when the temperature rose from 18 to 28 °C [36]. However, the rule of an enhanced OCR (and its outcome, ATP) with raised temperature is not universal. For example, in a non-active state, the ATP content of carp sperm remained unchanged when comparing 2 and 20 °C [33].

Apart from fish spermatozoa, numerous studies have also been conducted on various tissues and organs to explain the thermal response of mitochondrial functioning. In contrast to the above-mentioned thermal response or "general rule," a greater mitochondrial density, higher OCRs, and higher mitochondrial enzymatic activity have all been observed at low temperatures [4–6]. This phenomenon is well known in some ectotherms and is referred to as "thermal compensation" [7]. No such response was seen in this study. Altogether, it seems that the adaptation of mitochondrial functioning to varying thermal conditions is species-specific and cannot be generalized based on the thermal habitat.

Furthermore, at 15 °C, at 30 and 60 s, there were significantly lower OCRs in AM and NAM compared to the control after exposure to NaN_3, an inhibitor of OXPHOS (Table 2).

Thus, this study reveals that, in the burbot, the role of mitochondrial respiration in energy production becomes more pronounced at high temperature, whereas it is barely detectable (low OCR and no NaN_3 inhibition) at low temperature. However, to more deeply explain the adaptation strategies in the sperm mitochondria of cold-water fish at high temperatures, detailed studies of the changes in mitochondrial morphology and membrane potential, and of modulations in the energy-supplying pathways other than OXPHOS at different temperatures, are the footsteps for the future.

We compared the sperm energetics of the burbot at its CTmax with that of the Siberian sturgeon—using a similar study published by our research group [10]—because the burbot's CTmax is exactly at the Siberian sturgeon's spawning temperature. Even at the same temperature (15 °C), the sturgeon's motility duration (a few minutes) was longer than that of the burbot (<1 min).

The burbot sperm OCR at 15 °C, 60 s post-activation (5.3 ± 0.8 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$: the last studied time point for the burbot but the first for the sturgeon), was 2.5 times lower than that of the sturgeon (13.2 ± 1.0 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$) at the same temperature [10]. Similar results were obtained in a non-activation mediums: burbot, 4.9 ± 1.9 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$; sturgeon, 9.0 ± 0.4 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$. Additionally, in burbot sperm, as in Siberian sturgeon sperm, OXPHOS was inhibited by NaN_3 in AM and NAM (Figure 3c) [10]. Although the sperm mitochondrial activity was enhanced and played an important role in the energy production of our cold-water fish at this "high" temperature, the OCR level did not reach the level attained by temperate fish at the same temperature. The results show evidence of mitochondrial adaptation in the sperm of fish acclimatized to a particular thermal regime.

Furthermore, there might be a relationship among the number and size of mitochondria and the bioenergetic requirements of sperm. For example, salmonids have a low OCR range (0.6–3.9 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$) and one mitochondrion or a pair of mitochondria, whereas sturgeon sperm, which has a higher OCR range (8.5–13 nmol O_2 min^{-1} $(10^9$ spz$)^{-1}$), has a greater number of mitochondria (3–6) [8,10,65,66]. A similar quantitative study of burbot sperm mitochondria will reveal detailed bioenergetic features.

4.6. Future Directions

Future studies may also include assessments of the direct molecular turnover of amino acids (e.g., de novo bioconversions of creatine from arginine and arginine from proline [67,68]) or fatty acids (e.g., in PUFAs/SFAs + MUFAs and ω6/ω3 fatty acid ratios [64,69,70]), or of the metabolizable losses (catabolism) of amino and fatty acids when producing energy [71]. Considering the recent arguments surrounding NaF as an ineffective glycolysis inhibitor [72,73] (see discussions in [10,74,75]), additional techniques, such as evaluating the mitochondrial membrane potential, could provide clarity.

To the best of our knowledge, this study is the first to explain the relative contributions of energy-supplying pathways in a fish species that spawns in icy conditions. Determining the role of mitochondrial respiration in the burbot at higher temperatures and comparing the results with those of species with varied thermal regimes helped explain the basics of mitochondrial functioning in fish spermatozoa in different thermal habitats.

5. Conclusions

The results on sperm motility and the OCR observed at 4 and 15 °C suggest that burbot spermatozoa at the spawning temperature (ice conditions) are metabolically depressed with a low OCR and no capacity to enhance oxidative metabolism either by motility activation or by uncoupler treatment. The role of OXPHOS became more prevalent at CTmax; however, at both temperatures, the energy generated via OXPHOS during motility was not efficient enough to fulfil the high energy demand. Therefore, irrespective of the temperature, the energy production and usage strategy remained the same: most of the spermatozoon energy was derived from stored ATP that had been synthesized via OXPHOS during a quiescent but bioenergetically active state.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10080739/s1, Figure S1: Method used for motility assessment, Figure S2: General experimental plan.

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CHAPTER 4

TALE OF MOTILITY EXHAUSTED SPERM: BIOENERGETIC PATHWAYS DURING NON-ACTIVATION, POST-ACTIVATION, AND REACTIVATION OF STERLET (*ACIPENSER RUTHENUS*) SPERMATOZOON

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Tale of motility exhausted sperm: bioenergetic pathways during non-activation, post-activation, and reactivation of sterlet (*Acipenser ruthenus*) spermatozoon

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Abstract

The most important bioenergetic pathways involved in energy supply during quiescent and motile state of spermatozoa, possibility of sperm reactivation, fertilizing ability of reactivated sperm, possibility of improvement in fertilization rate of reactivated sperm by supplementation with metabolites, the most important bioenergetic pathways during reactivation of sterlet (Acipenser ruthenus) spermatozoa were studied.

Out of all studied pathways (glycolysis, mitochondrial respiration, and phosphocreatine-creatine – PCr-Cr shuttle system), only PCr-Cr shuttle system was found to be the most important pathway during quiescent state. While after motility activation, no studied pathway had significant energy supply contribution. After termination of first round of motility, the spermatozoa of sterlet were able to become motile and to fertilize the egg cells after 1-hour incubation in reactivation medium (RM). The motility rate, motility duration, and curvilinear velocity (VCL) of reactivated sperm were significantly lower than ones of the fresh sperm. However, the neurulation and hatching percentages after using reactivated sperm were similar to that after using the fresh sperm and significantly higher when motility exhausted sperm were used for fertilization. Motility rate, motility duration, and VCL after reactivation period were significantly decreased when reactivation and activation media were supplemented with inhibitors of mitochondrial respiration and creatine kinase. Similar results were observed at 1- and 2-hour post incubation.

Based on the obtained results, we suggest that PCr-Cr shuttle system is the most requested energy supplying pathway during quiescent state of spermatozoa. During motility, energy is derived by a pre-stored ATP synthesized during a quiescent state. Also, sturgeons belong to the group of fishes that possess an ability of sperm reactivation, and the second motility period is supported by the energy derived by PCr-Cr shuttle system and mitochondrial respiration. The presented study is the first report on sturgeon sperm reactivation, and it will be helpful to enhance our fundamental knowledge in fish spermatology field. At applied level, this novel approach could be helpful to achieve a better fertilization success, as poorly motile spermatozoa could be revived after incubation in reactivation medium.

Keywords: *fish, sperm motility, phosphocreatine-creatine shuttle system, regeneration, fertilization.*

1. Introduction

It is generally known that spermatozoa of externally fertilizing fishes initiate their motility only after exposure to external environment. The sperm motility that is a pre-requisite for a successful fertilization is of short duration for most teleosts but not for sturgeons. However, albeit the species specificity, energy demand for sperm motility is supported by ATP hydrolysis. To understand the energy budgeting pattern, the chronological utilization of ATP during sperm motility has been well reported in spermatozoa of many fish species, including sturgeon (Billard et al., 1999; Dreanno et al., 1999a; Perchec et al., 1995). However, the source of ATP (various energy supplying pathways – oxidative phosphorylation, glycolysis, fatty acid oxidation, etc.) and its role for energy supply in motile and immotile spermatozoa among different fish species are found to be not common and debatable (Bencic et al., 1999; Ingermann, 2008). In fact, the debate is not only restricted to the spermatozoa of fish but also a topic of discussion in the higher animals (Storey, 2004).

Additionally and surprisingly, even though the sperm motility duration of sturgeons is incomparably longer than in teleosts, the ATP utilization pattern (sudden fall in ATP content after motility activation and then keeping up at a certain level) is common and a range of ATP content at quiescent state in sturgeon spermatozoa (60–80 nmol $(10^9 \text{ spz})^{-1}$) is similar to that in salmonids (18–60 nmol $(10^9 \text{ spz})^{-1}$) and cyprinids (80–150 nmol $(10^9 \text{ spz})^{-1}$) (Billard et al., 1999; Ingermann, 2008). Moreover, the energy budgeting strategy of sturgeon sperm is also similar to that of teleosts (Rahi et al., 2020). According to that, ATP is produced and accumulated while spermatozoa are residing in quiescent state in seminal plasma. Right after gaining the motility energy is rapidly utilized, and spermatozoa become exhausted for the maintenance of motility. While this strategy could fit well in spermatozoa of most of the teleosts with brief motility duration. However, the mentioned strategy is ambiguous in sturgeon spermatozoa with incomparably longer motility duration for which bioenergetic reasons behind this unique character is still imperfectly understood.

Additionally, in spermatozoa, the site of energy production (mitochondria located in the middle piece) and utilization (axoneme dynein ATPase located in flagellum) is not the same. And the transport of ATP from mitochondria to flagellum could be strictly constrained due to the narrow space of flagellum that is majorly occupied by structural components of axoneme in addition to the low diffusion rate of ATP (Dzyuba and Cosson, 2014; Takao and Kamimura, 2008). Therefore, an alternative pathway called phosphocreatine-creatine (PCr-Cr) shuttle system is known to transport high energy phosphates from the site of production to the site of utilization. The diversity in the flagellum length and the distance between the middle piece and the end of the flagellum may create a difference in the relative importance of the PCr-Cr shuttle system among spermatozoa of different fish species. However, presently the availability of information regarding the importance of this pathway is restricted to the spermatozoa of very few fish species, and its role for energy transport during different states (before/after motility) is poorly studied.

Further, sperm reactivation is the ability of spermatozoa to regain motility after completion/termination of the first motility period. The mentioned phenomenon was first time reported in 1980 in spermatozoa of rainbow trout (*Oncorhynchus mykiss*) followed by carp (*Cyprinus carpio*) 28 years later (Benau and Terner, 1980; Linhart et al., 2008). These studies have shown that if the motility exhausted spermatozoa are incubated in their non-activation medium (NAM), they can not only be activated for the second time, but the reactivated spermatozoa also have the capacity to restore ATP content and possess fertilization capacity. However, the capability to regenerate energy and regain motility has never been explored in spermatozoa of fish groups other than cyprinids and salmonids, including endangered sturgeons.

Therefore, the present study was designed to determine following:
(1) the major role of specific energy supplying pathway(s) (glycolysis, oxidative phosphorylation (OXPHOS), PCr-Cr shuttle system) in sturgeon spermatozoa during quiescent and motile state;
(2) possibility for reactivation of sturgeon sperm and the most important bioenergetic pathway(s) required for energy supply during reactivation state of spermatozoa;
(3) fertilization capacity of reactivated sperm;
(4) the effect of metabolites involved in bioenergetic pathways on enhancing fertilization capacity of reactivated or motility exhausted spermatozoa.

2. Materials and methods

2.1. Broodstock, fish handling and gamete collection

Experiments were conducted in compliance with the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice. Mature male brood stock (n = 18, six per experimental phase, see section 2.4) were kept at the Genetic Fisheries Centre at the Faculty of Fisheries and Protection of Waters. Prior to the experiments, mature sterlet males were transferred from an outdoor pond (water temperature 3 °C) to a closed water recirculation system at the hatchery of the South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic. The water temperature in recirculation system was increased from 3 to 16 °C with a 1 °C rise per day. Holding tank was maintained at a temperature of 16 °C, water flow rate of 0.2 L s^{-1}, and near 100% oxygen saturation. Spermiation was induced by intramuscular injection of carp pituitary powder dissolved in a 0.9% (w/v) NaCl solution at 4 mg kg^{-1} body weight 24 h before sperm collection. For the collection of sperm, a 4 mm plastic catheter was inserted into urinogenital tract of the fish, and stripping was done by gentle abdominal massage. Semen was collected in 250 mL tubes and then placed on ice. For experimentation, sperm samples with motility > 80% were used within 5 hours.

For the induction of ovulation, six female fishes were injected intramuscularly with carp pituitary extract dissolved in a 0.9% (w/v) NaCl solution: 0.5 mg kg^{-1} body weight was injected as a prime dose and 4.5 mg kg^{-1} body weight was given as a second dose 12 h later. Eggs were collected approx. 30 h after the second dose of injection. Eggs were stripped by following the standard procedures and were kept in plastic bowls. Eggs from three different females were pooled and used for fertilization assay.

2.2. Basal solutions for activation, non-activation, and reactivation media

The buffered sperm motility activation medium (AM) and non-activation medium (NAM) were consisted of 10 mM Tris-HCl supplemented with 0.25% pluronic acid (pH 8.0) and 16 mM NaCl, 3 mM KCl, 0.19 mM $CaCl_2$ and 10 mM Tris (pH 8.0) respectively. Hundred times concentrated solution of NAM (reactivation medium, RM) – 1600 mM NaCl, 300 mM KCl, 19 mM $CaCl_2$ was used to make reactivation condition for spermatozoa activated in AM (see section 2.4.3.).

2.3. Reagents used in the experiment – Inhibitors and metabolites

The bioenergetic pathways i.e., glycolysis, OXPHOS, and phosphocreatine-creatine (PCr-Cr) shuttle system were targeted by the inhibitors of their key enzymes. For this purpose, 2-deoxy-d-glucose (DOG), oligomycin (OLIGO), and fluorodinitrobenzene (FDNB) were used to inhibit glucose-6-phosphatase, ATP synthetase, and creatine kinase (CK) respectively (Magdanz et al., 2019; Tombes and Shapiro, 1985; Wick et al., 1957). A preliminary experimental trial was conducted to finalize the inhibitor concentration. Finally, DOG, OLIGO, and FDNB were used in AM and NAM at a final concentration of 2 mM, 5 μM, and 50 μM, respectively in all related experiments. Additionally, glucose, pyruvate, and lactate (final concentration of 1 mM of each) were used in the fertilization assay as additives during sperm reactivation.

2.4. Experimental design

The experimental design of the present research was divided into three different phases representing three different questions. 1. Which are the most essential bioenergetic pathways involved in energy supply during quiescent and motile state of sturgeon spermatozoa? 2. Do sturgeons belong to the group of fish species that possess capacity of sperm reactivation after termination of first motility period? 3. If yes, which are the most important bioenergetic pathways that supply energy during the second activation of sturgeon sperm? For the better understanding, the experimental design is showcased in Fig. 1.

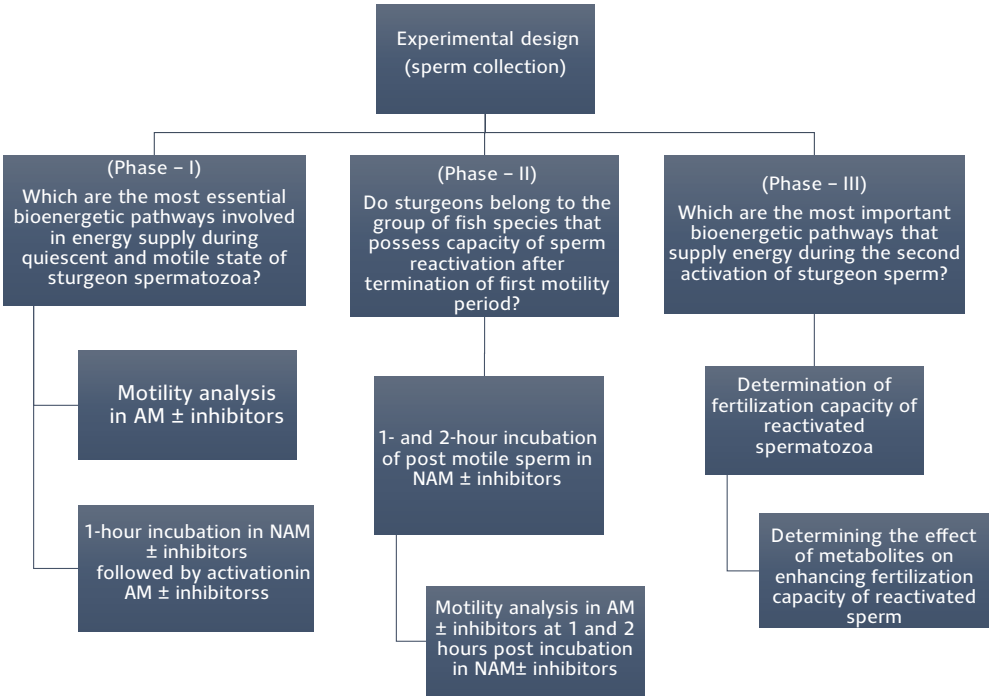


Figure 1. *Experimental design. AM, activation medium; NAM, non-activation medium.*

2.4.1. Phase – I. Influence of inhibitors on sterlet sperm during motility period and before motility activation

Preliminary, to understand the energy budgeting strategy (production and utilization of energy by specific energetic pathway) of sterlet sperm in quiescent and motile state, our previously established methodology was used (Rahi et al., 2020). Firstly, spermatozoa were activated in AM supplemented with inhibitors (in the text the abbreviation of "AM±inhibitor name" will be used: "AM+DOG", "AM+OLIGO", "AM+FDNB"). For this, 40 µL drop of AM± inhibitor was put onto microscopic slide and mixed with 0.5 µL sperm to obtain the appropriate cell concentration. Secondly, to study the bioenergetic pathways in quiescent state, sperm were incubated in NAM supplemented with inhibitors followed by motility activation in AM supplemented with corresponding inhibitors (in the text the abbreviation of "NAM±inhibitor name" will be used: "NAM+DOG", "NAM+OLIGO", and "NAM+FDNB"). Control groups (without any supplementation) used for direct activation and for activation after 1-hour incubation in NAM were named "AM" and "NAM", respectively.

All the motility records were taken at 16 °C. The desirable temperature in media was achieved and maintained by a thermoblock (HLC BO50/15, Germany). A temperature-controlled microscope stage (SEMIC® BIOELEKTRONIKA, Krakow, Poland) was used for temperature control during sperm motility assessment. The adjusted temperature was monitored via copper-constantan thermocouple (Omega, L-044T, Taiwan) using a data logger thermometer (Omega, HH127, Taiwan). Video records were made microscopically under 10x lens, negative phase-contrast condenser with an Imaging Development Systems (IDS) digital camera equipped with uEye Cockpit software, set at 25 frames/s. Afterwards, records were saved in AVI format and motility analysis was done by integrated system for semen analysis (ISAS software; PROISER, Spain). For the selection of the most representative motility parameters correlation matrix between five parameters – curvilinear velocity (VCL; µm s^{-1}), straight-line velocity (VSL; µm s^{-1}), average-path velocity (VAP; µm s^{-1}), straightness of track (STR; %) and linearity of track (LIN; %) was calculated. The least correlated parameters (that are VCL and STR) were selected as representatives of spermatozoon motility. Results for motility duration and motility percentage were also presented. Spermatozoa with VCL less than 20 µm s^{-1} were considered as non-motile. Motility duration was noted when approximately 90% of the spermatozoa stopped moving (in the text the abbreviation of this study point will be used as "end of motility"). Spermatozoon VCL and STR (with and without incubation) were analysed at 15 s post-activation and at the end of motility.

2.4.2. Phase – II. Sperm reactivation

During the second phase of experiment – sperm reactivation, 20 µL sperm were activated in 780 µL AM containing inhibitors, and motility duration was determined for each treatment. After termination of motility, 8 µL of RM was added to the suspension, and sperm samples were incubated. After addition of RM, ionic composition of medium become the same as NAM and similar to one of sterlet seminal fluid (Dzyuba et al., 2014). Due to this design sperm were prepared for the following incubation without inhibitors (control) and in presence of inhibitors DOG, OLIGO, and FDNB as was described in 2.3. Sperm motility activation and following incubation were performed at 16 °C. Within 1–2 min after addition of RM, each sample was tested to be non-motile. Further, at 1 and 2 hours post incubation, motility of sperm samples in each treatment was recorded in AM ± corresponding inhibitors. Later, motility percentage, VCL, and STR of spermatozoon were analysed at 15 s post activation from video records.

2.4.3. Phase – III. Fertilizing ability of reactivated sterlet spermatozoa

Preliminary to ensure our protocol, the fertilization rate in AM was confirmed to be non-significantly different from fertilization rate in water. Prior to the fertilization assay, the correct motility duration was determined by diluting the sperm sample in an eppendorf tube and checking motility at every 20–30 s interval till termination of motility. The dilution ratio of sperm to AM (1:39) and then diluted sperm to RM (100:1) was done in a similar way as explained in section 2.4.2. Following treatments were used in such a way that the final sperm:egg ratio at fertilization trial and correspondingly concentration of sperm in AM, NAM, and metabolite content should remain constant in all groups.

Description of conditions during fertilization trials:

(a) Control-I = fresh sperm and eggs were used for fertilization at the beginning of the experiment.
(b) Control-NAM = sperm were diluted in NAM and then the mixture was used for fertilization.
(c) Reactivation = sperm were activated in AM, then after termination of motility RM was added to the diluted mixture. After 60 min of incubation, the mixture was used for fertilization.
(d) Reactivation+Met = sperm were activated in AM, then after termination of motility RM containing metabolites (glucose, pyruvate, and lactate, final concentration of 1 mM of each) was added to the diluted mixture. After 60 min of incubation, the mixture was used for fertilization.
(e) End of motility = sperm were activated in AM, then after termination of motility RM was added to the diluted mixture. The mixture was used for fertilization without incubation.
(f) End of motility+Met = sperm were activated in AM, then after termination of motility RM containing metabolite syrup was added to the diluted mixture. The mixture was used for fertilization without incubation.
(g) Control-II = fertilization with use of untreated sperm was done at the end of experiment (90–120 min after egg collection).

For fertilization, 2 g eggs (~150 eggs) were taken in 25 mL beakers, that were placed on shaking table (150 rpm). Sperm volume used for fertilization (with and without incubation) was calculated according to sperm concentration for each male, to get 10^5 spermatozoa per egg during trial. For this, established protocol was used (Linhart et al., 2020). Calculated volume of sperm (with and without incubation) was pipetted at the bottom of the beaker. Eight mL of AM was added to the beaker in parallel. The process was repeated for all treatments. Mixture from each beaker was divided into two petri dishes and then transferred to the permeable screened cage of flow-through hatchery tank. Hatchery tank was designed in such a way that 300 L of water was continuously recirculating, along with temperature controlling system (at 16 °C), and germicidal UV protection light. Thirty four plastic cages (with netted/screened bottom) were attached to the hatchery tank. Each cage contained two petri dishes (with fertilized eggs) with gentle and continuous flow of water.

Petri dishes were incubated in flow-through hatchery tanks till hatching of all larvae (~7–8 days). Non-viable eggs were counted and removed daily. To determine the fertilization rate, data of embryo viability was observed at neurulation stage (72 hours post fertilization) and hatching stage (168 hours post fertilization). Neurulation rate was calculated for each petri dish (thus for each treatment and male) by (viable eggs/total eggs) * 100. The calculated values were expressed in percentage. Likewise, hatching rate was also calculated for each cage as (hatched larvae/total eggs) * 100 and expressed in percentage.

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

2.5. Statistical analysis

Statistical analyses for each phase of the experiment and for each group (activation medium, non-activation medium, reagents, control, post-activation time points, post-incubation time points, fertilization assay with and without metabolites, with and without reactivation) were done in STATISTICA v12 (Statsoft Inc., USA) and R (R Development Core Team 2012). Statistical data for each observation were collectively obtained by the average values of six male fish with two replicates. Interactions were considered statistically significant at $p \leq 0.05$.

The data distribution characteristics and homogeneity of dispersion were evaluated by using Shapiro-Wilk's and Levene's test respectively. For normally distributed data with similar dispersion values, parametric tests were followed. One-way ANOVA followed by Tukey's honest significant difference (HSD) test was applied for comparison of groups treated by inhibitors with control group for phase – I experiment. While in phase – II experiment, to study the effect of incubation time (1 and 2 hour) compared to fresh sperm as well as the effect of inhibitors on control group (no inhibitor) at each studied time point, two-way ANOVA followed by Tukey's honest significant difference (HSD) test was applied. The data obtained by phase – III of the experiment (fertilization) was not normally distributed. Thus, nonparametric Kruskal Wallis test followed by multiple comparison of mean ranks for all groups was used to compare the neurulation and hatching percentage among different groups.

3. Results

3.1. Phase – I. Influence of inhibitors on sterlet sperm during motility period and before motility activation

After activation of motility by AM containing inhibitors, spermatozoon velocity (VCL) in any "AM+inhibitor" group did not differ from control ($p > 0.05$) at both studied post-activation time points (Fig. 2A). After 1-hour incubation in NAM supplemented with inhibitors and then activation in AM supplemented with inhibitors, the significant decrease compared to control was observed only after treatment with FDNB at 15 s post activation (Fig. 2B). For STR of sperm, no changes compared to control ($p > 0.05$) were observed at any studied time point during direct sperm activation or activation after incubation in presence of any inhibitor (Fig. 2C and 2D).

Motility duration in AM, similar to VCL, was not changed ($p > 0.05$) after sperm activation in AM containing any inhibitor (Fig. 3A). After 1-hour incubation in NAM+inhibitors, the motility duration was reduced compared to control ($p < 0.05$) only in "NAM+FDNB" group (Fig. 3B).

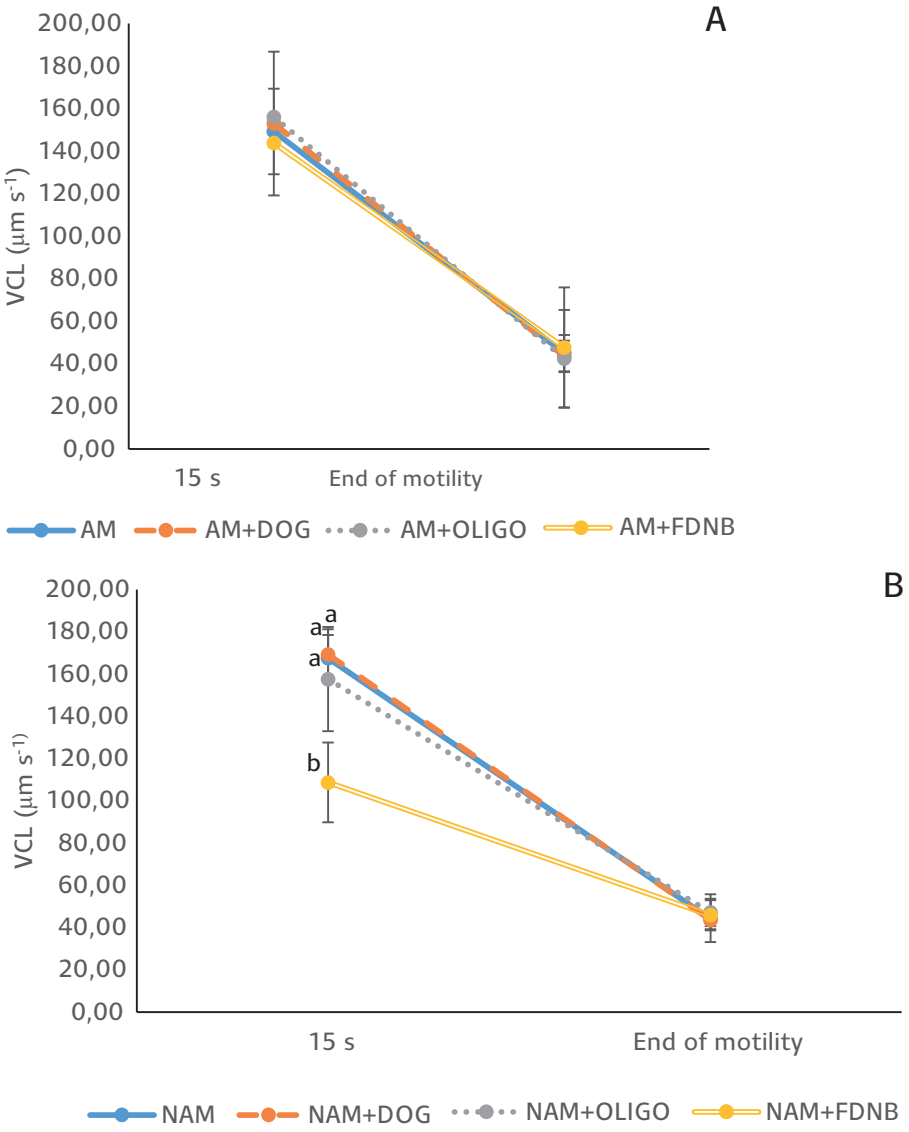


Figure 2. *Effect of inhibitors on spermatozoon motility parameters at 15 s post activation and at the end of motility. (A) VCL at 15 s post activation and at the end of motility in sperm samples activated in AM containing inhibitors; (B) VCL at 15 s post activation and at the end of motility in sperm samples pre-incubated for 1 h in NAM containing inhibitors followed by activation in AM; containing (C) STR at 15 s post activation and at the end of motility in sperm samples activated in AM containing inhibitors; (D) STR at 15 s post activation and at the end of motility in sperm samples pre-incubated for 1 h in NAM containing inhibitors followed by activation in AM. VCL (µm s^{-1}), curvilinear velocity; STR (%), straightness of track; AM, activation medium; AM+DOG, activation medium containing 2-deoxy-d-glucose; AM+OLIGO, activation medium containing oligomycin; AM+FDNB, activation medium containing fluorodinitrobenzene; NAM, non-activation medium; NAM+DOG, non-activation medium containing 2-deoxy-d-glucose; NAM+OLIGO, non-activation medium containing oligomycin; NAM+FDNB, non-activation medium containing fluorodinitrobenzene. Values with different letters are significantly different at the same post-activation time (one-way ANOVA, Tukey's HSD test, $p < 0.05$). Data are presented as mean ± S.D.*

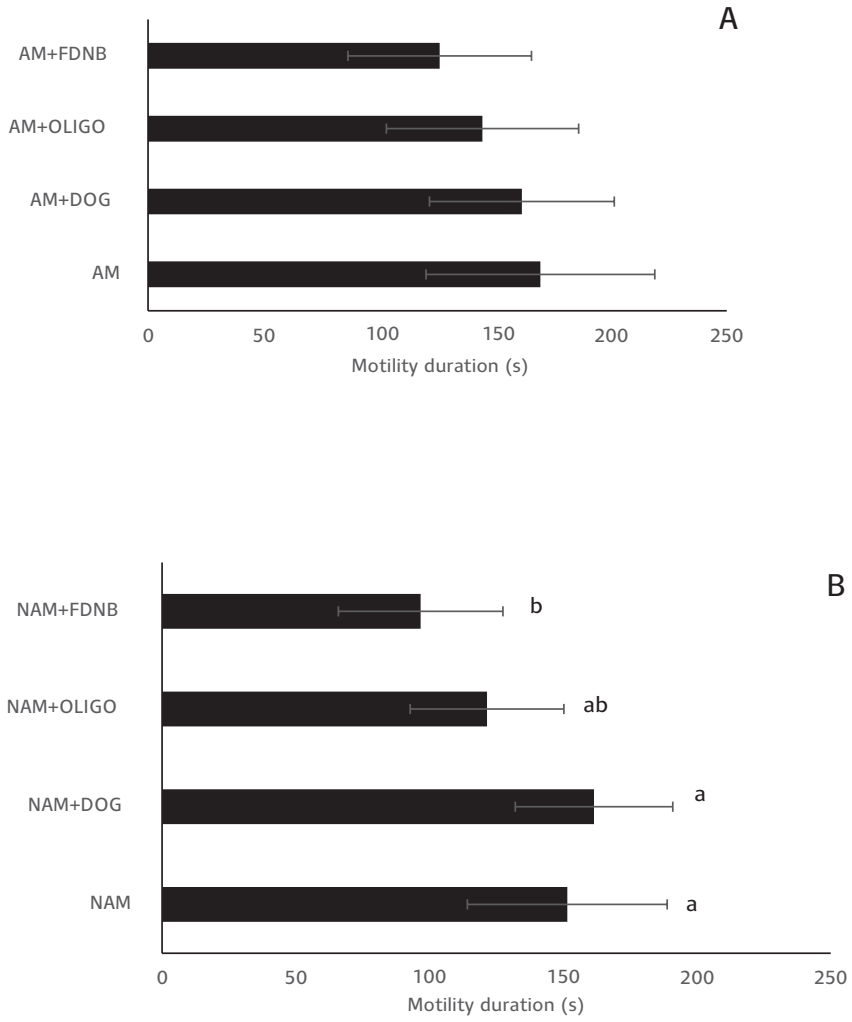


Figure 3. *Spermatozoon motility duration after treatment by inhibitors. (A) sperm samples activated in AM containing inhibitors (AM, activation medium, no inhibitor; AM+DOG, activation medium containing 2-deoxy-d-glucose; AM+OLIGO, activation medium containing oligomycin; AM+FDNB, activation medium containing fluorodinitrobenzene; (B) sperm samples pre-incubated for 1 h in NAM containing inhibitors followed by activation in AM supplemented with the same inhibitor (NAM, non-activation medium, no inhibitor; NAM+DOG, non-activation medium containing 2-deoxy-d-glucose; NAM+OLIGO, non-activation medium containing oligomycin; NAM+FDNB, non-activation medium containing fluorodinitrobenzene). Values with different letters are significantly different (one-way ANOVA, Tukey's HSD test, $p < 0.05$,). Data are presented as mean ± S.D.*

3.2. Phase – II. Sperm reactivation

Spermatozoon curvilinear velocity (VCL)

Right after completion of the first round of motility, RM was added to each sperm sample. Spermatozoa were found to be completely immotile around two min after addition of RM. After 1 hour of incubation, sperm were able to initiate the second round of motility. However, initial spermatozoon velocity (VCL at 15 s post activation) in all these groups was lower as compared to the value for groups of the first round of motility ($p < 0.05$) (Fig. 4A). No difference in initial spermatozoon velocity was found between control (not treated with inhibitor) and treated with DOG groups after 1 and 2 hours of incubation. At the same time, initial spermatozoon velocities in control and treated with DOG groups were significantly higher in comparison with groups treated with OLIGO and FDNB at each incubation time (Fig. 4A).

Straightness of spermatozoon track (STR)

No significant difference in straightness of spermatozoon track (STR) at 15 s post activation was found among all experimental groups at the first round of motility initiation and at the second round of motility activation in control group (not treated by inhibitors) or in groups treated with DOG after 1 and 2 hours of incubation. Initial spermatozoon STR after 1-hour incubation and then activation in AM containing OLIGO was lower compared to fresh sperm ($p < 0.05$) and higher compared to 2-hour incubation in AM containing OLIGO ($p < 0.05$). Similar results were obtained for FDNB group (Fig. 4B).

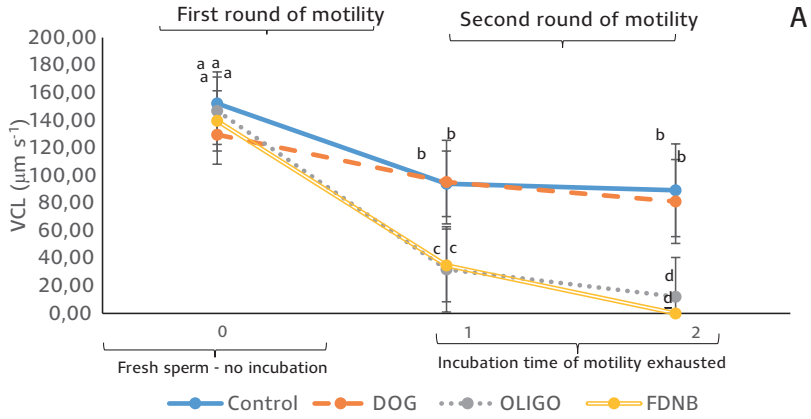
Motility percentage

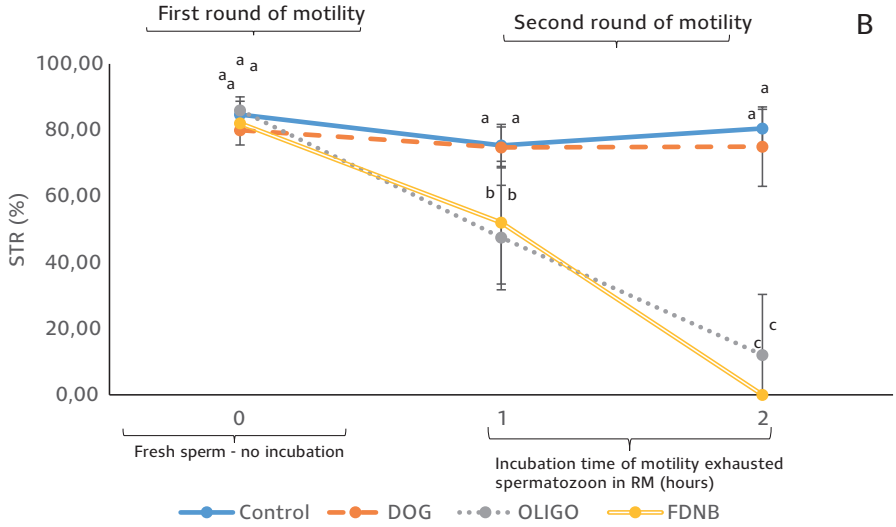
No significant differences in motility percentage among experimental groups were observed at the first round of spermatozoon motility activation. Motility percentage of secondly activated spermatozoa at 15 s post activation was lower compared to the first round of motility activation in all experimental groups. Motility percentage in control group was not different from one in group treated with DOG after 1 and 2 hours of incubation. Treatment with OLIGO and FDNB led to significant decrease in motility percentage of secondly activated spermatozoa after 1 and 2 hours of incubation compared to one in control or treated with OLIGO groups (Fig. 4C).

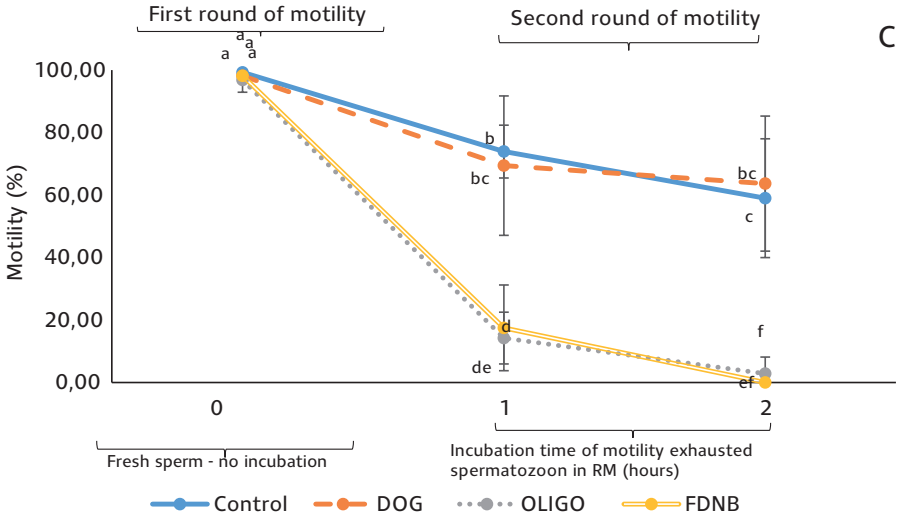
Motility duration

Motility duration in control group (AM without inhibitors) at 15 s post activation after 1-hour incubation was lower compared to fresh sperm ($p < 0.05$) and similar compared to 2-hour incubation ($p > 0.05$). These results were also similarly obtained in DOG, OLIGO, and FDNB groups.

Motility duration at 15 s post activation after 1-hour incubation in RM for control group (AM without inhibitors) was similar to DOG group ($p > 0.05$) and higher than OLIGO ($p < 0.05$) and FDNB ($p < 0.05$) groups. After 2-hour incubation in RM and then activation in AM±inhibitors, similar results were obtained. Motility duration in control group was similar to DOG group ($p > 0.05$) but higher than in OLIGO ($p < 0.05$) and FDNB ($p < 0.05$) groups (Fig. 4D).


A
First round of motility
Second round of motility
VCL (μm s-1)
200,00
180,00
160,00
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
0
1
2
Fresh sperm - no incubation
Incubation time of motility exhausted
Control
DOG
OLIGO
FDNB


B
First round of motility
Second round of motility
STR (%)
100,00
80,00
60,00
40,00
20,00
0,00
0
1
2
Fresh sperm - no incubation
Incubation time of motility exhausted spermatozoon in RM (hours)
Control
DOG
OLIGO
FDNB


C
First round of motility
Second round of motility
Motility (%)
100,00
80,00
60,00
40,00
20,00
0,00
0
1
2
Fresh sperm - no incubation
Incubation time of motility exhausted spermatozoon in RM (hours)
Control
DOG
OLIGO
FDNB

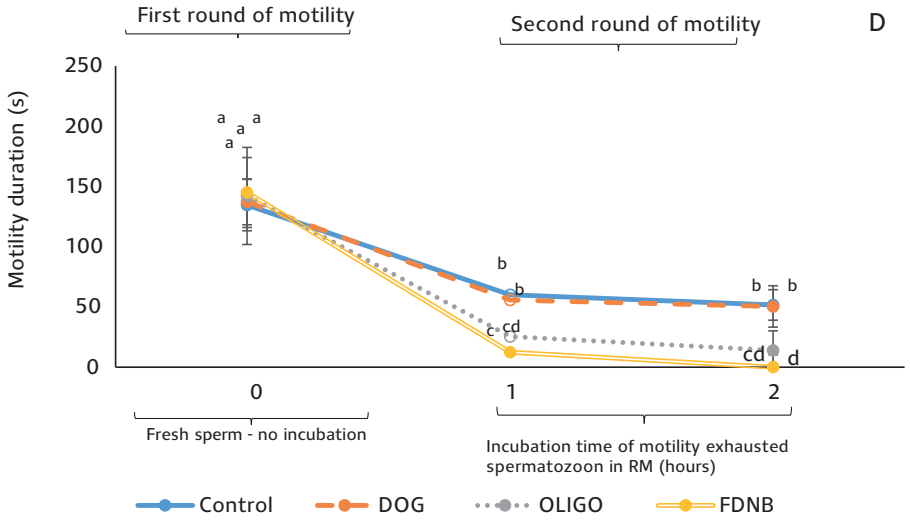
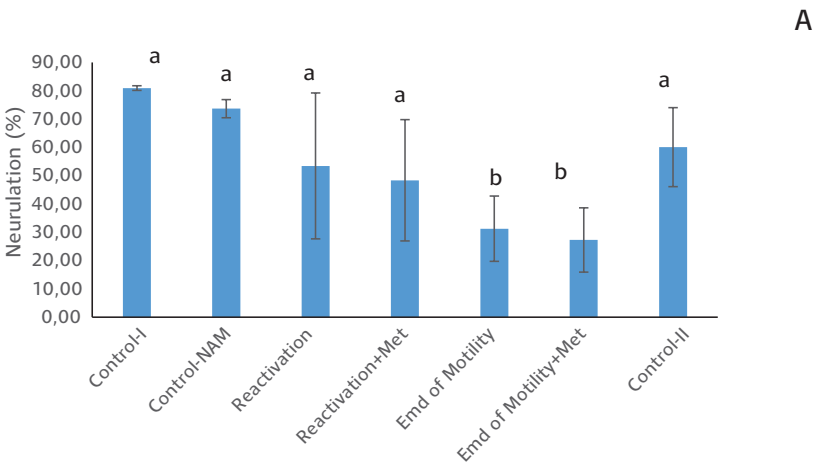


Figure 4. *Effect of inhibitors on motility parameters at 15 s post activation of fresh and secondly activated (after 1- and 2-hour incubation of motility exhausted spermatozoa in RM) sperm. (A) VCL (μm s^{-1}); (B) straight-line velocity (%); (C) motility percentage (%); (D) motility duration (s). VCL, curvilinear velocity; STR, straightness of track; RM, reactivation medium; AM, activation medium; DOG, activation medium containing 2-deoxy-d-glucose; OLIGO, activation medium containing oligomycin; FDNB, activation medium containing fluorodinitrobenzene. Values with different letters are significantly different (two-way ANOVA, Tukey's HSD test, $p < 0.05$). Data are presented as mean ± S.D.*

3.3. Phase – III. Fertilizing ability of reactivated sterlet spermatozoa

Both neurulation and hatching percentage obtained from reactivation groups (with and without metabolites) were similar to control values (control-I, control-NAM, control-II; see description in section 2.4.4) ($p > 0.05$). Neurulation and hatching percentages obtained from motility finish groups (with and without metabolites) were lower than controls ($p < 0.05$) and reactivated groups ($p < 0.05$). Both neurulation and hatching percentage were similar at the beginning and end of the fertilization experiment ($p > 0.05$) (Fig. 5A and 5B).



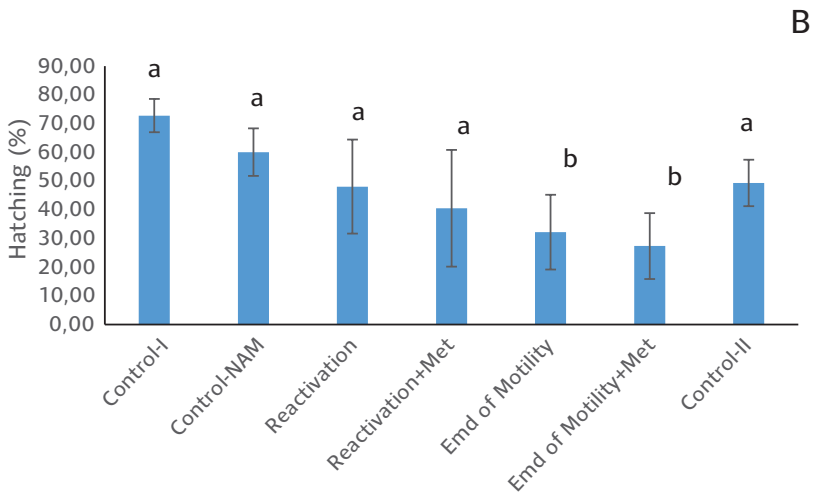


Figure 5. *Fertilization rate of reactivated and fresh spermatozoa presented as (A) neurulation % and (B) hatching %. Control-I = fresh sperm was used for fertilization at the beginning of experiment. Control-NAM = sperm were diluted in NAM and then the mixture was used for fertilization. Reactivation = sperm were activated in AM, then after termination of motility RM was added to the diluted mixture. After 60 min of incubation, the mixture was used for fertilization. Reactivation+Met = sperm were activated in AM, then after termination of motility RM containing metabolites was added to the diluted mixture. After 60 min of incubation, the mixture was used for fertilization. End of motility = sperm were activated in AM, then after termination of motility RM was added to the diluted mixture. The mixture was used for fertilization without incubation. End of motility+Met = sperm were activated in AM, then after termination of motility RM containing metabolites was added to the diluted mixture. The mixture was used for fertilization without incubation. Control-II = fertilization was done at the end of experiment (after 90–120 min after egg collection). AM, activation medium; NAM, non-activation medium; RM, reactivation medium. Mixture of metabolites was comprised of glucose+pyruvate+lactate (final concentration of 1 mM). Values with different letters are significantly different (multiple comparison of mean ranks for all groups test, $p < 0.05$). Data are presented as mean ± S.D.*

4. Discussion

So far, it is clear that despite having diversity in sperm ultra-structure, motility duration, level of metabolites content, relative role of various bioenergetic pathways for energy supply or mode of sperm motility activation among various taxa of fish, the energy for sustaining motility is derived from ATP hydrolysis by dynein ATPase (Cosson, 2012). Additionally, although it is well accepted that spermatozoa possess the capacity to perform glycolysis or fatty acid oxidation (e.g. in salmonids and cyprinids), the role of these pathways in energy supply for motility is minor. The major source of ATP is mitochondrial respiration. The mentioned pathway synthesizes ATP at quiescent and motile state of the spermatozoa. But energy synthesized during motility period is insufficient to compensate very high energy demand needed for sustaining motility. Therefore, ATP synthesis through OXPHOS and ATP accumulation during quiescent state of spermatozoa remain the predominant processes in energy supply of sperm motility. This statement is common for the spermatozoa of most teleosts that possess almost similar motility duration of less than a minute.

In our first attempt to understand the reason behind the long-lasting sperm motility of sturgeons, that is unusual for freshwater, externally fertilizing fishes, the following points

became clear: (a) despite exhibiting the rare character of long motility duration, the energy budgeting strategy of sturgeon sperm is similar to that in teleosts; (b) similar to teleosts, glycolysis and fatty acid oxidation have least contribution for energy supply in sturgeon spermatozoa at both non-motile and motile state; (c) sturgeons are among species which possess capacity to enhance sperm oxidative metabolism after motility activation or uncoupler treatment; (d) the level of OCR in sturgeon spermatozoa is higher compared to coldwater species trout, burbot (motility duration – less than a minute) (Ingermann et al., 2003, Rahi et al., 2021) but lower compared to temperate species turbot (*Psetta maxima*) (motility duration – 8–10 min) (Dreanno et al., 1999b). However, the given information still does not elucidate the reason/mechanism behind the longer motility duration of sturgeon sperm.

Additionally, there are available data indicating that sperm of cyprinids and salmonids possess the ability to be reactivated after exhaustion of the first motility period (Cosson, 2004). The mentioned phenomenon of gaining motility after exhaustion of the first motility period, thus gaining the capacity to fertilize eggs, is realized due to reloading of ATP in immotile spermatozoa (Benau and Terner, 1980; Linhart et al., 2008). We believe this fascinating idea of sperm reactivation could be of great importance in hatchery practices especially when dealing with poor quality sperm. Nevertheless, the two most important questions related to sperm reactivation have been unanswered till now. Whether sperm reactivation is a species specific phenomenon, or could it be achieved for spermatozoa of all species? What is the bioenergetic mechanism (the most important energy supplying pathways) during sperm reactivation? Therefore, the present research was taken up to study the most important energetic pathways – glycolysis, OXPHOS in addition to PCr-Cr shuttle system at non-active, active, and reactivated spermatozoa in sterlet. In addition, the capacity of reactivated spermatozoa to fertilize eggs was tested. Furthermore, the effect of metabolites to enhance the fertilization capacity of reactivated or motility exhausted sperm was also studied.

The value obtained for spermatozoon VCL in AM at 15 s post activation was well in the range detected in the previous studies performed on sterlet sperm (Boryshpolets et al., 2013; Dzyuba et al., 2014). Spermatozoon VCL and motility duration were insignificantly changed in AM after being supplemented with any studied inhibitors (DOG, OLIGO, FDNB) compared to control (without inhibitor) values (Fig. 2A and 3A). Similar results for VCL were obtained at the beginning (15 s post activation) and the end of motility. These results align with our previously published results on Siberian sturgeon (*A. baerii*) (Rahi et al., 2020) and are similar to the data for other fish species (Perchec et al., 1995, Rahi et al., 2021). After 1-hour incubation in NAM ± inhibitors of bioenergetic pathways followed by activation in AM supplemented with the same inhibitors, VCL as well as motility duration were decreased only when AM and NAM were supplemented with FDNB (Fig. 2B and 3B). These novel results suggest an active contribution of PCr-Cr shuttle system for the transport of ATP molecules from middle piece of spermatozoa to flagella during quiescent state of sterlet sperm, while insignificant contribution of the same was observed once spermatozoon motility is activated. Similar to the obtained results, an insignificant effect of CK inhibitor iodoacetamide on sperm motility was observed when zebrafish (*Danio rerio*) spermatozoa were exposed to the reagent during motility phase. While significant decrease in motility was observed after 30 min incubation in NAM containing the same reagent (Ingermann et al., 2011). Apart from spermatozoa of this model species, the PCr-Cr shuttle system has known to be important for the spermatozoa of aquatic animal like sea urchin (*Pseudocentrotus depressus*) and fishes such as cyprinids and salmonids as the level of PCr was shown to decrease with onset of motility (Kamp et al., 1996; Lahnsteiner et al., 1996; Robitaille et al., 1987; Saudrais et al., 1998; Takao and Kamimura, 2008; Tombes and Shapiro, 1985). Recently, the importance of mentioned pathway during final maturation process of sturgeon sperm was demonstrated (Fedorov et al., 2015).

Additionally, the presented study revealed for the first time that sturgeon spermatozoa have the capacity for reactivation after termination of the first motility period. However, sperm motility duration, motility percentage, and VCL studied at 15 s post reactivation were significantly lower than the values obtained at 15 s post activation of fresh sperm (Fig. 4A, C, and D). In agreement to the obtained results, the curvilinear velocity of second time activated carp sperm was almost half compared to the VCL of fresh sperm (Linhart et al., 2008). Almost five times lower motility duration and half of the motility percentage were gained during the second activation of turbot sperm (Dreanno et al., 1999b; Linhart et al., 2008). These results on the diversity of fish sperm reactivation success suggested the dependency of success in fish sperm reactivation on media composition and studied time point after reactivation. Also, the second activation in carp sperm and resting period needed to achieve it were demonstrated to be dependent on temperature of fish holding tanks (Boryshpolets et al., 2009a). Altogether, the phenomenon of the second activation of fish sperm and its success rate seems to be species specific and vary with different experimental conditions. In the future, it will be interesting to study the dependency of reactivation success on sperm samples collected during different seasons, fish feed, or other qualitative parameters that ultimately determine the sperm quality. The whole subject area holds a potential in the improvement of sperm handling and in vitro gamete manipulation and therefore should be thoroughly investigated in various fish species in the future.

Apart from an ability to regain fertilization rate or reloading ATP content in the reactivated spermatozoa, few studies have also shown that incubation/resting period of motility exhausted spermatozoa also allows the recovery of ultra-structure that is compromised during the first motility period (Alavi et al., 2012; Linhart et al., 2008). This might help spermatozoon to recover the motility and fertilization capacity. Cosson (2004) suggested an active contribution of ion pumping during reactivation process. These parameters (additional to the inputs of bioenergetic pathways for energy supply) might help spermatozoon to recover the motility and fertilization capacity. However, such parameters were not explored during our study performed on sturgeon sperm, and it remains future steps of the study.

The results of fertilization (neurulation and hatching percentages) by reactivated spermatozoa were found to be insignificantly different from fresh sperm (Fig. 5A and B). As a second control apart from fresh sperm, neurulation and hatching percentages were found to be significantly higher compared to group when fertilization was done with motility exhausted spermatozoa mixed with RM but without allowing them to rest. These results allow to assume that a resting period is needed to reload the ATP needed for motility reinitiation. However, the presented research was not performed on measuring metabolites level in fresh and reactivated sperm, which is a future study step of our study. Contrastingly, the lower fertilization rate was observed for reactivated sperm of carp and turbot compared to fresh sperm (Dreanno et al., 1999b; Linhart et al., 2008). The differences in fertilizing ability of reactivated sterlet, carp and turbot spermatozoa indicate the species-specific efficiency of reactivation process.

To determine the role of the most important bioenergetic pathways during reactivation state, the second activation of motility exhausted and incubated spermatozoa was done in presence and absence of inhibitors of studied pathways. Compared to the control (without inhibitor), spermatozoon VCL, motility duration, and motility percentage were significantly lower when AM used for the second activation was supplemented with oligomycin or FDNB. Similar results were obtained at both studied time points – 1- and 2-hour post incubation. Our results are in line with the results obtained at quiescent state of spermatozoa in the presented study and our previously published study performed on Siberian sturgeon suggesting that mitochondrial OXPHOS and PCr-Cr shuttle system are the most significantly

contributing pathways while glycolysis has insignificant role for energy supply (Fig. 4A, C, and D). The OCR of turbot spermatozoa during reactivation state was observed to be higher than OCR at quiescent state (Dreanno et al., 1999b). The mentioned study has also demonstrated the recovery of almost 60% of ATP content after resting period, however the recovered level of ATP was still significantly lower than the ATP content in fresh sperm. Presented results suggests that mitochondrial respiration is the most requested energy supplying pathway during reactivation period of fish spermatozoa. However, the ratio between the rate of ATP synthesis and rate of ATP utilization during reactivation period in sturgeon is still unknown.

Additionally, the results obtained for motility parameters were insignificantly different at 1- and 2-hour post incubation. This can indicate that at least 1 hour is enough to reload a sufficient amount of ATP to reinitiate the motility, and at the same time, no lethal damage (immotile or broken spermatozoon structure) to the reactivated spermatozoa was observed even if samples were incubated for 2 hours. Till now, a wide range of resting period duration varying from minutes to hours have been demonstrated in different studies (Boryshpolets et al., 2009a; Dreanno et al., 1999b; Perchec et al., 1995). However, unlike other studies, including the presented one that demonstrates a resting period of few minutes to hour required to reinitiate motility, the study of Benau and Terner (1980) performed on salmonid sperm using addition of phosphodiesterase inhibitor was the only one that exhibited reactivation of post motile spermatozoon without any resting period.

Furthermore, despite demonstrating the sturgeon sperm reactivation success in the existing study, it is also important to mention that the second or even subsequent in vitro activation of fish spermatozoa can also be related to an entirely different approach than sperm bioenergetics (ATP reloading after exhaustion in post motile sperm), as we have discussed till now. After termination of the first motility period, the second and third motility activations in Eurasian perch (*Perca fluviatilis*) sperm were observed after a step wise decrease in osmolality of AM (Boryshpolets et al., 2009b). The mentioned study did not observe reloading of ATP during succeeding activation and concluded this model of reactivation is related to the existence of several subpopulations of sperm activated at different times. The initiation and termination of motility in zebrafish spermatozoa were achieved several times by decreasing and increasing K^+ ion concentration and thus osmolality of AM (Takai and Morisawa, 1995). Vice versa results were obtained in marine teleost puffer fish (*Takifugu niphobles*) (Takai and Morisawa, 1995). Apart from above-mentioned studies in fishes that involves changes in osmotic pressure of the external environment for their sperm motility activation, spermatozoa in another group of fish such as salmonids initiate their sperm motility after changes in the ionic composition of external media. This has been extensively reviewed (Alavi and Cosson, 2006).

Apart from these, sturgeons are the group of fishes that possess both ionic and osmotic mode of sperm motility activation (Alavi et al., 2011). The second and third motility activation in sterlet sperm was achieved by decreasing the osmolality of AM followed by addition of calcium solution to the already diluted and motility ceased spermatozoa (Dzyuba et al., 2013). Additionally, sturgeon urine contains a high concentration of calcium (~0.7 mM) and the process of sturgeon sperm maturation that includes the passage of sturgeon spermatozoa via urinary duct (thus mixing of sperm with urine) is an essential part of sperm maturation (Dzyuba et al., 2017; Krayushkina and Semenova, 2006). Therefore, if the spermatozoa get an exposure to urine for some time during the sample collection, this might lead to an increase in the internal calcium concentration of spermatozoa that is sufficient for motility reactivation after coming in contact to hypotonic solution. Apart from this endogenous source of increased calcium concentration, there are an appreciable amount of calcium ions also present in the ovarian fluid of sturgeons that may act as external source of calcium and may also integrate to the internal concentration of calcium in spermatozoa during the fertilization process

(Kholodnyy et al., 2021). Therefore, until demonstrating the results on regeneration of ATP level after incubation of motility and ATP exhausted spermatozoa, it cannot be said with a definite notion that sturgeons come in the group of fish species that possess the ability of reactivation by means of ATP reloading/regeneration. Studies on metabolites content at the non-active, active and reactivated state of sturgeon spermatozoa with and without inhibitors of the mentioned pathways are our immediate future study steps.

Furthermore, the effect of metabolites on enhancing fertilization capacity of motility exhausted spermatozoa and motility exhausted spermatozoa after a resting period in RM was tested. As it was anticipated, the addition of metabolites during resting period of motility exhausted sperm did not enhance fertilization rate of either studied groups of spermatozoa. It could be explained by the absence of exogenous nutrients uptake in the spermatozoa of externally fertilizing fish in contrast to fishes that possess internal fertilization and spermatozoa of which are well known to derive energy by metabolizing exogenous substrates. Spermatozoa of numerous freshwater and externally fertilizing fish species such as zebrafish or African catfish justify this statement (Ingermann et al., 2011; Mansour et al., 2003). Nevertheless, there are handful of studies performed on spermatozoa of trout, sunfish (*Lepomis* sp.), salmon (*Salmo salar*), cod (*Gadus morhua*), Danube bleak (*Chalcalburnus chalcoides*) demonstrating the positive effects of exogenous nutrients to improve sperm motility or fertilization capacity (Lahnsteiner et al., 1999; Mounib, 1967; Terner and Korsh, 1963). Therefore, the overall mechanism to uptake, transport, and metabolize exogenous nutrients and the presence of specific membrane transporters can be species specific and are still far from final elucidation.

5. Conclusion

The results presented in the study confirmed that phosphocreatine-creatine shuttle system is the most demanded pathways during quiescent state of sturgeon spermatozoa. Once the motility is initiated, simultaneous activation of any bioenergetic pathway is least contributing to energy supply as the efficiency is too low to compensate the high energy demand. Therefore, pre-stored energy is the central contributing factor for energy supply in sturgeon spermatozoa, similar to the teleosts. Additionally, sturgeons are the group of species that possess the ability of the second motility activation after termination of the first round of motility, and the reactivated spermatozoa can fertilize the egg cells at a similar success rate as fresh sperm. Mitochondrial respiration and phosphocreatine-creatine shuttle system are the most crucial energy supplying pathways during the reactivation state of the sturgeon spermatozoa. However, to study the mechanism behind sperm reactivation through reloading of ATP and comparing the level of regenerated ATP to the level in fresh sperm are the future steps of our study.

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CHAPTER 5

General discussion

Spermatozoa are microscopic and poorly protected cells compared to counter gamete – egg. During the life of fish sperm, motility is the most energy demanding event apart from other housekeeping functions such as spermatogenesis. To understand the mechanism behind fulfilment of the high energy demand of fish sperm motility, various energetic pathways have been studied so far in different fish species. However, the relative contribution of these pathways for energy supply of spermatozoa at quiescent or motile state is not common for different fish species and imperfectly understood. The wide variation in sperm motility duration and spawning temperatures among various taxa of fish makes this understanding even more complicated.

Sperm reactivation after exhaustion as a result of the first activation period is a rarely explored phenomenon. Spermatozoa of cyprinids and salmonids have known to regain the capacity of motility, ATP regeneration, and fertilization after termination of the first motility round (Benau and Terner, 1980, Linhart et al., 2008). However, bioenergetics (most important energy supplying pathways, level of metabolites content etc.) during sperm reactivation has scarcely been explored among different fish species.

Therefore, the presented research aimed to assess the difference in energy budgeting (supply and utilization) strategy in spermatozoa of fish species that possess differences in their motility duration and spawning temperature – Siberian sturgeon (*Acipenser baerii*) and burbot (*Lota lota*). In addition to this, the prospect of sperm reactivation or second activation in vulnerable sturgeon species – sterlet (*A. ruthenus*) was also explored. For this, motility parameters, most important bioenergetic pathways, and reattainment of fertilizing capacity during sperm reactivation were studied.

Despite sturgeons and burbot belonging to different evolutionary era and having a huge variation in sperm motility duration, the energy budgeting strategy in the spermatozoon of sturgeon was found to be similar with the budgeting strategy of burbot and most of the teleosts. We assessed that, albeit the species specificity or thermal habitat history, oxidative phosphorylation (OXPHOS) is the major contributor to the energy supply in fish sperm. The OXPHOS is active during immotile and motile state of the spermatozoon. However, once motility is initiated the demand for energy to sustain motility is so much high that OXPHOS running during motility cannot compensate it. Therefore, the pre-stored ATP remains the main energy source in fish spermatozoa during motility phase.

Additionally, it was observed that sturgeons are the group of fish that possess the ability of sperm reactivation, and reactivated sperm can fertilize the egg cells at an equal rate as fresh sperm. Second round of motility activation is sustained mainly due to the functioning of OXPHOS and phosphocreatine-creatine (PCr-Cr) shuttle system. To the best of our knowledge, mentioned results on sturgeon sperm energetics during reactivation are the first report. Obtained results will help to enhance a basic understanding of fish sperm bioenergetics and potentially to improve in vitro gamete manipulation.

Sperm bioenergetic facet and its relationship with motility duration

Spermatozoa are unique cells because of their highly polarized nature, physiologically inactive transcriptional and translational machinery, and diverse requirements for successful fertilization. The discovery of spermatozoa happened in a concomitant era with the discovery of the microscope. In the late 1600s, Antoni van Leeuwenhoek saw the human spermatozoa for the first time, named it 'animalcula', and described very few but the most distinctive characters – the presence of head and tail, and motility (Egerton, 2006). Since then till today,

there has been an incessant advancement in various study areas of spermatology (Birkhead and Montgomerie, 2009). However, the research on fish sperm became popular quite late, in the late 1900s (Cosson, 2021), despite the fact that induced breeding in fish started in 1930–1950 (Harvey and Hoar, 1979). Fortunately, studies regarding fish sperm energetics – extracellular nutrient uptake, intracellular nutrients and their role for energy supply, ATP content dynamics before/after motility, or most important bioenergetic pathways, and so on also emerged during very initial years of fish spermatology (Christen et al., 1987; Gardiner, 1978; Mounib, 1967; Terner and Korsh, 1963).

When it comes to the comparison between fish and mammalian sperm, it is quite evident that the major energy supplying pathway(s), energy budgeting strategies, and resultant motility duration in fish sperm are very different from the latter ones that can remain motile for several hours (Ferramosca et al., 2012; Ferramosca and Zara, 2014). Unlike spermatozoa of fish, where OXPHOS contributes majorly to energy supply, both glycolysis and OXPHOS are known to be the major players in mammalian sperm (Cosson, 2012; Piomboni et al., 2012; Storey, 2004).

Among fishes, sperm motility duration in externally fertilizing fish is far shorter than in internally fertilizing fish, spermatozoa of which can remain motile from several minutes to hours or even till months (Billard, 1978; Ingermann, 2008; Winge, 1937). The longer sperm motility duration of internally fertilizing fish such as shark (*Triakis scyllia*), guppy (*Poecilia reticulata)*, or shiner surfperch (*Cymatogaster aggregate*) could be justified as their spermatozoa have capacity to uptake female derived or exogenous saccharides to fuel their motility (Billard, 1978; Gardiner, 1978; Minamikawa and Morisawa, 1996) similar to human sperm (Hereng et al., 2011). Higher endogenous glycogen content and more developed middle piece with several mitochondria were found in the spermatozoa of internally fertilizing fish (Billard et al., 1973). However, the mechanism to uptake exogenous nutrients or the presence of membrane transporters can be species specific and are poorly studied in the spermatozoa of externally fertilizing fish.

Among freshwater and externally fertilizing fish species, not very contrasting differences for motility duration can be found. Spermatozoa of most of the teleosts are motile for almost a minute or less. However, spermatozoa of few fish groups, such as sturgeons (Acipenseridae), are motile in water or activation medium (AM) for up to half an hour (Ginzburg et al., 1968; Linhart et al., 1995). In our study, the contribution of glycolysis, OXPHOS, and fatty acid oxidation was studied at motile and immotile states of Siberian sturgeon spermatozoa to explore the reason behind their long-lasting motility. Studies on sperm motility in AM and motility in AM after one-hour incubation in non-activation medium (NAM) were done in addition to oxygen consumption rate (OCR) measurement in AM and NAM. All the investigations were carried out by exposure of the sperm to the widely used inhibitors of glycolysis, fatty acid oxidation, and OXPHOS in addition to an uncoupling agent of OXPHOS.

The obtained results suggested that OXPHOS is active during the quiescent and motile phase of Siberian sturgeon spermatozoa. Additionally, like in teleosts the energy needed for prolongation of sturgeon sperm motility is derived majorly from pre-stored ATP that is produced via OXPHOS during quiescent state (Dzyuba et al., 2017). Similar results were concluded in the spermatozoa of freshwater fish – carp (*Cyprinus carpio*) and trout (*Oncorhynchus mykiss*) and marine fish – turbot (*Psetta maxima*) (Christen et al., 1987; Dreanno et al., 1999; Perchec et al., 1995). Ingermann et al. (2011) performed a similar study on zebrafish (*Danio rerio*) spermatozoa and concluded that right after activation of motility, ATP pre-stored during the quiescent state of spermatozoa is the most contributing factor for energy supply. The nascent ATP production during the motile phase becomes necessary short time (60–90 s) after motility activation.

In the presented study, the OCR level was significantly increased after exposure to an uncoupling agent in NAM and after motility activation. It suggests that spermatozoa have capacity to enhance their oxidative metabolism in both states: quiescent and after activation of motility. Similar results were obtained in the spermatozoa of warmwater and temperate fishes – common carp and turbot (Boryshpolets et al., 2009; Dreanno et al., 1999). While no capacity of enhancement in OXPHOS at motile or immotile state of spermatozoa was observed in trout (Ingermann et al., 2003). It is important to mention that spermatozoon respiration can be measured using whole intact cells or extracted mitochondria. Till now, in fish sperm most of the available studies demonstrating measurement of OCR have been performed on whole spermatozoa. The mitochondria extraction process has once been tried in a marine fish spermatozoa (Chauvigné et al., 2015). However, so far not a single study has been attempted on OCR measurement from freshwater fish sperm mitochondria. In fish sperm, mitochondria are confined only in the middle piece and tightly wrapped around axoneme – the typical arrangement that can make the process of mitochondria extraction difficult.

Our data on Siberian sturgeon spermatozoa (Chapter 2) exhibited no significant alteration in OCR during 1–5 min in AM and NAM (Rahi et al., 2020). Likewise, a constant level of ATP in the quiescent state of Siberian sturgeon spermatozoa was observed, which reduced within 5 s after motility activation (Billard et al., 1999). These results on Siberian sturgeon spermatozoa indicate that energy demand during motility is not sufficiently fulfilled by OXPHOS. Similar results after 2 min of motility activation were observed in carp spermatozoa (Perchec et al., 1995). In contrast to these species where OXPHOS is the only major source of energy, in trout spermatozoa, ATP content was reduced during the first minute after dilution in AM, but the endogenous store was then increased to its original level within 15 min (Christen et al., 1987). ATP content was found to be constant before and after motility activation in African catfish spermatozoa (Mansour et al., 2003). The possible explanation for the maintenance of ATP level during motility in the spermatozoa of African catfish and quick restoration of ATP stores after motility stop in trout could be the active role of other energetic pathways in addition to OXPHOS during motility. In trout spermatozoa, glycolysis occurs in the initial 30 s of motility period, followed by a shift to triglyceride metabolism (Lahnsteiner et al., 1993). In African catfish spermatozoa, glycolysis, lipid catabolism, and β-oxidation of fatty acid actively contribute to energy supply during motility (Mansour et al., 2003).

The present study exhibited no significant inputs for the glycolysis and fatty acid oxidation in energy supply of motile and immotile sturgeon spermatozoa. In contrast, glycolysis, fatty acid oxidation, and OXPHOS are known to participate in energy supply at motile and immotile states in the spermatozoa of cyprinids and salmonids (most important aquaculture species and most studied species in fish spermatology) (Lahnsteiner et al., 1992, 1993, 1994, 1996, 1999). However, the efficacy of glycolysis and fatty acid oxidation in the spermatozoa of mentioned species during motility is low. The present study aimed for the determination of relative importance of bioenergetic pathways before and after motility activation was the first attempt ever in sturgeon spermatozoa. Future studies of enzymatic activities involved in bioenergetic pathways or the dynamic changes in the content of lipids/amino acids/metabolites before and after motility activation in sturgeon spermatozoon will surely reveal more details. For reference, it is important to mention here that the bioenergetic strategies to synthesize and utilize energy efficiently in fish spermatozoa are entirely different from mammals where glycolysis plays a significant role for energy supply (Takei et al., 2014). Glycolytic enzymes are located in principle piece of spermatozoon that is located next to middle piece (Visconti, 2012). In fish spermatozoa, on the other hand, glycolysis is believed to occur in the cytosol of middle piece. However, no information regarding the location of glycolysis or glycolytic enzymes in the spermatozoon has been unravelled in fish sperm till now.

In the present study (Chapter 2), it was shown for the first time that chondrostean fishes despite being evolutionary more primitive than teleosts and possessing comparatively longer sperm motility duration, are characterized by the energy budgeting strategy that is similar to one in spermatozoa of teleosts (Dzyuba et al., 2017; Perchec et al., 1995). One possible explanation for the long-lasting motility of sturgeon spermatozoon could be its ultra-structure. From the evolutionary perspective, structure of sturgeon spermatozoon possesses similarities with mammalian one. It possesses acrosome, 6–8 mitochondria (and their number varies among sturgeon species), mitochondria are arranged helically (Psenicka et al., 2007). In general, mitochondria in fish sperm are cylindrical, spherical, or irregular. The number of mitochondria in sturgeon sperm are comparatively higher than in trout sperm which possess shorted motility duration and 1–2 mitochondria (Figueroa Villalobos et al., 2021).

Sperm energetics in under-ice spawning fish and its thermal dependency

Most fishes are ectotherms or poikilotherms. The body temperature of these groups of fishes corresponds to the environmental temperature or undergoes changes with respect to fluctuation in environmental temperature (Guderley, 2004). Moreover, in externally fertilizing fish, the spermatozoa are directly released into the water for fertilization purpose. Due to this, water temperature (apart from osmotic pressure or ionic composition of water) has a direct and major influence on sperm physiology. The bioenergetic pathways and overall sperm metabolism that is being regulated due to numerous enzymatic activities involved have shown to be influenced by temperature in a non-standard manner among spermatozoa of different fish species (Alavi and Cosson, 2005; Lahnsteiner and Mansour, 2012).

Generally, with temperature rise, there is a rise in sperm motility percentage, velocity, flagellar beat frequency, ATP consumption rate, and this is compensated by a shorter motility duration (and vice versa) (Dadras et al., 2017). The rise in these sperm parameters at higher temperature is because of enhanced activities of the enzymes that are involved in energy supplying pathways. Prolonged motility duration after exposure to low temperature was observed in spermatozoa of carp, trout, hake (*Merluccius merluccius*), European perch (*Perca fluviatilis*) (Billard and Cosson, 1992; Cosson, 2010; Lahnsteiner, 2011; Perchec et al., 1995). However, the trend of increased motility parameters with the rise in temperature (as mentioned) is not universal in all the species. Also, studies performed on few species reject the classic bell-shaped form of relation between enzymatic activity and temperature. Among four studied temperatures in the spermatozoa of Siberian sturgeon – 10, 12.5, 15, and 17.5 °C, the highest and lowest motility percentage was obtained at 10 and 17.5 °C (Williot et al., 2000). The most extended motility duration in the spermatozoa of streaked prochilod (*Prochilodus lineatus*) was observed at 17 °C, which was decreased by either decreasing or raising the temperature (Romagosa et al., 2010).

The present study (Chapter 3) was designed to determine the role of the major bioenergetic pathway(s) crucial for energy supply in burbot sperm at its spawning temperature 4 °C along with changes in the role of mitochondrial respiration at maximum critical temperature (CTmax) 15 °C. It is important to mention that the spermatozoa of burbot exhibit rare physiological character compared to the sperm of other teleosts: (a) spermatozoon motility and velocity were significantly increased rather than decreased at low temperature (Lahnsteiner and Mansour, 2012); (b) spontaneous activation of spermatozoa in seminal plasma at temperature 5–30 °C was observed (Lahnsteiner et al., 1997; Dadras et al., 2019); (c) activities of ATPase, adenylate kinase, pyruvate kinase, malate dehydrogenase, and peroxidase were decreased with rise in temperature (Lahnsteiner and Mansour, 2012).

In the present study, motility parameters of burbot sperm were shown to follow the general rule followed by spermatozoa of most teleost fish (Alavi and Cosson, 2005). Motility duration was decreased, and spermatozoon curvilinear velocity (VCL) and OCR were increased significantly when temperature of AM was raised from 4 to 15 °C. At spawning temperature, no significant reduction in any studied motility parameter was observed after exposure to inhibitors and uncoupler of mentioned bioenergetic pathways, suggesting burbot spermatozoa to be metabolically depressed in icy conditions. At 15 °C, VCL was increased significantly compared to 4 °C, while no significant enhancement in spermatozoon VCL was observed when sperm were activated in the presence of inhibitor of OXPHOS – NaN_3. Additionally, reduction in spermatozoon velocity post activation was faster at 15 °C compared to 4 °C. These results indicate that at enhanced temperature (in contrast to icy conditions), there is an active participation of OXPHOS for energy supply in burbot spermatozoa.

Our study on burbot spermatozoa showed no significant effect of sperm exposure to the inhibitors of glycolysis or fatty acids oxidation on any studied motility parameter. Glycolytic machinery was studied in cyprinid and salmonid spermatozoa, and glycolytic capacity in the spermatozoa of these fish groups was found to be low compared to that of OXPHOS (Lahnsteiner et al., 1993, 1999). In the spermatozoa of another coldwater fish – rainbow trout, an important role of triglyceride metabolism during motility period was determined (Lahnsteiner et al., 1993), and a high level of triglycerides has been reported in trout seminal plasma (Lahnsteiner et al., 2009). In fact, trout sperm viability, motility, and fertilization rate were enhanced after the addition of fatty acids to the sperm storage media or AM (Lahnsteiner et al., 2009). However, so far, there is no explanation for the mechanism to uptake and transport exogenous nutrients especially heavy molecules such as triglycerides in fish sperm. To get more details on the role of glycolysis, and fatty acid oxidation in burbot (or other coldwater fish) sperm, further studies on dynamic changes of metabolites in spermatozoon and seminal plasma, before and after motility activation must be performed.

Furthermore, the role of amino acid metabolism and importance of specific essential and non-essential amino acids in fish sperm motility are highly unexplored area. Additionally, the influence of environmental temperature on amino acid metabolism is totally untouched. In recent years, few studies performed on fish have shown the positive, negative, and species-specific effect of amino acids on sperm quality (Alavi et al., 2012; Cabrita et al., 2011; Kanyilmaz and Inanan, 2020; Kwasek et al., 2014; Lahnsteiner, 2010; Martínez-Páramo et al., 2013). Taking hints from higher vertebrates (Qiao et al., 2017; Zhao et al., 2018), the metabolic profiling and species-specific role of different metabolites in fish sperm should be the future line of study.

Furthermore, in the presented study (Chapter 3), at spawning temperature, burbot spermatozoal OCR could neither be enhanced by motility activation nor by uncoupler of OXPHOS at activated or non-activated state. Also, the OCR level in burbot spermatozoa was observed to be at lower level while comparing to that in other teleosts (Ingermann, 2008). In future, it will be interesting to study the ATP content (before and after motility activation) in burbot spermatozoa and compare it with values in spermatozoa of other coldwater species. The obtained results suggest that the burbot spermatozoa at its spawning temperature, do not possess the capacity of enhanced oxidative metabolism and OXPHOS at activation or non-activation is functioning at a low level compared to other teleosts. Moreover, the amount of ATP synthesized by OXPHOS during motility is not high enough to satisfy the required energy to maintain or prolong the motility period. Similar results were obtained in the spermatozoa of another coldwater fish – trout (Ingermann et al., 2003). These data are different from that in the spermatozoa of warmwater or temperate fish (carp and turbot) where significant enhancement in sperm OCR after treatment with uncoupler at quiescent state was observed (Boryshpolets et al., 2009; Dreanno et al., 1999).

At 15 °C, significant reduction in OCR after exposure to NaN_3 was observed in both AM and NAM at 30 and 60 s. Results suggest that the OXPHOS in burbot spermatozoa in both AM and NAM becomes more intensive at a higher temperature. However, the major energy source for burbot sperm motility is still derived from OXPHOS at quiescent state. Despite the exposed temperature to the burbot spermatozoa, this energy budgeting (production and utilization) strategy is similar to that in spermatozoa of most of the teleosts (Christen et al., 1987; Perchec et al., 1995). The modulations in the role of other pathways that are glycolysis and fatty acid oxidation for energy supply at a higher temperature should be the future steps of study. So far, no such study has been performed in the spermatozoa of any fish species.

Phosphocreatine-creatine (PCr-Cr) shuttle system supplementing OXPHOS for energy supply

So far, it is clear that a major part of the energy for sperm motility in most teleosts is derived from OXPHOS. However, the role of OXPHOS in fish sperm is temporal, species specific, and not essential during the motility period. Spermatozoa of some teleosts have exhibited the presence of an alternative/additional pathway – PCr-Cr shuttle system. It involves mitochondrial creatine kinase (CK), creatine, and phosphocreatine and mediates the reversible generation of ATP from PCr and homogenous distribution of ATP throughout the flagellar length (Cosson, 2012; Dzyuba and Cosson, 2014).

Our study performed on sterlet spermatozoa (Chapter 4) together with the previous study done on Siberian sturgeon spermatozoa (Chapter 2) found that sturgeon spermatozoa derive energy mainly via OXPHOS as well as PCr-Cr shuttle system at quiescent state. After motility activation, the relevance of both OXPHOS and PCr-Cr shuttle system for energy supply is negligible, since the energy derived is insufficient to compensate for high ATP demand of flagellar machinery. A similar study performed on zebrafish spermatozoa demonstrated no significant effect of CK inhibitor iodoacetamide on sperm motility when spermatozoa were activated in AM. At the same time, reduction in sperm motility and velocity was observed after 30 min incubation in NAM containing iodoacetamide (Ingermann et al., 2011). The PCr level in salmonid and cyprinid spermatozoa was decreased after motility activation suggesting a vital role of the same during sperm motility of these species (Kaldis et al., 1997; Robitaille et al., 1987; Saudrais et al., 1998; Tombes and Shapiro, 1985). In sterlet, the mentioned pathway had a significant participation for energy transport during sperm maturation (Fedorov et al., 2015). However, the presented study indicated for the first time that PCr-Cr shuttle system is one of the most demanded pathways during quiescent state of sturgeon spermatozoa.

In contrast to the above-mentioned studies demonstrated on fish sperm, the sperm motility of an aquatic animal sea urchin was impaired due to the damping of flagellar waves after exposure to fluorodinitrobenzene (FDNB) – an inhibitor of creatine kinase. These lesions were repaired when spermatozoa were demembranated and allowed to swim in ATP-containing medium (Tombes et al., 1987). In human spermatozoa, the function of CK enzyme was found to be unrelated to flagellar movement or ATP content (Vigue et al., 1992). Yeung et al. (1996) reported that ATP produced by OXPHOS and glycolysis both independently provide energy for human sperm motility. However, the role of CK was supplementary to these pathways and functionally became more important when supported with respiratory substrates or under the condition when glycolysis predominates. Altogether, these studies performed on various animals and different species of fish suggest the species-specificity in the role of PCr-Cr shuttle system in energy supply of spermatozoa.

Reactivation of sterlet spermatozoa and major bioenergetic pathways involved

Since the depletion of intracellular ATP content in fish spermatozoa is not quantitatively correlated with the termination of motility (Ingermann, 2008), it means after motility stop spermatozoa are not metabolically exhausted. Several postulates have been proposed to explain the motility termination in fish sperm. Cosson et al. (1991) proposed that the distribution of ATP in spermatozoon body at the end of motility is in such a way that the left-over ATP molecules after consumption for motility are localized away from dynein ATPase, hence they are not available for flagellar movement. One minute after motility activation in the spermatozoa of Siberian sturgeon, the flagellar waves were seen only at the proximal part of the flagellum, that is close to the sperm head (Billard et al., 1999). Another explanation is the accumulation of ADP (after ATP hydrolysis) at the motility termination stage. Since ADP is a competitive inhibitor of dynein ATPase, the accumulation of the same could lead to inhibition of flagellar movement (Perchec et al., 1995).

Our study (Chapter 4) demonstrated that after the termination of the first round of sterlet sperm motility in AM, motility was inhibited entirely a few minutes after adding reactivation medium (RM) to the motility exhausted spermatozoon. After 1-hour incubation of motility ceased spermatozoa in RM, the spermatozoa were once again able to be motile. Nevertheless, the motility percentage, motility duration, and spermatozoon VCL after 1-hour incubation were significantly lower than in the fresh sperm. Similar results were obtained in previous studies of sperm reactivation in freshwater (Benau and Terner, 1980; Linhart et al., 2008) and marine fish species (Dreanno et al., 1999).

Presented results on motility parameters of reactivated sperm were not different at 1- and 2-hour post incubation. The time of incubation of motility exhausted spermatozoa in RM to regain the motility can vary from species to species or experimental conditions. Sperm motility of trout was related to K^+ concentration in AM. Motility terminated spermatozoa were incubated in NAM solution with a higher concentration of K^+ for 30 min and then reactivated in low concentrated K^+ solution (Cosson, 2004). A similar time was achieved for the sperm reactivation of marine fish – turbot (Dreanno et al., 1999). Another study performed on trout spermatozoa demonstrated that motility was regained in water after 1–17-hour post dilution in ovarian fluid (Cosson, 2004). The most recent study regarding reactivation of carp sperm revealed that the time for incubation of motility exhausted spermatozoa in RM also varies with temperature. Incubation of 23 min and 37 min was taken to achieve the second activation in carp sperm when fish were kept at 20 °C and 15 °C for seven days, respectively (Boryshpolets et al., 2009). Here, it is important to mention that so far, there have been very few studies performed on sperm reactivation, and they were restricted only to cyprinids and salmonids. The phenomenon of sperm reactivation required resting period, and its success rate seems to be species and temperature specific. Other factors such as male specificity, fish feed, habitat history etc. might also influence the sperm reactivation. To the best of our knowledge, no such studies have been ever attempted on mammalian or any other higher animal sperm. The phenomenon of sperm reactivation is most likely restricted to fish groups only.

Furthermore, since the values of mentioned motility parameters at 15 s post activation after incubation for 1- and 2-hour were comparable, the incubation period for fertilization assay was kept only for 1 hour. The neurulation and hatching percentages after fertilization with reactivated sperm were similar to ones when fresh sperm was used and lower than the values obtained for the group when motility exhausted sperm before reactivation was used. Additionally, to study the effect of metabolites on enhancing the fertilization rate of reactivated sperm or motility exhausted sperm, a metabolite syrup of glucose+pyruvate+lactate was added to the RM with and without incubation, respectively. However, no differences in the neurulation and hatching percentages were observed with and without addition of metabolites, either in

motility exhausted group or in reactivated group of sperm. These results suggest that uptake of exogenous metabolites does not take place during incubation of spermatozoa needed for reactivation, in contrast to spermatozoa of internally fertilizing fish that are known to uptake exogenous or female derived nutrients to sustain their motility. The similar phenomenon is not well understood in the case of externally fertilizing fish species.

Additionally, the presented study revealed that application of oligomycin and FDNB during incubation of post motile sterlet sperm resulted in significant decrease of motility percentage, motility duration, and VCL compared to the values obtained after incubation without inhibitors. In contrast, no significant changes in the motility parameters were observed when DOG was added during incubation. The results clearly show that the OXPHOS and PCr-Cr shuttle system are the most important pathways, while glycolysis is least/not involved in energy supply to sustain motility during the second activation of sterlet sperm. A similar effect of respiratory inhibitors on turbot sperm flagellar beat frequency during reactivation was observed (Dreanno et al., 1999). Also, spermatozoa OCR during reactivation was significantly higher than OCR in the quiescent state but lower than in the activation state, suggesting mitochondrial respiration to be the most crucial pathway to support the second motility activation of turbot sperm (Dreanno et al., 1999). Although the phenomenon of ATP reloading after exhaustion of the first sperm motility has been proved in several fish species, the most important bioenergetic pathways supporting this ATP reloading are still imperfectly understood and need further thorough investigation.

Conclusions

This thesis was designed with an intention to study the role of most important energy supplying pathways in the spermatozoa of fish species that possess extreme/rare characteristics: having sperm motility duration that is unlikely longer than in other freshwater fish species, and another species that spawns at icy conditions. The obtained results were thoroughly discussed and compared with available data on spermatozoon characters of other fish species.

The main conclusions from these studies were:

1. A major part of the energy for motility of the spermatozoa in studied fish species (sturgeons and burbot) is derived from oxidative phosphorylation, while glycolysis and fatty acid oxidation are least contributing pathways.
2. The oxidative phosphorylation in these fish species is actively participating in the energy supply of spermatozoa during quiescent and motile periods. However, a major part of the energy is derived from oxidative phosphorylation at the quiescent state. During motility, the energy demand of spermatozoon is too high to be satisfied by oxidative phosphorylation occurring at that time.
3. Irrespective of the sperm motility duration or temperature of the external environment, the above-mentioned energy budgeting strategy is common in the spermatozoa of studied fish species.
4. The ability to enhance an oxidative metabolism after activation of motility or exposure to an uncoupling agent is clearly a species-specific phenomenon, which cannot be explained only by the thermal habitat history of a specific fish species.
5. After termination of the first round of motility, sturgeon spermatozoa can be reactivated. Despite the main sperm motility parameters of reactivated spermatozoa are significantly lower than ones of the fresh sperm, they possess high fertilizing ability.

6. Mitochondrial respiration along with phosphocreatine-creatine shuttle system are the majorly contributing pathways for energy supply in sturgeon sperm at quiescent and reactivated state.

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English summary

Fish sperm respiration: species specificity and effect of environmental temperature

Deepali Rahi

The evolutionary diversity of fish and a wide range of spawning conditions determine specific properties of fish gametes. Spermatozoa of externally fertilizing fishes are characterized by species-specific differences in structure and motility characteristics that add complexity to the generalization of the energy budgeting strategy of these cells before and after motility activation. Therefore, the presented study was designed to investigate the role of mitochondrial respiration as well as glycolysis and fatty acid oxidation in the energy supply of spermatozoa in freshwater, externally fertilizing fish species possessing essential differences in motility duration and spawning temperature.

The study performed on Siberian sturgeon (*Acipenser baerii*, Acipenseriformes) spermatozoa (Chapter 2) has shown that oxygen consumption rate (OCR) was constant (studied for 5 min) during non-motile and motile states. The OCR was found to be higher than the one detected for coldwater teleosts, for example, trout, and lower compared to marine species, for example, turbot. The presented study also revealed that sturgeons belong to the group of fishes whose spermatozoa possess the capacity to enhance oxidative metabolism after motility activation and uncoupling of oxidative phosphorylation (OXPHOS). Inhibitory analysis revealed negligible importance of glycolysis and fatty acid oxidation for energy supply of spermatozoa at both states – before and after attaining motility. It was concluded that mitochondrial oxidative phosphorylation is the primary pathway of energy production in Siberian sturgeon spermatozoa, with OXPHOS taking place in both immotile and motile states. Nevertheless, mitochondrial respiration is insufficient to meet high energy demands during motility. It means that in sturgeons, despite they are taxonomically different, evolutionary primitive, and possess unmatched long-lasting sperm motility compared with teleosts, the major source of energy required for sustained sperm motility is ATP generated and stored during the quiescent state.

In the next study (Chapter 3) conducted on spermatozoa of coldwater species burbot (*Lota lota*, Gadiformes), the functioning of the most important bioenergetic pathways (mitochondrial respiration, glycolysis, and fatty acid oxidation) was investigated at spawning temperature (4 °C) and maximum critical temperature (CTmax, 15 °C). At spawning temperature, similar to the results revealed for Siberian sturgeon spermatozoa, the role of glycolysis and fatty acid oxidation for energy supply was found to be insignificant at motile or immotile states of burbot spermatozoa. Mitochondrial respiration was detectable at both quiescent and motile states, but no inhibition was observed after exposure of sperm to the respiratory inhibitor. Also, the OCR range was the lowest compared to the available data for OCR in teleost spermatozoa at motile or immotile state. Additionally, the OCR was not enhanced after motility activation or after treatment with an uncoupling agent. Contrastingly, at CTmax, the activity of oxidative phosphorylation became predominant. The OCR was enhanced, and that could be inhibited by using a respiratory inhibitor. Therefore, irrespective of the temperature, the energy production and usage strategy remained the same: most of the spermatozoon energy was derived from stored ATP that had been synthesized via OXPHOS during a quiescent but bioenergetically active state.

Furthermore, to explore the long-lasting motility of sturgeons, successive steps were taken by studying the phosphocreatine-creatine (PCr-Cr) shuttle system apart from the previously studied pathways – OXPHOS, glycolysis, and fatty acid oxidation (Chapter 2). The

results obtained for sterlet (*A. ruthenus*, Acipenseriformes) (Chapter 4) revealed that PCr-Cr shuttle, known for maintaining a homogenous concentration of ATP molecules throughout the flagellar length, plays an active role while spermatozoa are at a quiescent state. No significant contribution of this shuttle in energy supply after sterlet sperm motility activation was detected.

In addition, the possibility of sperm reactivation or the second activation after termination of the first motility period was explored in sterlet spermatozoa (Chapter 4). The presented study was the first step ever to investigate this process in an endangered or vulnerable group of fishes – sturgeons. Our findings testify that sturgeon spermatozoa, similar to carp and trout sperm, can be reactivated after the termination of the first motility period, and these secondly activated spermatozoa can successfully fertilize eggs. Furthermore, for the first time, it was revealed that OXPHOS and PCr-Cr shuttle systems are the most crucial bioenergetic pathways during the sperm reactivation process.

In conclusion, the results obtained from the presented research added information and enhanced our understanding of fish sperm bioenergetics and its species specificity. The data on sturgeon sperm reactivation and the relative importance of bioenergetic pathways involved in energy supply during reactivation state could be helpful for fundamental and applied research.

Czech summary

Dýchání spermií ryb: druhová specifičnost a vliv teploty prostředí

Deepali Rahi

Evoluční rozmanitost ryb a široká škála podmínek jejich výtěru určují specifické vlastnosti rybích gamet. Spermie ryb s vnějším oplozením se vyznačují druhově specifickými rozdíly ve struktuře a vlastnostech pohyblivosti, které přispívají ke složitosti zobecnění strategie energetické bilance těchto buněk před a po aktivaci pohyblivosti. Předkládaná studie byla proto zaměřena na zkoumání úlohy mitochondriální respirace, jakož i glykolýzy a oxidace mastných kyselin v energetickém zásobování spermií sladkovodních druhů ryb s vnějším oplozením, které mají zásadní rozdíly v délce motility a teplotě při výtěru.

Studie provedená na spermiích jesetera sibiřského (*Acipenser baerii*, Acipenseriformes) (kapitola 2) ukázala, že rychlost spotřeby kyslíku (OCR – oxygen consumption rate) byla u nepohybujících i pohybujících se spermií konstantní (studováno po dobu 5 min). Bylo zjištěno, že OCR je vyšší než OCR zjištěná u studenovodních kostnatých ryb, např. pstruha, a nižší ve srovnání s mořskými druhy, např. kambalou. Předložená studie rovněž odhalila, že jeseteři patří do skupiny ryb, jejichž spermie mají schopnost zvýšit oxidační metabolizmus po aktivaci motility a rozpojení oxidativní fosforylace (OXPHOS). Inhibiční analýza odhalila zanedbatelný význam glykolýzy a oxidace mastných kyselin pro zásobování spermií energií v obou stavech – před dosažením motility i po ní. Byl učiněn závěr, že mitochondriální oxidativní fosforylace je primární cestou produkce energie ve spermiích jesetera sibiřského, přičemž OXPHOS probíhá jak v nepohyblivém, tak v pohyblivém stavu. Nicméně mitochondriální respirace je nedostatečná k pokrytí vysokých energetických nároků během motility. Znamená to, že u jeseterů, přestože jsou taxonomicky odlišní, evolučně primitivní a mají ve srovnání s kostnatými rybami bezkonkurenčně dlouhotrvající pohyblivost spermií, je hlavním zdrojem energie potřebné pro trvalou pohyblivost spermií ATP generovaný a skladovaný během klidového stavu.

V další studii (kapitola 3) provedené na spermiích studenovodního druhu mníka jednovousého (*Lota lota*, Gadiformes) bylo zkoumáno fungování nejdůležitějších bioenergetických drah (mitochondriální respirace, glykolýza a oxidace mastných kyselin) při výtěrové teplotě (4 °C) a maximální kritické teplotě (CTmax, 15 °C). Při výtěrové teplotě byla podobně jako u výsledků zjištěných u spermií jesetera sibiřského zjištěna nevýznamná úloha glykolýzy a oxidace mastných kyselin pro zásobování spermií mníka energií v pohyblivém nebo nepohyblivém stavu. Mitochondriální respirace byla zjistitelná jak v klidovém, tak v pohyblivém stavu, ale po vystavení spermií respiračnímu inhibitoru nebyla pozorována žádná inhibice. Také rozsah OCR byl nejnižší ve srovnání s dostupnými údaji pro OCR u spermií kostnatých ryb v pohyblivém nebo nepohyblivém stavu. Kromě toho se OCR nezvýšila po aktivaci motility, ani po ošetření činidlem rozpojujícím oxidativní fosforylaci. Naopak při CTmax začala převažovat aktivita oxidativní fosforylace. OCR byla zvýšena, bylo možné ji inhibovat použitím inhibitoru dýchání. Bez ohledu na teplotu tedy zůstávala strategie produkce a využití energie stejná: většina energie spermií pocházela z uloženého ATP, který byl syntetizován prostřednictvím OXPHOS během klidového, ale bioenergeticky aktivního stavu.

Dále byly pro zkoumání dlouhotrvající pohyblivosti spermií jeseterů učiněny další kroky, kdy byl kromě již dříve studovaných drah – OXPHOS, glykolýzy a oxidace mastných kyselin (kapitola 2) – studován také systém fosfokreatin-kreatin (PCr-Cr). Výsledky získané u jesetera malého (*A. ruthenus*, Acipenseriformes) (kapitola 4) odhalily, že systém fosfokreatin-kreatin, známý tím, že udržuje homogenní koncentraci molekul ATP po celé délce bičíku, hraje aktivní roli,

zatímco spermie jsou v klidovém stavu. Nebyl zjištěn žádný významný podíl tohoto systému na zásobování energií po aktivaci motility spermií.

Kromě toho byla u spermií jesetera malého zkoumána možnost reaktivace nebo druhé aktivace spermií po ukončení prvního cyklu motility (kapitola 4). Předložená studie byla vůbec prvním krokem ke zkoumání tohoto procesu u ohrožené nebo zranitelné skupiny ryb – jeseterů. Naše zjištění svědčí o tom, že spermie jesetera, podobně jako spermií kapra a pstruha, mohou být po ukončení první periody motility reaktivovány a tyto sekundárně aktivované spermie mohou úspěšně oplodnit jikry. Kromě toho bylo poprvé zjištěno, že OXPHOS a PCr-Cr jsou nejdůležitější bioenergetické dráhy během procesu reaktivace spermií.

Závěrem lze říci, že výsledky získané v rámci prezentovaného výzkumu obohatily naše znalosti o bioenergetiku rybích spermií a její druhovou specifičnost. Údaje o reaktivaci spermií jeseterů a relativním významu bioenergetických drah podílejících se na zásobování energií během reaktivačního stavu by mohly být užitečné pro základní i aplikovaný výzkum.

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List of publications

Peer-reviewed journals with IF

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Rahi, D., Dzyuba, B., Policar, T., Malinovskyi, O., Rodina, M., Dzyuba, V., 2021. Bioenergetic pathways in spermatozoa of an under-ice spawning fish burbot (*Lota lota*): role of mitochondrial respiration in varying thermal environment. Biology 10, 739. (IF 2021 = 5.079)

Knowles, J., Boryshpolets, S., **Rahi, D**., Vysloužil, J., Muselík, D., Stejskal, V., Kouřil, J., Podhorec, P., 2021. Effect of poly(lactic-co-glycolic acid) microparticles and carp pituitary on northern pike (*Esox lucius*) spermiation stimulation and its effect on quantity and quality of sperm. Animal 16, 100430. (IF 2021 = 3.240)

Rahi, D., Dzyuba, B., Xin, M., Cheng, Y., Dzyuba, V., 2020. Energy pathways associated with sustained spermatozoon motility in the endangered Siberian sturgeon *Acipenser baerii.* Journal of Fish Biology 97, 435–443. (IF 2020 = 2.038)

Under review

Dadras*, H., Golpour*, A., **Rahi, D.**, Lieskovská, J., Dzyuba, V., Gazo, I., Policar, T., 2021. Effects of cryopreservation on spermatozoon motility, plasma membrane integrity, antioxidant status, DNA integrity, and ultrastructure in sterlet *Acipenser ruthenus*. Frontiers in Marine Science. Under review.
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Rahi, D., Dzyuba, B., Sotnikov, A., Cheng, Y., Herrera, F., Tučková, V., Rodina, M., Dzyuba, V. Tale of motility exhausted sperm: bioenergetic pathways during non-activation, post-activation, and reactivation of sterlet (*Acipenser ruthenus*) spermatozoon. Aquaculture. Under review.

Abstracts and conference proceedings

Rahi, D., Xin, M., Cheng, Y., Dzyuba, B., Dzyuba, V., 2019. Inhibitory analysis of energy supplying pathways of motile and immotile spermatozoa in Siberian sturgeon (*Acipenser baerii*). In: Book of abstracts "7th International Workshop on the Biology of Fish Gametes", 2–6 September 2019, Rennes, France, p. 63.

Training and supervision plan during study

Name	Deepali Rahi
Research department	2018–2022 – Laboratory of Reproductive Physiology of FFPW
Supervisor	M.Sc. Viktoriya Dzyuba, Ph.D.
Period	18th January 2018 until 30th March 2022

Ph.D. courses	**Year**
Basics of scientific communication	2018
Pond aquaculture	2018
Applied hydrobiology	2018
Ichthyology and fish taxonomy	2018
English language	2018
Czech language	2019
Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2019 2020 2021 2021
International conferences	**Year**
Rahi, D., Xin, M., Cheng, Y., Dzyuba, B., Dzyuba, V., 2019. Inhibitory analysis of energy supplying pathways of motile and immotile spermatozoa in Siberian sturgeon (*Acipenser baerii*). In: Book of abstracts "7th International Workshop on the Biology of Fish Gametes", 2–6 September 2019, Rennes, France.	2019
Rahi, D., Dzyuba, B., Dzyuba, V., 2021. Fish sperm respiration: species specificity and effect of environmental temperature. "Over the horizon and for mutual acquaintance", 3–4 November 2021, Ceske Budejovice, Czech Republic.	2021
Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Klaus Reinhardt, School of Science, Faculty of Biology, Applied Zoology, TU Dresden, Germany (3 months)	2020
Pedagogical activities	**Year**
Announcing the project entitled Determination of enzymatic activities involved in energy production in sturgeon spermatozoa: a tale of long-lasting motility at Summer school	2019
Announcing the project entitled Comparative study of basic characteristics of mitochondrial respiration in spermatozoa and somatic cells of common carp at Summer school	2020
Laboratory training on sperm motility analysis and measurement of oxygen consumption rate by polarographic system	2020

Curriculum vitae

PERSONAL INFORMATION

Name: Deepali
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Languages: English (TOEFL – score 88), Hindi
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EDUCATION

2018 – present Ph.D. student at Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic
2015–2017 M.Sc., Kerala Agricultural University, Kerala, India
2010–2015 B.Sc., Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan, India

COMPLETED COURSES

Basics of scientific communication, Pond aquaculture, Applied hydrobiology, Ichthyology and fish taxonomy, English language, Czech language

RESEARCH STAY AND COLLABORATIONS

01.02–30.04.2020 Prof. Klaus Reinhardt, School of Science, Faculty of Biology, Applied Zoology, TU Dresden, Germany