FACULTY OF FISHERIES AND PROTECTION OF WATERS
UNIVERSITY OF SOUTH BOHEMIA IN ČESKE BUDĚJOVICE

Energetic and motility of fish spermatozoa

Energetika a motilita rybích spermií

Sergii Boryshpolets

Czech Republic, Vodňany, 2011

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I thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. SPERM STRUCTURE

Fish reproduction absolutely depends on successful fertilization of eggs which, in turn, is related to the metabolism and appropriate function of spermatozoa and oocyts. A spermatozoon has the crucial function to transport the male haploid chromosomes set into the oocyte.

A fish spermatozoon consists of several parts, a head, a midpiece and a flagellum with the typical 9 + 2 arrangement of microtubules. The head contains the nucleus and therefore the paternal DNA material. The midpiece is firmly linked to the head and it contains centriolar and mitochondrial segments. In the midpiece of spermatozoa occur metabolic reactions leading to appearance of macroergic phosphates required for beating of flagella. The flagellum itself represents the motor of the spermatozoon. Teleostean fish spermatozoa have no acrosome, while chondrostean fish have sperm with acrosome (Ginsburg, 1968; Cherr and Clark, 1984). The acrosome has lytic activities to enable the entrance of the spermatozoon into the oocyte through egg envelope. It has been already demonstrated that there are family-, species- and subspecies- specific differences in terms of the fine structure of spermatozoa with functionality (motility and fertility) and physiological and biochemical characteristics of spermatozoa (Alavi and Cosson, 2006; Morisawa and Morisava, 1986; Cosson, 2004).

1.2. METABOLIC PATHWAYS

Elucidation of the metabolic processes of these highly differentiated and simplified cells is a key component to gain comprehensive understanding of fish reproduction.

Several authors reported the presence of numerous organic molecules in fish sperm which can be metabolized for energy and demonstrated that enzymatic activities for their metabolism are present (Terner and Korsh, 1963; Mounib and Eisan, 1968; Lahnsteiner et al., 1993). At the same time Terner C. and Korsh G. (1963), Billard et al. (1995) demonstrated that the role of glycolytic metabolism becomes less important in the mature spermatozoa. According to Lahnsteiner et al. (1993) and Mansour et al. (2003) oxidative phosphorylation and cycle of tricarboxylic acid is the key metabolic pathway in fish spermatozoa.

1.3. OXYGEN CONSUMPTION

As was mentioned above, the midpiece of fish spermatozoa contains mitochondria and fish spermatozoa demonstrate oxygen consumption. Immotile fish spermatozoa respiration ranges among species from about 1 to 70 nmol O_2/min/$l0^9$ spermatozoa. Incubation of sperm samples with cyanide or under nitrogen atmosphere (anaerobic conditions) results in a decrease both of ATP level and motility (Christen et al., 1987; Perchec et al., 1995). Therefore the oxygen consumption in non activated fish spermatozoa is a metabolic pathway that produces and maintains stores of ATP for further activation.

1.4. SPERM MOTILITY ACTIVATION

In the fish seminal tract and testes spermatozoa remain immotile. The high osmolality and ionic composition of the seminal plasma prevent sperm motility in fish sperm ducts (Lahnsteiner, 1995). But during natural reproduction, fish sperm becomes motile after discharge into the aqueous environment due to external factors e.g. low K^+ concentrations in acipenserids or hypo-osmotic shock in freshwater

teleost fishes (Linhart et al., 2003) or opposite for marine fish.

Cellular studies on mechanism of sperm motility in fish showed that spermatozoa use the external factors as the trigger for the intracellular cascade of events leading to sperm motility activation. A hypo-osmotic shock activates sperm motility in freshwater fish species (see reviews by Cosson et al., 1999; Morisawa et al., 1999). An extremely rapid transition of spermatozoa from an immotile to motile state is observed. Motility of spermatozoa is sustained thanks to the hydrolysis of ATP catalyzed by dynein ATP-ase, which is coupled to the sliding of adjacent microtubules leading to the generation of flagella beating (Gibbons, 1968). The activation process is followed accompanied by a quick reduction of the ATP content in sperm, an increase of spermatozoa volume due to hypotonic swelling, and a rise of respiration rate (Lahnsteiner et al., 1999; Perchec et al., 1995; Perchec-Poupard et al., 1997; Dzyuba et al., 2001).

1.5. RESPIRATION RATE DURING SPERM MOTILITY PERIOD

During motility activation, spermatozoa pass some changes in metabolic processes leading to an increase of their respiration rate (Robitaille et al., 1987; Lahnsteiner et al., 1999; Dreanno et al., 1997, 1999). Thus the spermatozoa have a reserve capacity for increased oxidative metabolism.

To estimate maximal oxygen consumption rates, uncouplers of oxidative phosphorylation are used. Uncoupler – treated spermatozoa are expected to show an enhanced rate of oxygen consumption if total oxidative capacity is greater than that demonstrated by quiescent spermatozoa. The results in the rates of oxygen consumption of activated and "uncoupled" spermatozoa suggest that fish spermatozoa have a limited capacity to increase their oxidative metabolism. Even with "uncoupled" respiration chain or in media without oxygen, spermatozoa were able to activate their motility (Inaba at al., 1988; Perchec et al., 1995). Bencic et al. (1999, 2000) demonstrated that salmonid spermatozoa after incubation under nitrogen show depletion of ATP content and motility, but reoxygenation resulted in an increase of ATP and motility. It probably means that oxidative phosphorylation is needed only for production of ATP store in non activated sperm. Altogether, it appears that fish spermatozoa do not need respiration during motility phase if motility is based on stored rather than newly synthesized ATP.

1.6. ENERGY CONSUMPTION AND SPERMATOZOA MOTILITY

In previous paragraph, it was mentioned that ATP store is probably the main source of energy for sperm motility. Correlation among ATP level before activation, motility percentage and fertilization rate in different fish species were found (Zilli et al., 2004; Bencic et al., 1999, 2000; Ingermann et al., 2003).

Very rapid and short motility of freshwater fish spermatozoa with 50–90 Hz initial frequency of flagella beating (Christen et al., 1983; Gibbons et al., 1985; Cosson et al., 1985, 1991, 1997, 2000; Perchec et al., 1995, 1998) also suggests a much higher level of energy consumption compared to mammalian spermatozoa. In fish species such as trout (Christen et al., 1987), carp (Perchec et al., 1995), catfish (Linhart et al., 2004), sea bass (Fauvel et al., 1999) or turbot (Dreanno et al., 1999), ATP stores were shown to shift down to values reaching one third to one tenth of the initial content during intensive motility phase. It proves that, in fish spermatozoa, the mitochondrial oxidative phosphorylation, which is highly requested to produce ATP required during motion, remains insufficient to sustain endogenous ATP stores (Dreanno et al., 1999), even though during activation it became higher than in immotile sperm (see 1.5.). In other words, in motile fish spermatozoa, the rate of energy production is

slower than the rate of consumption: this feature obviously results in energy limitation within the short period of motility. Nevertheless it is worth to remark that the final ATP concentration at the end of a motility phase is usually still high enough to fulfil the dynein ATPases requirements. It means that the ATP concentration is probably not the limiting factor for motility in that case (Cosson et al., 2004). An increase of spermatozoa volume due to hypotonic swelling and osmotic damage may be considered as one of the reasons why fish sperm motility stop.

1.7. REACTIVATION

Several examples among various species demonstrate that fish spermatozoa can become activated for a second time and previously activated spermatozoa remain metabolically active (Benau and Terner, 1980; Perchec et al., 1995; Christen et al., 1987). This effect indicates that cessation of motility is not the result of lethal osmotic damage.

For salmonids, the second motility phase could be achieved by adding the phosphodiesterase inhibitor isobutylmethylxanthine (which inhibits the degradation of cAMP to AMP) after motility has stopped (Benau and Terner, 1980) or by first activation in ovarian fluid followed by second activation in hypotonic conditions. Factors such as ATP or cAMP would be responsible for the initiation and possibly maintenance of motility. This was confirmed by Morisawa and Okuno (1982).

After motility arrest following the transferring of carp sperm into isotonic condition, the ATP level (Billard et al., 1995) and cellular volume (Perchec-Poupard et al., 1997; Dzyuba et al., 2001) can be recovered together with the ability for the second motility activation in hypotonic condition. Thus the ability for recovering both ATP content and cell volume as well are the key parameters determining the possibility of reactivation of carp sperm (Perchec et al., 1995).

1.8. IDENTIFICATION OF THE STUDY DIRECTION

We know that most of freshwater fish with external fertilization spermatozoa possess a hypotonic mode of motility activation. Very rapid and short motility of fish spermatozoa is initiated and maintained by the hydrolysis of ATP stores, which were produced prior to activation. Respiration rate increases together with a sperm volume during motility activation. But in the same time they can be motile in media containing no oxygen. Incubation of sperm under anaerobic condition results in reversible decrease of both ATP level and further motility. That is why we suppose that the respiration of fish spermatozoa is involved in producing and maintaining high enough ATP store. Are external signals of motility activation similar to those for activation of oxidative phosphorylation? Is decrease of intracellular ATP a signal for respiration activation? These topics have not been studied thoroughly. In any case the respiration rate of sperm during resting period should be at maximum level (Chapter 2).

Carp as many as other fishes, is very sensitive to the temperature regime. The reproduction is directly associated with increasing water temperature during the reproduction season. However, the important question remains as follow: how the prespawning temperature could affect the process of spermatogenesis. Moreover, the mechanism of energy stores as well as its production during the spermatogenesis have not been well studied, yet. Probably, the different temperature regimes during the spermatogenesis could effect metabolic activity of spermatozoa or some other parameters of spermatozoa motility or their ability to be reactivated.

Sperm characteristics such as velocity or ATP content express behaviour of a sperm population, but inside this population, some dereference in physiological condition of spermatozoa could be present.

Different spermatozoa have different velocity, some of them stop earlier than other etc. It could be related to individual size of initial ATP stores or to capacity of oxidative phosphorylation in separate sperm cell during spermatozoa maturation (Chapter 2). If different spermatozoa are in different physiological stages, could they be activated separately? In that case we will be able to initiate motility only in part of spermatozoa when the rest of sperm cells will preserve the ATP and ability for further activation (Chapter 3).

The main role of spermatozoa is delivery of male haploid chromosomes set to the egg. Sperm become motile after discharge into the aqueous environment due to changes of external factors e.g. low K+ concentrations in acipenserids or hypo-osmotic shock in freshwater fishes. Other process during which sperm of fish can pass osmotic changes in environment exists: cryopreservation of sperm became very important for preservation of gene material. Osmotic changes (cryoprotectors as usual had high osmolality) or ice crystal growing could damage the spermatozoa what in tern leads to decreasing of sperm motility percentage and fertility. The reasons for the decrease in sperm velocity still are not clear (Chapter 4, 5). During process of freezing – thawing with slow cooling rates (commonly used for fish sperm), alterations of osmotic pressure is arising (Mazur, 1984). Could process of freezing – thawing somehow influence sperm motility or energy stores after thawing (Chapter 4)? Could it mean that this process is a species specific (Chapter 5)?

Better understanding of these processes will provide us new useful information for increase of artificial reproduction success and for use of sperm samples after thawing.

THE AIMS OF THIS STUDY WERE:

- Measure respiration and motility of activated and reactivated spermatozoa, and to compare the above between groups of common carp males kept at different temperature conditions.
- Study the dynamics of ATP content and sperm movement parameters in conditions of stepwise reduction of osmotic pressure of the activating solution.
- Examine spermatozoa activation during freeze-thawing process (spontaneous activation) and evaluate sperm motility percentage, spermatozoa velocity and ATP content changes associated with this phenomenon.
- Evaluate the influence of post-thaw storage time on ability of thawed sperm to fertilize after spontaneous activation.
- Investigate the presence of spontaneous motility activation in fish species presenting variant forms in their activation mechanisms.

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CHAPTER 2

PRE-SPAWNING WATER TEMPERATURE AFFECTS SPERM RESPIRATION AND REACTIVATION PARAMETERS IN MALE CARPS

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Pre-spawning water temperature affects sperm respiration and reactivation parameters in male carps

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Abstract Concentration, ability to be motile, motility during the second activation (reactivation), and endogenous respiration were studied in sperm from two experimental groups of carp males. Group 1 was maintained for 7 days at 15°C (cold water [CW] group), whereas the second group was subjected to a temperature of 20°C (warm water [WW] group) before sperm sampling. Reactivation was achieved after incubation of initially activated sperm in media with osmotic pressure adjusted up to 300 mOsm*kg^{-1} by increasing K^+ concentration. A statistically significant reduction in spermatozoa concentration in CW versus WW samples (from 46.0 ± 12.5 [15°C] to 59.3 ± 7 10^9 [20°C] spermatozoa/ml) was observed. The sperm of the CW group required a significantly longer incubation time (37 min) under isotonic conditions to achieve a maximum percentage of potent motility at repeated activation than the WW group (23 min). After activation of sperm motility, an increase in the respiration rate up to a maximum level was found; this level remained the same under conditions of recovering the potential for repeated activation. During the sperm movement respiration rate, in the CW group (6.1 nmol O_2/min/10^9spermatozoa) and the WW group (3.9 nmol O_2/min/10^9spermatozoa) were significantly higher than the nonactivated sperm (2.4 nmol O_2/min/10^9spermatozoa for CW and 1.1 nmol O_2 /min/10^9spermatozoa for WW). Keeping males at 15°C for 7 days increased the respiration rate of the sperm.

Keywords Carp · Sperm · Physiology · Aquaculture

Introduction

The motility of spermatozoa is initiated and maintained by the hydrolysis of ATP catalyzed by dynein ATPase, which is coupled to the sliding of adjacent microtubules, leading to the generation of flagellar beating (Gibbons 1968).

A hypo-osmotic shock triggers the initiation of sperm motility in most freshwater fish species (see review by Cosson et al. 1999; Morisawa et al. 1999). A rapid transition of spermatozoa from an immotile to a motile state is found, accompanied by a quick reduction in the ATP content of sperm, an increase in the spermatozoa volume due to hypotonic swelling, and a rise in the respiration rate (Lahnsteiner et al.

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1999; Perchec et al. 1995; Perchec-Poupard et al. 1997; Dzuba et al. 2001).

It has been demonstrated that the motility of spermatozoa of freshwater species is of short duration, and is associated with a large, but not complete, depletion of ATP levels (Ingermann 2008; Cosson 2004). In addition, mitochondrial oxidative phosphorylation, which is needed to produce energy required during sperm movement, remains insufficient to sustain endogenous ATP stores (Dreanno et al. 1999).

Keeping initially activated fish spermatozoa in a medium with high osmolality, such as K^+-rich medium, where motility is prevented, can regenerate the ATP of cyprinid spermatozoa within 10 min post-incubation. It has recently been shown that:

1. Morphological alterations in reactivated spermatozoa can be reversed
2. Reactivated sperm have the potential to fertilize eggs
3. Sperm velocity and percentage motility differ between initially activated and reactivated spermatozoa (Billard et al. 1995; Linhart et al. 2008)

Fish spermatozoa, as mentioned above, contain mitochondria, and quiescent spermatozoa show measurable oxygen consumption. The oxygen consumption of quiescent fish spermatozoa represents the basal metabolism that maintains ionic exchanges across plasma membrane (Ingermann 2008). Any correlation between the respiration of spermatozoa and their motility in initially activated and reactivated spermatozoa will aid in the understanding of the metabolism of energy in a medium with high osmolality during regeneration of ATP.

The objectives of the current study were to measure respiration and motility of initially activated and reactivated spermatozoa, and to compare the above between groups of common carp males kept at either 15 (CW) and 20°C (WW).

Materials and methods

Broodstock

The experiment was conducted at the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of the Ukraine, Kharkov, Ukraine (IPC&C) between February and April 2007. Mature male carp *C. carpio* L. (3 years old, 1–3 kg body weight) were introduced into the laboratory of the aquatic system of IPC&C in November 2006 and kept in 4000-l holding tanks. The stocking density was 40 fish per tank. Approximately 30% of the water volume was renewed every second day. Fish were fed daily with commercial carp pellets at 0.5% of their body weight, and the photoperiod was kept at 14 h light to 10 h dark. Water temperature, initially between 15 and 18°C, was gradually decreased to reach 15°C 1 month before the experiment started.

Seven days before hormonal injections were applied, two groups of males ($n = 8$ in each group) were transferred to two 1,000-l tanks, kept at water temperatures of 15 (CW—cold water group) and 20°C (WW—warm water group) respectively, with 30% of the water replaced every second day.

Sperm sampling

Males were intraperitoneally injected with a single dose (5 mg/kg body weight) of carp pituitary extract dissolved in 0.65% NaCl solution. Twenty-four hours after injection, sperm were stripped into dry plastic tubes without contamination by water, feces or urine, with the use of light abdominal massage, and were stored for not more than 1 h at 18°C.

Sperm motility analysis

Prior to motility estimation, the sperm in each tube were thoroughly mixed. To trigger sperm motility, some of the sperm were transferred with a dissecting needle tip (approximately 0.01 μl as estimated from an observation screen) onto a glass slide containing 10 μl of the activating medium studied (see next section). Sperm was mixed with the medium and covered with a cover slip. To remove the stickiness of the sperm, slides and cover slips were coated with 1% bovine serum albumin (BSA) solution.

The motility of the sperm was observed under a microscope (Carl Zeiss CS 25 series) using X16 positive-phase contrast objectives and a green filter. Approximately 150 spermatozoa in a microscope vision field were detected.

Images were captured using a CCD monochrome video camera (Panasonic WV-BP 330), recorded for

2 min using a TV tuner (AVerMedia Technologies, Milpitas, CA, USA), and stored on a computer.

The percentage of sperm motility was measured by video image analysis using Light Alloy software with a start–stop function starting from 5–7 s post-activation. All evaluations of fresh sperm were performed on samples with more than 90% motile spermatozoa.

Media used in experiments

- Activating medium (AM): 5 mM KCl, 45 mM NaCl, 30 mM Tris–HCl, pH 8.0 (Saad and Billard 1987)
- Nonactivating medium (NAM): 200 mM NaCl, 30 mM Tris–HCl, pH 8.0 (Saad and Billard 1987)
- Incubation medium (IM): 1 M KCl, 30 mM Tris–HCl, pH 8.0

Procedure for the second activation (reactivation)

Fifty microliters of fresh sperm were initially activated by mixing with 1 ml of AM. Fifty seconds post-activation (when approximately 99% spermatozoa had stopped), 100 µl of IM was added to the suspension of the initially activated sperm (final salt concentrations after dilution were 95 mM KCl, 41 mM NaCl, 30 mM Tris–HCl), and the resulting mixture was incubated at 22°C for 2 h. Thereafter, 1 µl of suspension was mixed with 10 µl of pond water at intervals of 5–8 min for 2 h, and the percentage of motile spermatozoa (reactivation percentage) was determined, as described above. This enabled the calculation of the optimal incubation time, resulting in the maximal percentage of sperm reactivation for every sperm sample (reactivation time).

Measuring the sperm concentration

Sperm concentration was measured using a spectrophotometric method at a wave length of 610 nm (Dzuba et al. 2001).

Oxygen consumption measurements

Fifty microliters of fresh sperm were transferred into a 1-ml thermostatically controlled chamber (22°C) equipped with a galvanic oxygen electrode 5972 (01) of a universal 5221 type oxygen meter (MERA–ELWRO, Wrocław, Poland). Rates of endogenous respiration were measured in AM and NAM in the absence and presence of an uncoupling agent of oxidative phosphorylation 2,4 dinitrophenol (DNP), with a final concentration of 0.25 mM. Respiratory rates were expressed in nmol O_2/min/10^9spermatozoa. The respiration rate was measured after 2–4 min of transferring the sperm sample into an oxymeter chamber or adding DNP, when the oxygen consumption curve observed became linear. After linear shape gaining no changes in the oxygen consumption curve were observed. The ratio of the respiration rate in nonactivating medium prior to and after DNP was calculated as an index of the uncoupling extent of oxidative phosphorylation.

Data presentation and statistical analysis

The initial respiration rate and sperm concentration data obtained were pooled for all experiments and presented as scatter plots with trend lines. Thereafter, data were stratified according to groups, and mean values (Mean), standard deviations (SD), and standard errors of the mean (SE) were presented in figures. The distribution and homogeneity of dispersion results were evaluated using Shapiro-Wilk's and Levene's tests. Abnormal distributions and an absence of the homogeneity of dispersions (for respiration rate values) necessitated the use of nonparametrical methods to compare groups. The presence of an abnormal distribution of values for these parameters in the groups or the absence of homogeneity of dispersions (for respiration rate values) determined the need to use nonparametrical methods of comparing the groups. In the case of paired comparisons we used the Mann–Whitney U test ($P < 0.05$ was considered to be significant). In the case of multiple comparative procedures the Kruskal–Wallis test was used with following consideration of the significance of differences between the groups by means of the Mann–Whitney U test with Bonferroni correction ($P < 0.015$ was considered to be significant). For normally distributed values with similar dispersion parameters (movement time) the parametric method of comparing using the t test for independent groups was applied. To represent the results as box and whisker plots and for calculation of the above-mentioned tests of the significant

differences between the groups, Statistica software (V6.0, Statsoft UK, Bedford, UK) was used.

Results

Motility, sperm movement time, and concentration

In both the WW and the CW groups the initial percentage of motility was 90–100%, with a short motility time (40–50 s). No significant differences between the WW and CW groups were found ($P > 0.05$, Mann–Whitney test). A high temperature during pre-spawning resulted in a rise in sperm concentration from 46.0 ± 12.5 (15°C) to 59.3 ± 7 10^9 (20°C) spermatozoa/ml (Fig. 1).

Parameters of reactivated sperm

A significant difference in the optimal incubation time (23 min in WW and 37 min in CW) resulting in the maximum percentage of reactivated spermatozoa has been found between the groups (Fig. 2a). However, the maximum percentage of the reactivated sperm (54% in WW and 46% in CW) did not differ ($P > 0.05$) between groups (Fig. 2b), with this parameter related to post-activation time ($P > 0.05$, Mann–Whitney test).

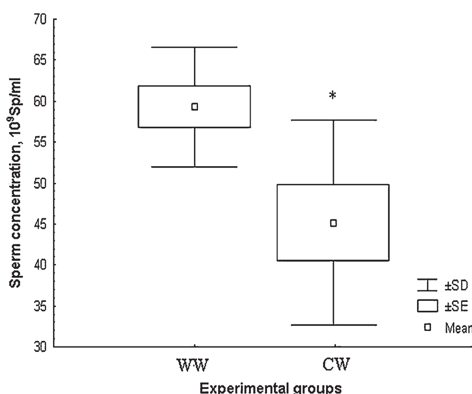

Fig. 1 Sperm concentration of the two groups of male carps kept for 7 days before hormonal injection either in cold water at 15°C (*CW*) or in warm water at 20°C (*WW*). *Asterisk* denote a significant difference, $P < 0.05$, t test, $n = 8$

General characteristics of oxygen consumption

A scatter plot of all data, obtained during all the experiments in the two groups, on respiration rate and concentration of spermatozoa is presented in Fig. 3. The rate of endogenous respiration of sperm was within the range 1.2–11.5 nmol O_2/min/10^9spermatozoa. The data presented testify to the possible statistically significant differences in respiration rates with regard to both sperm concentration and activation condition.

Rate of endogenous respiration of sperm in experimental groups

The rate of endogenous respiration in the two groups was characterized by statistically significantly higher values under conditions of AM (3.9 nmol O_2/min/10^9spermatozoa for WW, 6.1 nmol O_2/min/10^9 spermatozoa for CW) compared with NAM (1.1 nmol O_2/min/10^9 spermatozoa for WW and 2.4 nmol O_2/min/10^9 spermatozoa for CW). In addition, keeping males for 7 days at 20°C significantly reduced the respiration rate. The uncoupling of the oxidative phosphorylation DNP resulted in a significant increase in the respiration rate in NAM, and under these conditions, the respiration rate did not statistically significantly differ from that in AM (Fig. 4).

After first movement activation and following the addition of KCl to AM, which resulted in increasing the osmotic pressure in the solution up to isotonic levels and thereby creating conditions for gaining potential for repeated activation (Fig. 4, R-WW and R-CW), respiration was at a high level, and did not differ ($P > 0.05$) from respiration obtained under the hypotonic conditions of AM (Fig. 4, AM-WW, AM-CW). No significant differences in the respiration rate were found between sperm obtained from males kept at different pre-spawning temperatures.

The addition of DNP to AM to a final concentration of 0.25 mM did not increase the respiration rate, and an increase in DNP concentration to 0.5 mM did not result in a reduction in oxygen consumption.

The ratio of respiration rates in NAM prior to and after the addition of DNP, characterizing the uncoupling of oxidative phosphorylation, is presented in Fig. 5. The results show that the extent of the uncoupling of oxidative phosphorylation is significantly higher in males kept at 15°C before spawning.

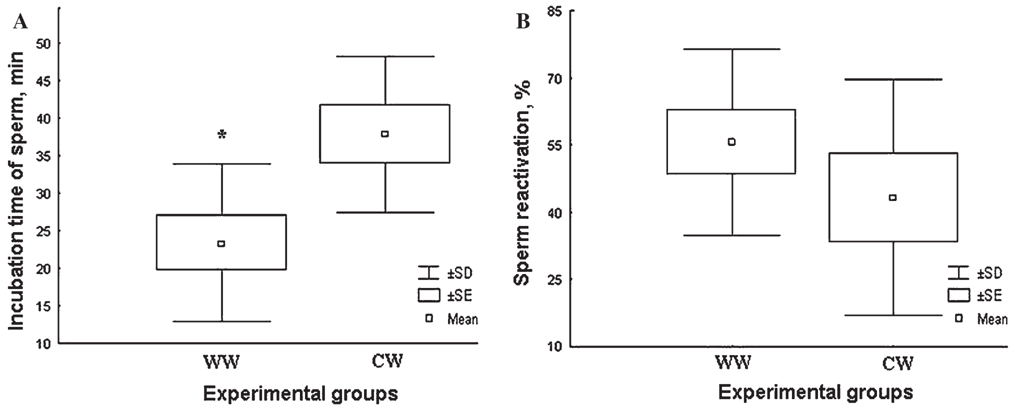

Fig. 2 **a** Incubation time necessary to reach the maximum percentage of motility for reactivated sperm, and **b** the maximum percentage of motile spermatozoa after incubation with incubation medium in the two groups of common carp males kept at a water temperature of 15°C (CW) and 20°C (WW) for 7 days before hormonal injection. For **a** the *asterisk* indicated that the difference was significant ($P = 0.04$, *t* test). For **b**, the absence of any statistically significant difference is shown ($P = 0.5$, Mann–Whitney test)

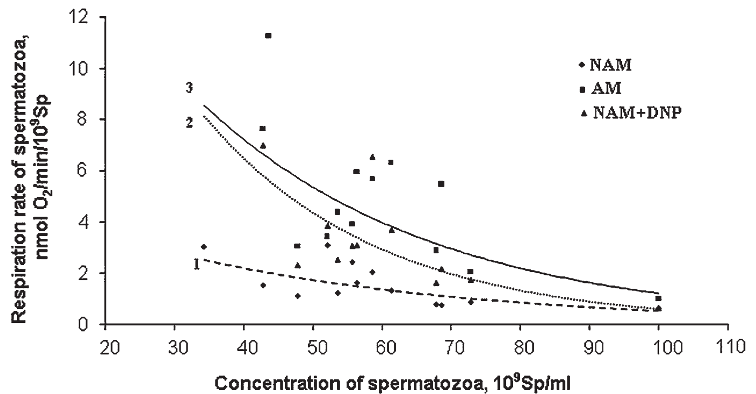

Fig. 3 Scatter plot of respiration rates versus concentration of carp spermatozoa. Measurements of the oxygen consumption rate are presented under the conditions of activating medium (*AM*), nonactivating medium (*NAM*), and after adding 0.25 mM 2,4 dinitrophenol (DNP) to NAM (*NAM+DNP*). Lines show exponential trends

Discussion

After first activation, carp spermatozoa reinitiate motility after incubation in hyperosmotic medium, called “reactivated” or “ready for second activation” spermatozoa, probably due to the regeneration of ATP (Perchec et al. 1995).

This study confirmed recent results published by Linhart et al. (2008) that during the second activation the character of sperm motility, either the percentage of motile spermatozoa or the sperm velocity, differs from those of initially activated spermatozoa. Changes in the percentage of motile spermatozoa during activation and reactivation are presented in Fig. 6. The percentage of motile carp spermatozoa rapidly decreases after activation in a hypo-osmotic medium, such as in AM in the present study (Fig. 6a). However, adding IM to the AM with activated spermatozoa (see Materials and methods) creates conditions for a second activation of sperm movement (reactivation). The reactivation of carp spermatozoa leads to changes in motility dynamics, meaning that 25–45 s post-reactivation the percentage of motility reaches the maximal value (Fig. 6b). This study showed similar dynamics in the percentage of reactivated sperm for all males from both

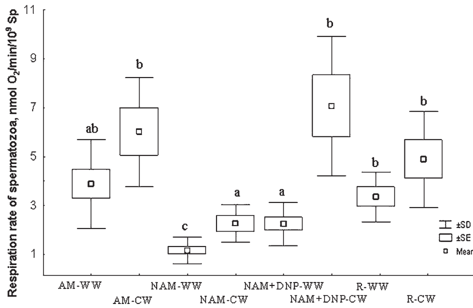

Fig. 4 Effect of water temperature (7 days of incubation before stripping) on oxygen consumption of spermatozoa of male carps. AM-WW—sperm respiration in AM for males kept at 20°C; AM-CW—sperm respiration in AM for males kept at 15°C; NAM-WW—sperm respiration in NAM for males kept at 20°C; NAM-CW—sperm respiration in NAM for males kept at 15°C; NAM+DNP-WW—sperm respiration in NAM, containing 0.25 mM DNP for males kept at 20°C; NAM+DNP-CW—sperm respiration in NAM, containing 0.25 mM DNP for males kept at 15°C; R-WW—sperm respiration after adding KCl to AM during reactivation of sperm for males kept at 20°C; R-CW—sperm respiration after adding KCl to AM during reactivation of sperm for males kept at 15°C. Values with the same superscript letter are not significantly different ($P > 0.015$, Mann–Whitney test)

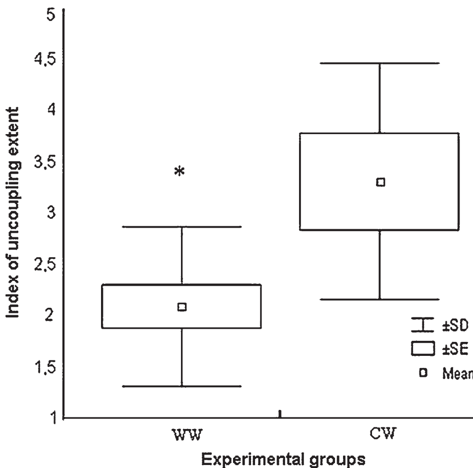

Fig. 5 Ratio of respiration rates in NAM prior to and after the addition of DNP (index of the uncoupling extent of oxidative phosphorylation) for sperm obtained from males kept at 15°C (CW) and 20°C (WW). *Asterisk* indicates the statistically significant difference between groups ($p = 0.015$, Mann–Whitney test)

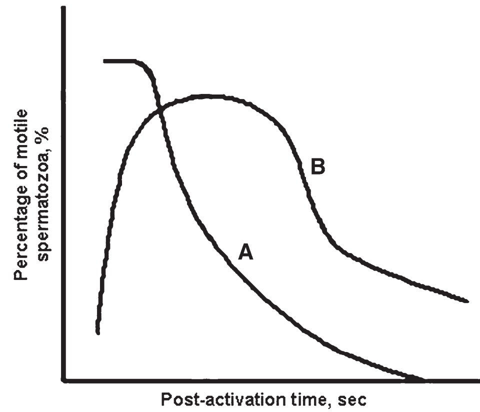

Fig. 6 Graph showing percentage of motile carp spermatozoa under the conditions of the first activation (*A*) and the second activation (reactivation) (*B*)

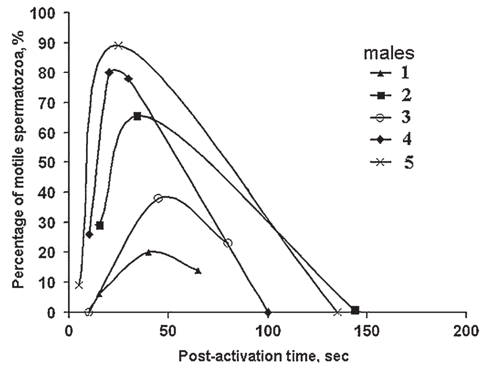

Fig. 7 Graph showing percentage of motile spermatozoa (%) at the second activation obtained for five different males

groups (CW, WW). The percentages of motile spermatozoa after 45 min incubation in IM are presented for five different males in Fig. 7. Since the percentage of reactivated sperm depends on the incubation time and individual properties of the sperm sample (Fig. 7), the differences among males can be related to the initial ATP content of spermatozoa in each individual.

Based on the literature on cellular mechanisms in fish sperm activation (Alavi and Cosson 2006), the existence of dynamics in percentages of secondarily activated sperm could not be properly explained. It might be postulated that the observed effect is related

to the inhibition effect of K^+ ions (Cosson 2004; Takai and Morisawa 1995), which is eliminated after sperm dilution and the timing reflects the activity of the cell regulatory processes.

Recovery of the initially activated spermatozoa cell volume under isotonic conditions is rapid, occurring within the first minute after the osmotic pressure has increased (Perchec-Poupard et al. 1997; Dzuba et al. 2001). Possibly, it leads to sperm metabolic activity resulting in the ATP accumulation needed for many functions in a cell, such as compensation for membrane permeability changes, ionic readjustment, etc. Finally, spermatozoa achieve the possibility of reactivating (Perchec et al. 1995). Reactivation time is supposed to reflect the rate of ATP production available for motility purposes, whereas the maximum percentage of reactivation shows sperm integrity after hypotonic shock. It should be emphasized that sperm capable of repeated activation also have fertilizing abilities, which is confirmation of their functional integrity (Linhart et al. 2008).

The study of motility and reactivation parameters from groups maintained at 15 and 20°C (CW and WW) showed that statistically significant differences were not observed in the percentage of motile spermatozoa and timing of their movement between groups. The sperm of the CW group require a significantly longer period to achieve the maximum percentage of motility (Fig. 2a); the percentage of reactivation remains statistically insignificant (Fig. 2b).

A significant reduction in the sperm concentration in males of the CW group might be attributed to the slowing-down of spermiogenesis caused by a lower water temperature during pre-spawning.

The energy metabolism of the sperm, either during movement or during recovery of the potential for reactivation, is a key parameter in sperm physiology. The participation of oxidative phosphorylation in the movement and recovery of the potential for movement during repeated activation has been indirectly demonstrated before (Perchec et al. 1995; Christen et al. 1987). However, there are no data available on respiration parameters of both initially activated and reactivated carp spermatozoa.

The influence of medium on the sperm respiration rate and sperm concentration has been illustrated in this study. In addition, an increase in the respiration rate due to the uncoupling of oxidative phosphorylation by DNP in NAM, and the absence of this effect in AM, could be related to a maximum sperm respiration rate achieved with the activating medium. The role of respiration in metabolic pathways providing fish sperm motility is currently not completely clear and its value can fall within quite a large range (0.6–280 nmol O_2/min/10^9spermatozoa) among species. In addition, the possibility of increasing the respiration rate in activating media is a species-specific parameter and carp possibly belongs to a species that does have reserve capacity for oxidative metabolism (Ingermann 2008).

Carp sperm gain the ability to move after passing the last stage of spermatogenesis, finishing with the yield of spermatozoa from the seminal ducts, where mature spermatozoa are mixed with seminal fluid (Morisawa et al. 1983). This process is connected to both changes in ions and pH of the surrounding spermatozoa medium and to the possible accumulation of macroergic compounds. With the assumption that the process of gaining the potential to move is similar under in vitro and in vivo conditions, we compared the intensity of oxygen consumption in sperm samples obtained from males of two experimental groups. Differences were found due to the pre-spawning water temperature, which might affect the rate of spermatozoa maturation after hormonal injection and, as a consequence, spermatozoa respiration.

We observed a statistically significant rise in the rates of endogenous respiration in AM compared with NAM for both experimental groups (Fig. 4, AM-CW versus NAM-CW and AM-WW versus NAM-WW). The absence of an increase in the respiration rates after adding DNP to AM proved that sperm respiration is at its maximum level during activation. The significant reduction in the sperm respiration rate in a nonactivating medium in the WW group could be related to the accumulation of an amount of ATP that is capable of causing inhibition of respiration. In sperm of the CW group ATP accumulation was probably not completed at the moment of sperm collection; therefore, there was increased consumption of oxygen in NAM (Fig. 4, NAM-WW versus NAM-CW). This could be attributed to a slowing-down in the maturation of the spermatozoa caused by the lower water temperature during pre-spawning. An important addition to the description of the respiration process is an increase up to the maximum level

of sperm respiration within the period after the first activation of movement (reactivation), as shown in Fig. 4 (RW-CW, RW-WW versus AM-CW, AM-WW). We assume that a sharp reduction in ATP content at the end of movement (Perchec et al. 1995; Cosson 2004) is the stimulus to respiration activation.

In conclusion, we postulate that a reduced water temperature during the pre-spawning period results in a slowing-down of spermiogenesis, manifesting in a decreased sperm concentration and differences in the respiration activity of spermatozoa. Therefore, sperm obtained from the males maintained at a lower temperature are characterized by lower sperm density and a higher level of endogenous respiration. In addition, the possible changes in the phospholipid fatty acids content of the plasma membrane of the sperm (Labbe et al. 1995) and in ion distribution (Emri et al. 1998) during fish adaptation to different temperatures might be related to our study phenomenon. Further research should utilize these findings to increase the efficiency of both native and cryopreserved carp sperm during artificial reproduction.

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CHAPTER 3

DYNAMICS OF ATP AND MOVEMENT IN EURASIAN PERCH (*Perca fluviatilis* L.) SPERM IN CONDITIONS OF DECREASING OSMOLALITY

Boryshpolets, S., Dzyuba, B., Stejskal, V., Linhart O., 2009. Dynamics of ATP and movement in Eurasian perch *Perca fluviatilis* L. sperm in conditions of decreasing osmolality. Theriogenology 72, 851–859.

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Dynamics of ATP and movement in Eurasian perch (*Perca fluviatilis* L.) sperm in conditions of decreasing osmolality

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Abstract

Repetitive activation of perch (*Perca fluviatilis* L.) sperm motility was investigated in this study. The first phase of sperm motility activation was initiated by dilution in a 260 mM glucose solution (75% motility). The second phase of motility was achieved by adding water to previously activated sperm, so that the glucose concentration dropped to 220 mM (24% motility). Finally, the third phase was obtained by further addition of water (down to 90 mM glucose) to the activated sperm suspension (15% motility). Parallel measurements of sperm ATP content were also made. The median value for nonactivated sperm was 43.9 nmol ATP/10^9 spermatozoa. The ATP concentration decreased significantly from 35 to 7 nmol ATP/10^9 spermatozoa after successive activations of motility in the above glucose solutions. Sperm velocity ranged in value from 25 to 330 μm/sec at 10 sec postactivation, from 10 to 290 μm/sec at 30 sec, and from 0 to 200 μm/sec at 45 sec. A model postulating several classes in the population of spermatozoa is developed, tentatively accounting for such successive activation. Possible further application of multiple sperm activation is discussed.

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Keywords: ATP; Eurasian perch; Fish; Fish biology; Osmotic pressure; Sperm motility

1. Introduction

Eurasian perch (*Perca fluviatilis* L.) is a new and promising aquaculture species for European countries [1]. Although an understanding of the mechanisms of sperm movement activation is an essential link in the development of methods for artificial reproduction in fish, the bioenergetic metabolic pathways of sperm movement have been insufficiently studied and there is a need for additional studies in this field [2].

Motility of spermatozoa is sustained by ATP hydrolysis catalyzed by dynein ATPases, which are coupled to the sliding of adjacent microtubules leading to the generation of flagellar beating [3]. It is clear that fish sperm use the energy of macroergic phosphates accumulated in the middle piece of spermatozoa (ATP and other energetic compounds are distributed the whole length of the flagellum as dyneins are present all along the length), and processes of their accumulation and hydrolysis during motility phases may be species specific [2]. Perch spermatozoa, as well as those of other freshwater fish, are activated for a short period right after their transfer into a hypotonic environment [4,5]. The significant dependence of perch sperm velocity and percentage motility on the osmolality of activating media [5] suggests that several sperm populations coexist within the same sample, each population requiring specific conditions for its motility activation.

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For freshwater fish, it has been shown that during sperm movement in hypotonic conditions, a reduction of the ATP concentration [6] and a rise in the sperm cell volume [7,8] are observed due to water penetration into a spermatozoon from the hypotonic environment, but the dynamics of ATP concentrations during the motility activation phase are not completely understood. Some observations of a rise of sperm ATP concentrations within several seconds after dilution in activating medium have been reported [9,10]. The characteristics of ATP expenditure during the motility period of perch spermatozoa have remained unstudied.

Our working hypothesis is that stepwise reduction of the osmotic pressure (osmolality) of the activating solution may positively affect the duration of sperm movement due to the decrease in the rate of ATP expenditure, and this feature may in turn be used for developing effective methods for artificial reproduction of this species. Therefore, our current research aim was to study the dynamics of ATP content and sperm movement parameters in conditions of stepwise reduction of osmotic pressure of the activating solution.

2. Materials and methods

2.1. Experimental design

2.1.1. Pilot study of repeated sperm motility activation by stepwise decreasing the osmolality of the activating medium

Fresh spermatozoa (2 μL) were diluted into 4 mL of the activating solution under test, and after rapid mixing (first activation), 10 μL suspension was placed on a glass slide under the microscope to visualize the motility. Osmolality of the activating solutions under test was within the limits of 150 to 290 mOsm/kg. The solutions with various osmolalities were obtained by diluting 300 mM glucose solution, which is nonactivating for perch spermatozoa [11]. After termination of movement (45 sec after activation) 0.1 to 1 mL distilled water (second activation of sperm motility) was added, and after rapid mixing, the motility was once again analyzed. After termination of sperm movement, 4 to 8 mL of distilled water (third activation of motility) was added. As a result, it was found that first activation with 260 mOsm/kg solution and second and third activations were possible. The ratio of sperm suspension volumes obtained in previous dilutions with distilled water being 1:0.2 and 1:1.7 at second and third activations, respectively, allowed the maximum motility percentage to be obtained. The mean osmolality values of media used in experiments were 260 ± 5 mOsm/kg, 223 ± 1 mOsm/kg, and 92 ± 3 mOsm/kg (mean ± SD, n = 6) for first, second, and third activations, respectively.

2.1.2. Percentage motility of perch spermatozoa in activation media of 90, 220, and 260 mOsm/kg

Fresh spermatozoa (2 μL) were diluted into 4 mL activating solution (90, 220, and 260 mOsm/kg), and after rapid mixing (first activation), 10 μL suspension was placed on a glass slide under the microscope to measure motility percentage (Fig. 1).

2.1.3. Velocity of sperm in the model of multiple activation of movement when using stepwise reduction of osmolality of activating solution

At the beginning of the study, 10 μL 90 mOsm/kg glucose activating medium was placed on a glass slide under the microscope, whereupon using the tip of a dissecting needle, the spermatozoa were introduced and were thoroughly mixed. Sperm velocity was recorded for analysis at 10, 30, and 45 sec postactivation (Fig. 2A). Such simple control of one-step activation was used for comparison with multiple activation (Fig. 2B).

Based on the results of the pilot study (see Section 2.1.1) of multiple activation of movement, for performance of further studies, the following procedure for three-step activation was used: 10 μL activating medium (260 mOsm/kg) was placed on a glass slide under the microscope, whereupon using the tip of a dissecting needle, the spermatozoa were introduced and

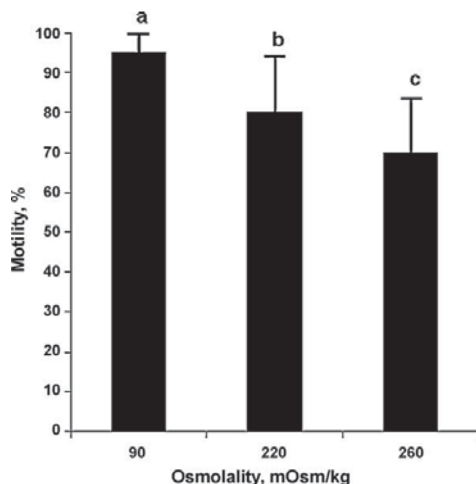

Fig. 1. Percentage of motile perch spermatozoa (mean ± SD) in different osmolalities of activation media. Values with different superscripts are significantly different (Mann-Whitney test, n = 6).

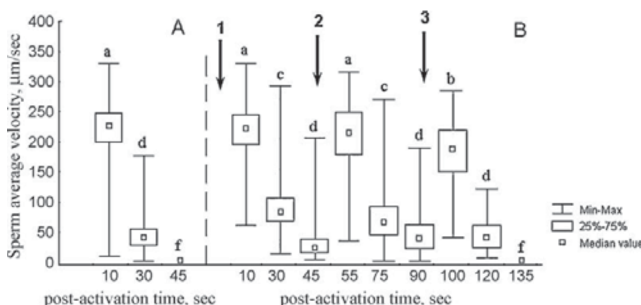

Fig. 2. Perch sperm average velocity during motility activation by (A) 90 mOsm media and (B) in the model of multiple activation. Arrow 1: activation in 260 mOsm/kg (first activation). Arrow 2: distilled water was added to decrease osmolality down to 220 mOsm/kg (second activation). Arrow 3: distilled water was added down to 90 mOsm/kg (third motility phase). Values with the same superscript are not significantly different (Mann-Whitney test, number of sperm analyzed = 34 to 325, n = 10). Min-Max, the levels of minimal and maximal values; 25%-75%, the values of 25th and 75th percentiles, respectively.

thoroughly mixed (first activation). Forty-five seconds after motility activation, 2 µL distilled water was added to the suspension with reduction of osmolality to 220 mOsm/kg (second activation) and again 45 sec later (at 90 sec after first activation) an additional 20 µL distilled water was added with final reduction of osmolality to 90 mOsm/kg (third activation). Recorded velocity of sperm in first, second, and third activation were analyzed at 10, 30, and 45 sec postactivation (Fig. 2B).

2.1.4. Sperm motility in the model of multiple activation of movement when using stepwise reduction of osmolality of activating solution

Ten microliters activating medium, osmolality 260 mOsm/kg, was placed on a glass slide under the microscope, whereupon using the tip of a dissecting needle, the spermatozoa were introduced and thoroughly mixed, and the percentage movement was measured at 10 sec postactivation. Later at 45 sec when sperm movement had ceased, 2 µL distilled water was added to decrease osmolality down to 220 mOsm/kg, and the second motility phase was initiated and analyzed at 55 sec after initial activation. Again 45 sec later when sperm movement ceased, 20 µL distilled water was added. Osmolality finally decreased down to 90 mOsm/kg. The third motility phase was initiated, and percentage of motility was measured at 100 sec after initial activation (Fig. 3).

2.1.5. Measurement of ATP content in sperm before and after activation in different conditions of activation

2.1.5.1. Nonactivated spermatozoa. To estimate ATP level in nonactivated spermatozoa, 2 µL sperm suspension was added to 5 mL boiling extraction medium and processed as described above

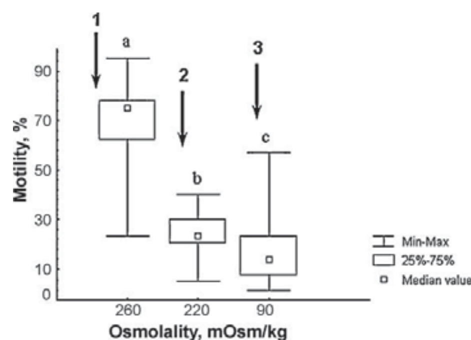

Fig. 3. Perch sperm percentage motility at different stages in the multiple activation model. Sperm motility was initiated in activating medium of osmolality 260 mOsm/kg (Arrow 1), percentage was measured at 10 sec of motility; 45 sec later (after first motility phase stop), distilled water was added to decrease osmolality down to 220 mOsm/kg (Arrow 2) and the second motility phase was initiated. Percentage of the second phase of motility was measured at 50 sec of experiment; 45 sec later (after the second motility phase stop), distilled water was added down to 90 mOsm/kg (Arrow 3) and the third motility phase was initiated. Percentage of the third phase of motility was measured at 100 sec of experiment. Min-Max, the levels of minimal and maximal values; 25%-75%, the values of 25th and 75th percentiles, respectively. Values with different superscripts are significantly different (Mann-Whitney test, n = 10).

2.1.5.2. Spermatozoa activated in 90 mOsm/kg activating medium. Twenty microliters semen was activated by mixing with 5 mL 90 mOsm/kg activating medium. Then at 10, 30, 45, 55, 60, 90, and 135 sec postactivation, 0.5 mL sperm suspension was added to 5 mL boiling extraction medium, and samples were processed for ATP content measurements.

2.1.5.3. Sperm activated in media of 260 mOsm/kg osmolality with second activation. Twenty microliters semen was activated by mixing with 5 mL 260 mOsm/kg activating media. Then at 10, 30, and 45 sec postactivation, 0.5 mL suspension was added to 5 mL boiling extraction medium. After 45 sec postactivation, 1 mL distilled water was added (to initiate second activation) and then at 55, 75, 90, 120, and 135 sec after first activation, 0.5 mL suspension was added to 5 mL boiling extraction medium, and samples were processed for ATP content measurement.

2.1.5.4. Spermatozoa activated in media of 260 mOsm/kg osmolality with second and third activations (model of multiple activation of movement). Twenty microliters semen was activated by mixing with 5 mL 260 mOsm/kg activating media. Then at 10, 30, and 45 sec postactivation, 0.5 mL suspension was added to 5 mL boiling extraction medium. After 45 sec post-activation, 1 mL distilled water was added (to initiate second activation), and at 55, 75, and 90 sec post-activation, 0.5 mL suspension was added to 5 mL boiling extraction medium. Ninety seconds after activation, 10 mL distilled water was added (to initiate third activation), and at 100, 120, and 135 sec postactivation, 0.5 mL suspension was added to 5 mL boiling extraction medium, and samples were processed for ATP content measurements.

2.2. Collection of sperm samples

Mature Eurasian perch (*Perca fluviatilis* L.) males were kept in a laboratory aquatic system. Spermatozoa from 16 males were collected into syringes after gentle abdominal massage during the natural spawning period. Care was taken to avoid sperm contamination by urine. Samples were stored in an icebox during the experiments.

2.3. Sperm concentration measurements

Spermatozoa were diluted in physiologic saline at a ratio of 1:2000 (sperm:saline). Sperm concentration was calculated after counting the number of spermatozoa in 12 cells of a Burker hemocytometer (Meopta, Prague, Czech Republic) at × 200 magnification with an Olympus BX 50 phase-contrast microscope (Olympus, Tokyo, Japan).

2.4. Osmolality measurements

Osmolalities of seminal fluid and of the activating media used were measured by vapor pressure osmometer (model 5520; Wescor, Logan, Utah, USA). Osmolality of seminal fluid was measured in sperm supernatants resulting from fresh sperm centrifugation at 10,000 × *g* for 10 min.

2.5. Analysis of sperm motility

Sperm motility was recorded after activation using a 3CCD video camera (Sony DXC-970MD; Sony, Tokyo, Japan) mounted upon both dark-field and phase-contrast microscopes (Olympus BX 50). Video recordings were performed with a video recorder (Sony SVHS, SVO-9500 MDP; Sony). Video records from the dark-field microscope with stroboscopic light were analyzed to estimate average sperm velocity (μm/sec), and records from the phase-contrast microscope were used to estimate the percentage of motility. To compute average sperm velocity, five successive frames were analyzed by a micro-image analyzer (Olympus Micro Image 4.0.1. for Windows; Olympus). Spermatozoa with velocity less than 3 μm/sec were considered as immotile and were omitted from calculation of motile spermatozoa. Sperm motility and velocity were measured after 10 sec of sperm dilution in activating media according to experiments described in Section 2.1.

2.6. Measurement of ATP content

Sperm samples were added to boiling extraction medium, which stops changes in the ATP content and extracts ATP from the cells. The composition of the boiling extraction medium was 100 mM Tris-HCl, pH 7.75, and 4 mM ethylenediamine tetraacetic acid (EDTA).

Samples of sperm suspension in boiling media (boiled 2 min at 100 °C) were subsequently centrifuged at 15,000 × g for 15 min using a Heraeus model 400-R centrifuge (Hanau, Germany). Supernatants were pipetted into 2-mL sterile vials, and kept at −18 °C before analyses were performed. ATP content in the supernatants was evaluated by bioluminescence using Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Basel, Switzerland). Luminescence was read with multifunctional microplate reader infinite M200 (TECAN, Mannedorf, Switzerland). ATP content was assessed by calibration as recommended in the kit manual and sperm density parameters. ATP content was expressed as nmol ATP/10^9spermatozoa.

2.7. Data presentation and statistical analysis

The distribution characteristics and homogeneity of dispersion results were evaluated using Shapiro-Wilk

and Levene tests, respectively. For normally distributed values (percentage of motility in media of different osmolarities, osmotic pressures of seminal fluids, and activating media used), results are presented as mean ± SD. For not normally distributed values (percentage of motile spermatozoa, sperm velocity, and ATP content during multiple activation), results are presented in the illustrations as box and whisker plots. These plots contain the values of median and 25th and 75th percentiles (''box'') and minimal and maximal ranges (''whiskers''). For multiple comparative procedures, the Kruskal-Wallis test was used with consideration of the significance of differences between the groups by means of Mann-Whitney U-test with Bonferroni correction ($P < 0.025$ was considered to be significant). To represent the results as box and whisker plots and for calculation of the above-mentioned tests of the significant differences between the groups, Statistica V8.0 (Statsoft Inc, Tulsa, Oklahoma, USA) software was used.

To represent the correlation between osmolality and median value (n = 15) of final content of ATP at the end of sperm movement after first, second, and third activations in the model of multiple activation, the equation of linear regression with confidence coefficient (R^2) was calculated using Microsoft Excel (Microsoft, Redmond, WA, USA).

3. Results

3.1. Pilot study

The osmolality of perch seminal fluid, in which spermatozoa were not active, was 298 ± 12 mOsm/kg (mean ± SD, n = 9), however, it was established that repeated activation of movement is possible only if the osmolality of the first activating solution was above 200 mOsm/kg. The increase in osmolality above 260 mOsm/kg resulted in a partial activation of the sperm population. The medium with an osmolality of 280 mOsm/kg had activating effects only in a very low proportion of the sperm samples. The consequence of using activating media with osmolalities of 260, 220, and 90 mOsm/kg resulted in the sequential activation of sperm movement, and we decided to apply exactly this protocol as the model of multiple activation of movement in our studies.

3.2. Percentage motility of perch spermatozoa in activation media of 90, 220, and 260 mOsm/kg

The results of initial percentage motility in activating solutions of different osmolalities are presented in Figure 1. The percentage of motile spermatozoa at 10 sec of motility significantly increased with decreased activation media osmolality. The highest percentage of motile spermatozoa (95%) was observed at 90 mOsm/kg. Use of 260 and 220 mOsm activators resulted in sperm activation with motility percentage of 75% and 90% (mean values), respectively. As soon as the highest motility percentage was observed in medium of 90 mOsm/kg, the average sperm velocities at different postactivation times were measured, and results are presented in Fig. 2A. Velocity of spermatozoa varied across a wide range (10 to 330 μm/sec at 10 sec, 5 to 170 μm/sec at 30 sec, and 0 to 10 μm/sec at 45 sec postactivation), showing a sharp significant decrease within first 45 sec of motility.

3.3. Velocity of spermatozoa and percentage motility in the model of multiple activation of movement when using stepwise reduction of osmolality of activating solution

Velocity of spermatozoa in the multiple activation model varied across a wide range (25 to 330 μm/sec at 10 sec, 10 to 290 μm/sec at 30 sec, and 0 to 200 μm/sec at 45 sec postactivation) and was significantly dependent on activator osmolality. No significant difference was found for sperm velocity median value at the 10th sec of motility between samples activated in 90 and 260 mOsm/kg (first activation) and second activation (Fig. 2B, 55 sec), whereas a significant decrease was observed for this value in case of the third activation (Fig. 2B, 100 sec).

Percentages of motile spermatozoa in the model of multiple activation are presented in Figure 3, showing the significant decrease of median percentage value from 75% down to 24% and 15% for first, second, and third activations, respectively. The values of motility percentages shown in Figure 3 correspond with the points of 10, 55, and 100 sec on Figure 2B.

3.4. Measurement of ATP content in sperm before and after activation in different conditions of activation

The values of ATP content in activated perch spermatozoa in 90 mOsm/kg activating medium and in spermatozoa activated in media of 260 mOsm/kg osmolality after second and third activations are presented in Figure 4. The median value of ATP content in nonactivated spermatozoa was 43.9 nmol ATP/10^9 spermatozoa (Fig. 4, activation time ''0''), whereas after motility activation it decreased down to

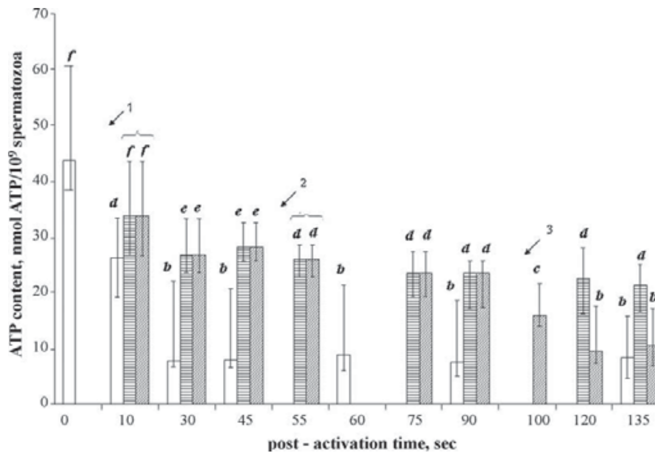

Fig. 4. ATP content in perch sperm after motility activations by sharp and stepwise decrease of activating medium osmolality. Spermatozoa were diluted with activating media of differing osmolality: □, activation in media of 90 mOsm/kg osmolality (sharp decrease of osmolality, one step activation); ▤, activation in media of 260 mOsm/kg osmolality with following second activation (two-step activation); ▨, activation in media of 260 mOsm/kg osmolality with following second and third activations (three-step activation, model of multiple activation). For two- and three-step activation, the first step of motility was initiated by dilution of sperm in media of 260 mOsm/kg (Arrow 1), the second phase of motility was initiated by addition of distilled water to decrease the osmolality down to 220 mOsm/kg (Arrow 2), and the third phase was initiated by addition of distilled water to decrease the osmolality down to 90 mOsm/kg (Arrow 3). Point "0" represents nonactivated sperm. Values with the same superscript are not significantly different (Mann-Whitney test, n = 15). Column, median value; whiskers, the values of 25th and 75th percentiles, respectively.

25 to 35 nmol ATP/10^9 spermatozoa, and at the end of motility, it shifted down to 8 to 21 nmol ATP/10^9 spermatozoa. These values were dependent on post-activation time and activation media used. Within 10 sec of motility activation, a significant decrease in sperm ATP content was observed, and the osmolality of media used for first activation had a significant positive influence on ATP level. A significant median ATP level decrease was observed after 30 sec of motility in both conditions of the first phase of motility activation. During the second phase of sperm motility activation in 260 mOsm/kg, the ATP level was significantly higher than at any postactivation time in comparison with those for sperm activated in osmolality 90 mOsm/kg. After the third phase of motility activation, when osmolality of activating media was decreased down to 90 mOsm/kg, the level of ATP became statistically indistinguishable in comparison with that for sperm activated in 90 mOsm/kg medium.

4. Discussion

4.1. Percentage of sperm motility in activating media of different osmolalities

Our measurements of seminal fluid osmolality and motility percentage in hypotonic activators of differing osmotic pressure are in good agreement with those previously reported for perch [4,5] and indicate a hypo-osmotic mode of sperm activation in perch, which is typical for freshwater species [12]. The phenomenon of increasing percentage of sperm motility with decreased osmolality of activating media (Fig. 1) could be explained by the speculation that several subpopulations of spermatozoa differing in their threshold value of activating media osmotic pressure coexist in one sperm sample. Some aspects of this hypothesis will be considered below.

4.2. Sperm motility parameters during first, second, and third activations in the model of multiple activation

We developed a model of multiple activation of movement when using stepwise reduction of osmolality of activating solution. This model takes into account our results showing that, due to stepwise decrease of activating media osmolality, it is possible to prolong the total duration of perch sperm motility from 45 sec up to 135 sec (Fig. 2).

We consider these results as demonstrating, not the fact that the same spermatozoa have a prolonged motility period, but that the total period of motility phase (more than 10% spermatozoa are motile in the

same time) became prolonged, possibly due to the activation of different fractions of spermatozoa at different time points. If increasing the period of sperm motility can also positively increase the fertilization rate [13,14], this phenomenon might be exploitable for applied use in perch breeding.

As we have demonstrated in our model of multiple sperm activation, repeated activation of perch sperm movement can be achieved when the osmolality of the activating solution used for the first activation is higher than 200 mOsm/kg. Use of an activating medium with osmolality of 260 mOsm/kg rendered possible the second (at 220 mOsm/kg) and the third (at 90 mOsm/kg) activation due to decreased osmolality (Fig. 2). We hypothesize that the observed phenomenon could be related to differences in the individual sensitivity of spermatozoa to the osmotic shock [8]. Higher osmolality of activating medium led to activation of the motility of selected sperm populations but without affecting the functional integrity of spermatozoa. Possibly, this level of swelling is not large enough for motility activation in another portion of the sperm population, which requires larger differences in osmotic pressure between ovarian fluid and activating media. A similar response of sperm motility on hypotonic influence was reported earlier for several freshwater species [15–17], and for the time being, there is no certain explanation of this phenomenon.

Our current data do not lead to firm conclusions but allow us to speculate that different sperm subpopulations can be activated in conditions of different osmotic pressure. Further, the different median values of sperm velocities could be considered as characteristics of these subpopulations. In that case, the sperm subpopulation for which motility could be initiated only after osmotic pressure is reduced down to 90 mOsm/kg shows significant decrease in average sperm motility at initial stages of activation, whereas for two other subpopulations, no significant differences in this parameter were found (Fig. 2B). Our findings differ from results observed on the repeated sperm activation in the carp model, when a second round of motility was achieved after 10 min sperm incubation in potassium-rich media [18]. In the latter case, the velocity was halved in spermatozoa, which were able repeatedly to initiate motility.

We suppose that during the first phase of motility activation, the major part of the sperm population is activated while the rest (not activated in this condition) of the spermatozoa preserve their potential for activation in media with lower osmolality (Fig. 3).

The importance of mechano-sensitive channel activity for freshwater fish sperm motility activation was shown early [19]. The activity of these channels could be dependent on cell environment osmotic pressure, but understanding of differences in activation conditions for different sperm populations requires further study.

4.3. ATP content during motility in the experiments

Because our interest is focused on the dynamics of ATP concentration, which are the main issue here for discussion, we used an external calibration method (mentioned in the kit manual).

It is well known that motility of spermatozoa is associated with macroergic phosphate metabolism, which comprises (1) production of ATP in the middle piece, (2) diffusion of ATP along the axoneme by the phosphocreatine shuttle, and (3) ATP hydrolysis by dynein ATPases spaced along the axoneme (see review by Cosson [20]). Although an intensive ATP consumption by activated spermatozoa could not be compensated by processes of ATP production, the decrease in ATP within motility phase is observed for several fish species (see review by Ingerman [2]). Therefore, our results of ATP content decrease in perch spermatozoa during motility phases (Fig. 4) are in good conformity with knowledge described earlier. As was shown earlier, the presence of ATP in swimming media is necessary for the motility of de-membranated carp sperm models. In addition, ATP level, beat frequency, and velocity of spermatozoa are strongly and positively correlated [7]. These findings suggest that sperm velocity could be strongly dependent on ATP content near the dyneins. The total ATP content could be associated with potential for sperm motility, but the distinct meaning of this parameter is unclear because usually after cessation of motility, the level of total ATP is still high enough for dynein activity but flagellum beating becomes arrested [21]. This flagellum beating arrest in freshwater spawning species could be attributable to several reasons. First is the hypotonic swelling in conditions of low osmolality, which leads to flagellum morphology changes [18,22]. Second is the decrease of cAMP levels for species demonstrating cAMP-dependent pathway responsible for flagellum beating [23]. The third reason could be associated with changes in sperm intracellular ionic composition occurring during sperm motility and leading to changes of membrane potential [24]. In addition, we speculate that low levels of total ATP that are found in spermatozoa after motility has ceased in conditions of hypotonic activation [6]

could reflect the absence of distribution of ATP among the sperm flagellum.

The possibility of inducing the second motility phase in vitro has been shown for several fish species differing in metabolic pathways for motility activation signaling. For carp, which show an osmotic pressure–dependent mode of sperm motility activation [12], the second round of motility was achieved, and then spermatozoa were transferred after cessation of motility to conditions of increased osmolality (300 to 400 mOsm/kg) for 10 to 20 min and then activated again in hypotonic condition [18]. Within the time of incubation in media of increased osmolality, the ATP concentration was increased, and it was supposed that this time is important for ATP level recovery needed for second motility phase [25]. The model of second activation described above differs from the model of multiple activation used in our experiments because no time of incubation in isotonic condition between the first and second activation phases was used in our experiment, and only a stepwise decrease in osmolality was applied (see Fig. 4). Other models of multiple activation have been described for zebra fish and salmonids. In zebra fish, the activated spermatozoa were artificially stopped using higher osmolalities (at the stage prior to motility stop onset) and then the spermatozoa were again transferred to hypotonic conditions to activate spermatozoa motility, but this procedure may be repeated several times [26]. For salmonids, the second motility phase could be achieved by adding the phosphodiesterase inhibitor isobutylmethylxanthine (which inhibits the degradation of cAMP to AMP) after motility has stopped [23] or by first activation in ovarian fluid followed by second activation in hypotonic conditions. Thus, the model used in our experiment is different from the models described earlier.

As shown in Figure 4, the reduction of total ATP level during motility phases is dependent on the osmolality of the activating media used. Whereas the distinct metabolic pathway responsible for these changes is still not clear (for review, see [2]), well-defined interpretation of the ATP content dynamics is complicated due to changed motility percentage and velocity, as well as the ATP consumption by immotile spermatozoa in the media with different osmolalities. That is why we could only suppose that the high ATP level in spermatozoa activated by 260 mOsm/kg and 220 mOsm/kg activating media (Fig. 5) could provide the ATP reserves for multiple sperm activation.

We propose that the high ATP level seen after cessation of motility in spermatozoa activated by 260 mOsm/kg and 220 mOsm/kg media (Fig. 4, two-step activation) could be one of the reasons for the multiple sperm activation providing the ATP reserves for second and third phase of motility, respectively (Fig. 4, three-step activation). Thus, the specific reasons for motility cessation in activating media of high osmolality are still not clear, but they do not appear to be related to ATP depletion.

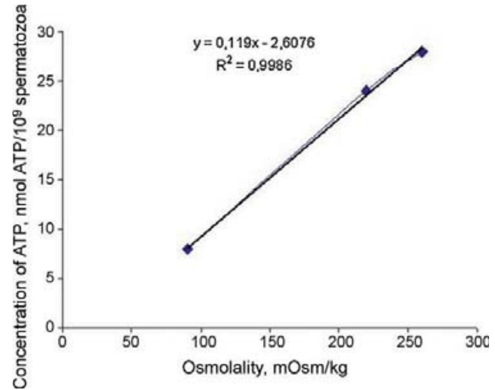

Fig. 5. Correlation between osmolality and final content of ATP at the end of sperm movement in the first, second, and third activations in the model of multiple activation.

In conclusion, we suppose that phenomenon of multiple and successive perch sperm motility activation is based on ability of several sperm subpopulations to be differentially activated by solutions of different osmolality. Furthermore, the use of decreased differences in osmotic pressure of seminal fluid and activating media spermatozoa preserves the level of ATP together with the ability for repeated activations.

Future studies on more precise identification and description of the hypothetical sperm subpopulations are needed and could be performed by measuring sperm head diameter in activated and nonactivated spermatozoa in the same osmolality condition. This procedure might show the differences in swelling rate for different sperm subpopulations. To our knowledge, our findings are the first report of stepwise fish sperm activation by hypotonic media of different osmolalities. This phenomenon requires future study to be useful for enhancement of fertilization success in fish artificial reproduction.

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CHAPTER 4

FREEZE-THAWING AS THE FACTOR OF SPONTANEOUS ACTIVATION OF SPERMATOZOA MOTILITY IN COMMON CARP (*Cyprinus carpio* L.)

Boryshpolets, S., Dzyuba, B., Rodina, M., Li, P., Hulak, M., Gela, D., Linhart, O., 2009. Freeze-thawing as the factor of spontaneous activation of spermatozoa motility in common carp (*Cyprinus carpio* L.). Cryobiology 59, 291–296.

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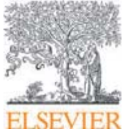

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Freeze-thawing as the factor of spontaneous activation of spermatozoa motility in common carp (*Cyprinus carpio* L.) ☆

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ATP

ABSTRACT

In the present study, we investigated the possibility of spontaneous carp spermatozoa activation by freeze-thawing. To evaluate this, the parameters of spermatozoa motility percentage, velocity, ATP content level and fertility rate of sperm were used. The motility and velocity of spermatozoa activated by freeze-thawing were characterized by motile spermatozoa with a median value of 16% and a velocity of 98 μm/s. In addition, the motility and velocity of sperm from the thawed samples were significantly lower than in the control (median value of 100% for sperm motility and 175 μm/s for sperm velocity). Furthermore, a spontaneously activated spermatozoa motility terminated within five minutes post-thaw time. After freeze-thawing the ATP level significantly decreased with post-thaw time (46 nmol ATP/10^9 and 10 nmol ATP/10^9 at 25 s and 10 min after thawing, respectively). Fertility of spermatozoa was not significantly affected within 10 min post-thaw. On the other hand, the fertility of frozen-thawed sperm was significantly lower if compared to fresh sperm. We conclude that the freeze-thawing procedure spontaneously activated spermatozoa motility in common carp. However, this activation did not negatively affect the fertility of frozen-thawed sperm.

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Introduction

The carps are the most cultivated species throughout the world [7]. Several successful methods have been reported for the cryopreservation of common carp sperm, e.g. [10,8,5,6]. However, the methods of fish sperm cryopreservation reveal several common problems, such as considerable variability in spermatozoa motility parameters as well as a wide range of fertility [9] in spermatozoa survival after thawing.

Spermatozoa in common carp possess a hypotonic mode of motility activation [17]. This activation leads to a sharp decrease in ATP content in parallel with spermatozoa velocity and motility percentage decreases. Moreover, it is possible to manipulate sperm motility by changing of the environmental osmolality. For example, sperm motility re-activation could be achieved after a subsequent increase of osmotic pressure of the environmental medium of activated spermatozoa up to the level of isotonicity, which would result in cessation of motility and restoration of ATP content together with the potential for a further motility phase [16,13,1]. Furthermore, the multiple motility activation could be obtained by a stepwise osmotic pressure decrease [2]. Changes in osmotic pressure outside and inside the cells following freeze-thawing processes with slow cooling rates (commonly used for fish sperm) [14], could provokes carp spermatozoa motility stimulation.

We suppose that alterations of osmotic pressure arising from freeze-thawing could activate spontaneous movement in carp sperm. "Spontaneous" here implies without any environmental changes made by an observer, in contrast to activation usually made by sperm dilution in hypotonic activating media (hypotonic activation).

The first objective of the present study was to examine spontaneous spermatozoa activation by freeze-thawing and evaluate sperm motility percentage, spermatozoa velocity and ATP content changes associated with this phenomenon in comparison with hypotonic activation. A second aim was to evaluate the influence of post-thaw storage time on fertility of thawed sperm after spontaneous activation before fertilization.

Materials and methods

Gametes collection

Fish maintained in a were transferred to individual 4 m^3 hatchery tanks. Water flow rate was maintained at 0.2 l s^{-1}. Temperature and oxygen ranged from 18–22 °C to 6–7 mg l^{-1},

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respectively. Fish were anesthetized before handling with a 1:1000 aqueous solution of 2-phenoxyethanol. Before stripping (24 h), males were injected with carp pituitary extract at 1 mg kg^{-1}. Females were injected with the same extract at 24 (0.4 mg kg^{-1}) and 12 h (2.1 mg kg^{-1}) before stripping. Sperm was obtained from 10 males by abdominal massage and collected directly into 20 ml plastic syringes. Special care was taken to avoid contamination with urine, mucus, feces or water. Sperm samples were not pooled and were stored under aerobic conditions at 0 °C. The females were stripped and ova stored under aerobic conditions at 17–19 °C. Ova were used within 1 h post-collection [12].

Experimental design

Percentage of motile cells, sperm average velocity and ATP content at different post-activation times in hypotonically activated fresh spermatozoa were determined. For freeze-thawed sperm the same parameters were estimated with both hypotonic and spontaneous activations. At least three straws per sperm sample were frozen. After thawing of the first straw, motility parameters and ATP content in hypotonically activated spermatozoa were estimated. Surplus spermatozoa were centrifuged and supernatant (CS-seminal fluid + cryoprotective medium) was obtained for the following spontaneous motility test. Immediately after thawing of the second straw, the spontaneous motility was determined using a dilution with CS. Dilution with CS was necessary to decrease the sperm concentration in order to obtain clear observations of spontaneous sperm motility. The sperm suspension from the second straw was transferred into an Eppendorf tube and stored on ice for motility parameters and ATP content determinations at different post-thaw time periods. The third straw was used for sperm fertility assessment of frozen-thawed samples, which were stored for different post-thaw time periods.

Evaluation of sperm concentration and osmolality

Concentrations of each sperm samples were estimated microscopically using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification and an Olympus BX 50 phase contrast microscope (Olympus, Japan). Seminal fluids (SF) and supernatants of sperm samples after freeze-thawing (CS) were obtained after samples were centrifugation at 10,000*g*, 10 min. Osmolalites of SF and CS were evaluated using a Vapor Pressure Osmometer 5520 (Wescor, USA), and expressed in mOsmol/kg.

Evaluation of motility parameters

A saline solution (AM—45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.2) was used as hypotonic activating media, and/or supernatants (CS) obtained after centrifugation (10,000*g*, 10 min) of thawed sperm samples were utilized as diluents for spontaneous motility observation.

For fresh sperm 20 μl AM was placed on a glass slide under a microscope. Sperm was added to the AM or CS using the tip of dissecting needle, whereafter the sperm suspensions were thoroughly mixed for 2 s. The same activation procedure was used for frozen-thawed sperm samples after 0, 5 and 10 min of post-thaw storage at 0 °C.

Directly after dilution (with AM or CS), motility was immediately recorded for 1 min post-activation using a CCD video camera (Sony, SSCDC50AP) mounted on a dark-field microscope (Olympus BX50, 200×) and illuminated with a stroboscopic lamp (Chadwick-Helmut, 9630, USA) set to a flash frequency of 50 Hz.

Video recordings were captured using a video recorder (Sony SVHS, SVO-9500 MDP, Japan), and analyzed to estimate average velocity (μm/s) and percentage of sperm motility. Average sperm velocity and percentage of sperm motility were calculated at 10, 30 and 45 s post-activation by analyzing five successive frames using a micro-image analyzer (Olympus Micro-Image 4.0.1. for Windows). Ten to 50 spermatozoa were evaluated from each frame. Spermatozoa with velocity less then 3 μm/s were considered as immotile and therefore excluded for further analysis [19].

Evaluation of ATP content

Sperm samples were added to a boiling extraction medium consisting of 100 mM Tris–HCl, pH 7.75 and 4 mM EDTA. After boiling for 2 min at 100 °C samples of the sperm suspension were centrifuged at 15,000*g* for 15 min using a Heraeus Model 400-R centrifuge (Hanau, Germany). Supernatants were pipetted into 2 ml sterile vials, and kept at −18 °C before analyses. ATP content in the supernatants was evaluated by bioluminescence, using a Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany). Luminescence was read with a multifunctional microplate reader Infinite M200 (Tecan, Austria). ATP content was expressed as nmol ATP/10^9 spermatozoa.

ATP content was measured after the following experimental procedures: (1) non-activated fresh sperm (2 μl of sperm was added to 5 ml of boiling extraction medium), (2) fresh, hypotonically activated sperm within the motility phase (20 μl of fresh sperm were activated with 5 ml of AM at 10, 20, 40, 60, 120, 300 s post-activation, and 0.5 ml of sperm suspension were added to 5 ml boiling extraction medium), (3) thawed sperm at different storage times (4 μl of thawed sperm was added to 5 ml of boiling media at 25, 45, 75, 120, 300, 600 s post-thaw, with thawing sperm stored on ice), and (4) fresh sperm diluted with cryoprotective medium (fresh sperm diluted with cryoprotective medium in a 1:1 ratio. Four microliters of this suspension was added to 5 ml of boiling media immediately and at 20 min after dilution).

Freezing and thawing procedures

Sperm from 10 individual males were frozen. Before freezing sperm were diluted (1:1) in an extender composed of the following: 59 mM NaCl, 6.3 mM KCl, 0.68 mM $CaCl_2$, 2.1 mM Mg_2SO_4, 27 mM $NaHCO_3$, 3.4 mM sucrose, 69 mM D-manitol, 118 mM Tris–HCl pH 8.1, 16% ethyleneglycol [8], modified). After dilution sperm samples were loaded into 0.5 ml straws (CRYO-VET, France). The straws were placed horizontally onto a 3 cm high styroframe raft, which was floating on the surface of liquid nitrogen (temperature of styroframe surface was about −130 °C). Straws were left on the raft for 20 min before being plunged into liquid nitrogen. After 10 days of storage in liquid nitrogen, samples were thawed in a 40 °C water bath for 6 s. The changes of temperature inside the straw during freezing and thawing were recorded with a copper constantan thermocouple connected to a calibrated analog recorder (KSP-4, Russia).

Hatching of embryos used in experiments

To assess hatching rates the pooled ova from three females were used. Egg samples (2 g) were inseminated in a dish with either (1) fresh sperm, (2) frozen-thawed sperm immediately after thawing, (3) frozen-thawed sperm stored 5 min after thawing, or (4) frozen-thawed sperm that had been stored for 10 min after thawing. Eggs were fertilized at a sperm/ova ratio of 10^5:1. Dishes were placed on an orbital agitator (200 rpm, 10 mm deflection). Gametes were activated with 10 ml of hatchery water, and after 1 min samples of ova were transferred to three Petri dishes. Approximately 100–200 eggs were placed into each dish and incubated with aerated, dechlorinated and UV-sterilized tap water (20–21 °C, 9 mg l^{-1} O_2).

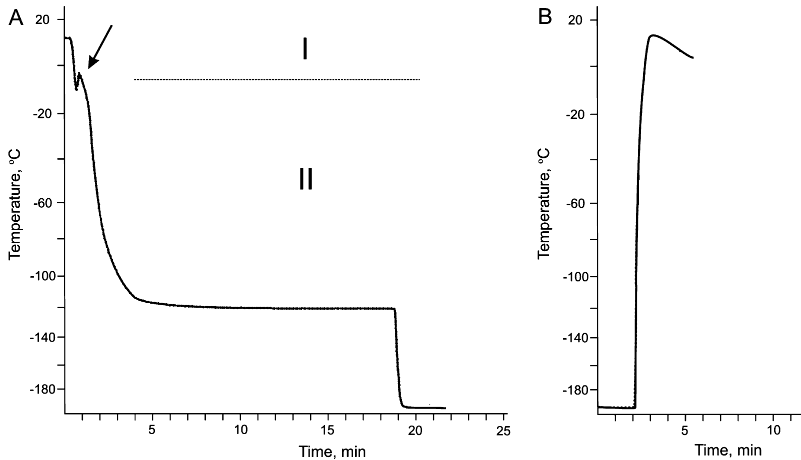

Fig. 1. Examples of thermograms of freezing (A) and thawing (B) regimes used in experiments. I—Cooling down to initial water crystallization (arrow shows the supercooling areas), II—cooling after initial water crystallization.

Living and dead eggs were counted in each Petri dish during incubation and dead eggs removed. Hatched fry were counted after 5 days of incubation at 20–21 °C. The hatching rate was calculated as the ratio of hatched larvae to the initial number of eggs incubated.

Data presentation and statistical analysis

Normality and homogeneity of dispersions in studied values were tested using Shapiro–Wilk's and Levene tests. Because of the presence of abnormal data distribution or the absence of homogeneity, we used non-parametric methods for the comparisons of: (1) motility percentage for carp sperm samples at different post-activation time before and after cryopreservation, (2) ATP content in samples of carp sperm before and after cryopreservation, and (3) hatching rate of carp embryos obtained with sperm stored at different time periods after thawing. For the above the Kruskal–Wallis ANOVA was used with consideration of the significance of differences between the groups by means of Mann–Whitney *U*-test with Bonferroni correction ($p < 0.01$ was considered to be significant). Values for median, minimal, maximal, 25 and 75 percentiles were presented as a box-wicker plot.

The percentages of carp spermatozoa motility after freeze-thawing and values of sperm velocity before and after cryopreservation were normally distributed with similar dispersion. For these two parameters the parametric ANOVA with subsequent post hoc Tukey's honest significant difference (HSD) test was applied ($p < 0.01$ were considered to be significant). Mean values (Mean), standard deviation (SD), standard error of a mean value (SEM) or 95% confidence interval are presented in figures. All analysis was performed using STATISTICA V8.0 software (Statsoft Inc., USA).

Results

Freeze-thawing rates

Thermograms were prepared showing the freezing-thawing rates for the protocol used. The calculated maximal rate ($n = 3$) for freezing was 100 °C/min before initial water crystallization (Fig. 1A, I) and 70 °C/min after initial crystallization (Fig. 1A, II). The thawing rate was considerably higher in the range 2000 °C/min (Fig. 1B).

Osmolality measurements

Mean (±SD; $n = 10$) osmolality of seminal fluid and CS samples were 272 ± 6 mOsm/kg and 1798 ± 39 mOsm/kg, respectively.

Motility of carp sperm samples before and after cryopreservation

Motility percentage of fresh hypotonically activated spermatozoa

The median value of percentage of hypotonically activated fresh sperm motility at 10 s post-activation was 100% (90% and 100% values for 25 and 75 percentiles, respectively). Median values for spermatozoa motility was significantly decreased (Mann–Whitney *U*-test, $p < 0.01$, $n = 10$) to 80% (65% and 95% values for 25 and 75 percentiles, respectively, Fig. 2, I) at 45 s post-activation time.

Influence of post-thaw storage time on hypotonic motility activation

Cryopreservation procedure significantly decreased (Mann–Whitney *U*-test, $p < 0.01$, $n = 10$) the median value for the percentage of spermatozoa motility at 10 s post-activation in AM from 100% (fresh sperm, Fig. 2, I) to 25% (21% and 36% values for 25 and 75 percentiles, respectively) for sperm immediately after thawing (Fig. 2, II). A significant effect (Mann–Whitney *U*-test, $p < 0.01$, $n = 10$) of storage after sperm thawing was found. In sperm stored for 10 min, the median value for spermatozoa motility decreased to 16% (3% and 16% for 25 and 75 percentiles, respectively, Fig. 2, II and IV). Five minutes post -thaw storage did not effect sperm motility (Fig. 2, II and III).

Spontaneous motility percentage

Just after thawing and following dilution in CS motile spermatozoa were detected, whereas no motility was observed in this medium after 5 and 10 min of storage. Before cryopreservation no motility was found using fresh sperm dilution with CS or cryoprotective media.

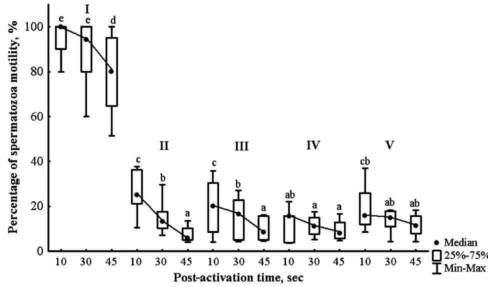

Fig. 2. Percentage of spermatozoa motility in hypotonically and spontaneously activated sperm samples. I–IV—Hypotonically activated sperm motility, V—spontaneously activated sperm motility. I—Motility percentage for fresh sperm samples, activating medium in AM; II—motility percentage for sperm samples after freeze-thawing, time of post-thaw storage is 0 min, activating medium is AM; III—motility percentage for sperm samples after freeze-thawing, time of post-thaw storage is 5 min, activating media in AM; IV—motility percentage for sperm samples after freeze-thawing, time of post-thaw storage is 10 min, activating media is AM; V—motility percentage for spontaneously activated sperm samples, time of post-thaw storage is 0 min. Min–max are minimum and maximum values, 25—75% are 25% and 75% percentiles values. Values connected with solid lines belong to the variants I–V. Values with the same superscript are not significantly different (Mann–Whitney *U*-test, $p > 0.01$, $n = 10$).

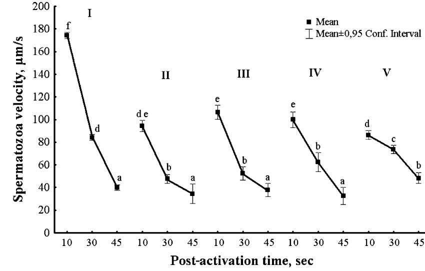

Fig. 3. Sperm velocity in hypotonically and spontaneously activated sperm samples. I–IV—Hypotonically activated sperm motility, V—spontaneously activated sperm motility. I—Average spermatozoa velocity values for fresh sperm samples, activating medium is AM; II—average spermatozoa velocity values for sperm samples after freeze-thawing, activating medium is AM, time of post-thaw storage is 0 min; III—average spermatozoa velocity values for sperm samples after freeze-thawing, activating medium is AM, time of post-thaw storage is 5 min; IV—average spermatozoa velocity values for sperm samples after freeze-thawing, activating medium is AM, time of post-thaw storage is 10 min; V—motility percentage for spontaneously activated sperm samples, time of post-thaw storage is 0 min. Values connected with solid lines belong to the variants I–V. Values with different superscript are significantly different (HSD test, $p \leqslant 0.01$, $n = 10$).

Freeze-thawing procedure activated spermatozoa motility, with a median value of 16% (12% and 26% values for 25 and 75 percentiles, respectively) for percentage of spermatozoa motility. The value for percentage of spermatozoa motility activated by freeze-thawing (spontaneous motility) was significantly lower (Mann–Whitney *U*-test, $p < 0.01$, $n = 10$) compared to median values for fresh sperm samples activated in AM (Fig. 2, I and V).

Carp sperm velocity before and after freeze-thawing

Spermatozoa velocity (ASV) significantly decreases (Tukey HSD test, $p < 0.05$, $n = 10$) within period of motility after both hypotonic (Fig. 3, I–IV) and spontaneous activations (Fig. 3, V). Cryopreservation procedure significantly decreased velocity at 10 s post-activation. With hypotonic activation a decrease from 175 ± 3 µm/s in fresh sperm (mean ± 95% confidence interval) (Fig. 3, I) to 95 ± 5 µm/s in samples activated just after thawing (Fig. 3, II) was found. Post-thaw storage time had no influence on velocity of hypotonically activated spermatozoa (107 ± 6 µm/s and 100 ± 6 µm/s in samples stored 5 and 10 min after thawing, respectively) (Fig. 3, III and IV). The mean ASV for spontaneously activated spermatozoa at 10 s after dilution with CS was significantly lower (88 ± 6 µm/s) (Fig. 3, V) than in hypotonically activated fresh sperm (Fig. 3, I). No significant difference was found between mean ASV in hypotonically activated just after thawing and spontaneously activated sperm, whereas the mean ASW of hypotonically activated sperm after 5 and 10 minutes storage (Fig. 3, III and IV) was significantly higher than in spontaneously activated sperm (Fig. 3, V).

ATP content in samples of carp sperm before and after cryopreservation

The initial level of total ATP content was found as 45 nmol ATP/10^9 spermatozoa. For fresh sperm the ATP level at 60 s after spermatozoa activation was 22 nmol ATP/10^9. After 60 s post-activation the ATP content remained constant and no significant differences were found compared to the minimal level of ATP at 300 s post-activation (13 nmol ATP/10^9) (Fig. 4A). The incubation of fresh sperm in cryoprotectants did not result in significant

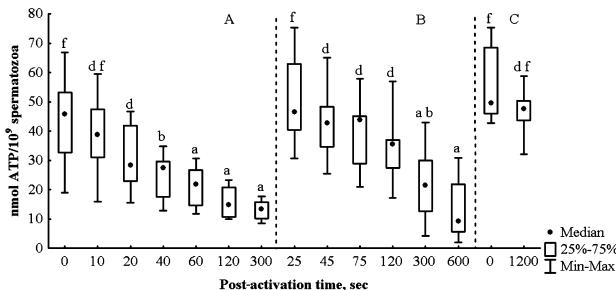

Fig. 4. ATP content in hypotonically and spontaneously activated sperm samples. (A) Fresh sperm activated AM (hypotonic activation), (B) after freeze-thawing, without activation in AM (after spontaneous motility activation), (C) after fresh sperm dilution in cryoprotective media. Min–max are minimum and maximum values, 25–75% are 25% and 75% percentiles values (down and up box side correspondingly). Values with the same superscript are not significantly different ($p > 0.01$, Mann–Whitney test, $n = 10$). *Note.* In (A) and (B) motility was initiated, while in (C) no motility was observed.

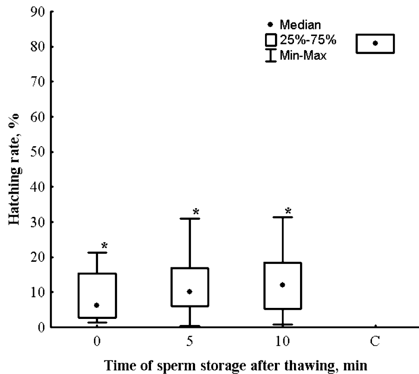

Fig. 5. Hatching rate of carp embryos after ova fertilization with fresh and thawed sperm stored at 0, 5 and 10 min of post-thaw time, activated with AM. Min–max are minimum and maximum values, 25–75% are 25% and 75% percentiles values. *Significant difference ($p \leqslant 0.01$, Mann–Whitney test, $n = 10$) in comparison with control, obtained with fresh sperm (C).

changes in ATP level at 20 min of incubation (Fig. 4C). ATP content significantly decreases ($p < 0.01$, Mann–Whitney test, $n = 10$) with time post-activation in all investigated samples (Fig. 4).

After freeze-thawing the ATP median level spontaneously decreased and reached a level of 36 nmol ATP/10^9 at 120 s post-activation. This level was significantly lower (Mann–Whitney test, $p < 0.01$, $n = 10$) compared to the initial value, but significantly higher than the ATP level in fresh sperm after 40 s post-activation in AM (Fig. 4 A and B).

Hatching rates control

Hatching rates for the control (78–83% min–max values) demonstrated the high quality of sperm and eggs used in the experiment (Fig. 5). Hatching rates for the experimental groups were significantly lower compared to the control, but insignificant differences were found among experimental groups (Mann–Whitney test, $p > 0.01$, $n = 10$, Fig. 5).

Discussion

The phenomenon of spontaneous sperm motility activation

The appearance of sperm motility in CS conclusively demonstrates the phenomenon of spontaneous activation of carp spermatozoa due to freeze-thawing. It is widely accepted that in cyprinids sperm motility can be achieved by decreasing osmotic pressure [17,3]). The sequence of events on osmotic pressure changes can be found in the model of cryodamage by "slow" freezing regimes [14]. This model has recently been accepted by scientific community [15,18,4]. According to this model, during cooling procedure the cells enter hypertonic conditions, which have resulted from the water freezing. During thawing the cells entered the conditions of decreasing osmotic pressure arising from the process of water crystals melting. With integrating both knowledge about carp sperm motility activation and osmotic pressure changes during freeze-thawing, we can speculate about the basis of the phenomenon of spontaneous motility activation after freeze-thawing.

We postulate that the mechanism of spontaneous activation could be similar to hypotonic activation. In both the decrease of environment osmolality could be the motility initiating factor. However, with spontaneous activation the event of osmolality decrease is difficult to measure because it appears during thawing. We suppose that immediately after quick thawing (in the procedures used the rate of thawing was significantly higher than cooling rate; see Fig. 1) the osmotic pressure inside the sperm is higher than in the environment (CS), resulting in the start of sperm motility. The following equilibration in osmotic pressure outside and inside of sperm to the level of CS (about 1800 mOsm/kg) leads to cessation of motility, but spermatozoa posses the possibility for hypotonic activation by AM. This could explain why, during post-thaw storage, the spontaneously activated spermatozoa motility ceases, while keeping the possibility for hypotonic activation for longer periods (at least 10 min in our experiment). More discussion of this possibility is provided in the section on "Carp sperm post-thaw motility parameters."

The cryoprotective media and freeze-thawing regime used in our study were proposed by several authors [8,5,6], and provided satisfactory results in post-thaw fertilization when hypotonic conditions for fertilization were used. Thus, it might be possible that the phenomenon of spontaneous motility activation could not be the limiting factor for cryopreserved sperm used.

Carp sperm post-thaw motility parameters

From the measurements of motility percentage in spontaneously and hypotonically activated sperm (Fig. 2), we concluded that cryopreservation led to cryodamages in part of the spermatozoa. Non-damaged cells were activated by freeze-thawing, and could be reactivated with activation solution with a lower osmolality. Re-activation of spermatozoa motility can be achieved by means of different experimental conditions [16,13,21], but hypotonic (less than 300 mOsm/kg) condition always was required for activation of carp spermatozoa motility. In our study we demonstrate, that this activation is possible in condition of extremely high osmolality of CS (1800 mOsm/kg). Moreover, so high osmolality is not the limiting factor for keeping quite long lasting spermatozoa motility as well as the potential for a second activation.

The reduced velocity during the spermatozoa movement period is a well known fact [3], as well as the decrease of spermatozoa velocity in AM activation solution caused by freeze-thawing [11,20]. Therefore, our results (Fig. 3) are in good agreement with previously reported results. However it can be hypothetized that, during movement activation by freeze-thawing, suppression of movement velocity is manifested to a lesser extent when using AM solution (Fig. 3). This could probably be related to different osmotic conditions for spermatozoa motility. With spermatozoa activation caused by freeze-thawing the sperm are in an environment of high osmotic pressure, even at the end of motility phase. That is why hypotonic cell swelling is impossible in condition of spontaneous activation, while activation in AM leads to hypotonic swelling, which could be the reason for the sudden ending of motility [17,3]).

ATP content on different stages of freeze-thawing in carp spermatozoa

Our results on ATP content dynamics (Fig. 4A) are in agreement with Perchec et al. [16]. In our study, we found that the freeze-thawing led to an ATP content reduction compared to sperm diluted with cryoprotective media and stored without freezing (Fig. 4B and C). This decrease could probable contributed to spontaneous motility activation. However, the reduction of total ATP content could be associated with ATP consumption by the processes of spermatozoa cellular volume changes or/and ATP hydrolysis in damaged cells. Thus, explicit interpretation of ATP decrease is complex due to the diverse pathways of ATP consumption. We

speculate that the drastic decrease in ATP content during post-thaw time could reflect the process associated with two kind of cellular events. One of them is motility of spontaneously activated (not cryodamaged) sperm and second one is processes of ATP hydrolysis in damaged sperm. Future studies of ATP distribution among live and damaged sperm with cryopreservation are required.

Post-thaw fertilizing ability of carp sperm

A low egg/sperm ratio has been used in the current study in order to avoid the possible masking among the effects of cryopreservation on carp sperm and their post-thaw fertilizing ability. The necessity to use this methodology has been described previously [12]. However, we did not find a negative influence of storage within 10 min after thawing. We assume that, even after a decrease of percentage motility with activation by AM solution (on 10th minute of post-thaw storage), the motile spermatozoa possess a high level of velocity, and are still able to fertilize.

Conclusion

The freeze-thawing procedure used in the present study results in spontaneous activation of spermatozoa motility. This activation, being an initial event of sperm thawing, did not lead to loss of fertilizing ability of sperm after motility ended. This was evident from obtaining the second phase of motility activation by sperm dilution in hypotonic media. Sperm freeze-thawing leads to a decrease of total ATP level, whereas fertilizing ability of spermatozoa remains stable within a 10 min post-thaw storage period. We assume that there is an ATP distribution among undamaged and cells damaged by the freeze-thawing processes. That might be the reason why, even after a drastic total ATP level decrease, part of the total amount of spermatozoa could preserve the intracellular ATP, which is essential for motility activation. Future studies should be focused on the possibility to avoid the motility activation of carp sperm caused by freeze-thawing in order to obtain maximum fertilization rate with thawed sperm.

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CHAPTER 5

SPONTANEOUS ACTIVATION OF SPERMATOZOA MOTILITY BY ROUTINE FREEZE-THAWING IN DIFFERENT FISH SPECIES

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Spontaneous activation of spermatozoa motility by routine freeze-thawing in different fish species

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Summary

In the present study, we investigate the possibility of spermatozoa motility activation resulted from freeze-thawing, defined by us as spontaneous sperm activation (SSA) in common carp (*Cyprinus carpio* L.), Eurasian perch (*Perca fluviatilis* L.), rainbow trout (*Oncorhynchus mykiss*), Northern pike (*Esox lucius* L.) and sterlet (*Acipenser ruthenus* L.) using cryopreservation methods described previously. To evaluate that, the motility percentage and velocity of frozen-thawed spermatozoa before and after dilution with activating media were used. The SSA phenomenon was found in carp and perch, but not in pike, rainbow trout and sterlet. The motility and velocity of spermatozoa for carp SSA were characterized by 16% (median value) and velocity 98 ± 2 μm s^{-1} (mean ± 0.95 CI), for perch 5% and 53 ± 5 μm s^{-1}, correspondingly. Significant decrease of sperm velocity was observed, when activation media were applied for thawed sperm in carp, perch, trout and pike, while for sterlet this parameter became significantly increased. We believe that the phenomena of spontaneous activation by freeze-thawing processes are species-specific ones.

Introduction

It is well known, that fish spermatozoa commonly immotile in seminal fluid, require environment changes to start motility. While distinct sequences of cytological events laying in the base of motility initiation are still unknown, we believe, that environment osmolality and ion composition are key points in this process (Cosson, 2004). It was shown, that for some freshwater fish the environment osmolality is the main motility inhibiting factor (for review see Alavi and Cosson, 2006). It is case for example for carp (Morisawa et al., 1983; Perchec-Poupard et al., 1997), perch (Lahnsteiner et al., 1995; Alavi et al., 2007) and pike (Babiak et al., 1999; Alavi et al., 2010). While for sturgeons and salmons the sperm motility initiation is under strong control of environment ionic composition. As an example here the phenomenon of inhibitory effect of K^+ in different species from these taxa could be mentioned (Gallis et al., 1991; Takai and Morisawa, 1995, Cosson et al., 1999).

Sperm cryopreservation as a tool for enhancing the fish farming effectiveness and rare fish species conservation is an extensively developing field of biology (Billard, 2001). Modern procedures of sperm freeze-thawing are quite empirically developed and for elaboration of more effective methods the study of basic changes occurring in sperm during freeze-thawing is required. Freeze-thawing leads to decrease of sperm fertility, which is associated with decreasing of sperm velocity and motility percentage. The reasons for the decrease in sperm velocity still are not clear and we hypothetize that elucidation of its mechanisms could be valuable for the development of new methods.

Water freezing occurring during cooling of cell suspension leads to changes of cellular environment. Among them are quite well known the increase of osmolality (Mazur, 1984) and pH change (for review see Fuller and Paynter, 2004; Petrunkina, 2007). However, direct influence of these processes on fish sperm is purely studied. We hypothesized that environment changes caused by freeze-thawing could themselves activate fish sperm motility. Furthermore this spontaneous activation could be species-specific and as a result, could be one of the factors, which influence the effectiveness of thawed sperm use. To check this hypothesis we investigated the presence of motility and velocity in thawed sperm before dilution with activating media in fish species possessing different sperm activation mechanisms. Perch, carp and pike were selected as the models characterized by motility activation due to osmotic pressure decrease, while sterlet and rainbow trout were selected as the ones of ion sensitive motility activation.

Materials and methods

Fish

Eurasian perch (*Perca fluviatilis*), common carp (*Cyprinus carpio*), Northern pike (*Esox lucius*), rainbow trout (*Oncorhynchus mykiss*) and sterlet (*Acipenser ruthenus*) were used in our experiments.

Males of Eurasian perch, common carp, Northern pike and sterlet, were kept during natural spawning season in 4 m^3 plastic tanks with constant pond water flow with the rate of 20 L min^{-1} located at the hatchery of the Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Males of rainbow trout were kept in ponds of the fish farm Anin, Klatovske Rybarstvi, Czech Republic. For all the species examined the hydrochemical parameters were the same as the ones used in aquaculture practice.

Gametes collection

Males of carp and sterlet were intramuscularly treated with carp pituitary extract at 1 and 4 mg kg^{-1} correspondingly, 24 h before stripping. Males of perch, rainbow trout and pike were not hormonally treated before sperm sampling. Sperm

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from carp (n = 10), perch (n = 5), sterlet (n = 5) and rainbow trout (n = 7) was obtained by abdominal massage and collected directly into plastic syringes, taking special care to avoid contamination with urine, mucus, faeces or water.

As soon as in pike the sufficient amount of sperm was not available using abdominal stripping, thus gonads were removed from body cavity and blood was eliminated. Later, gonads were transferred into dry Petri dish for 0.5 h on ice. Sperm leaking from gonads were collected from Petri dish and used in experiments (n = 3).

Sperm cryopreservation

We used the methods of cryopreservation described previously: sperm were diluted with cryoprotective medium (CM) and frozen in 0.5 ml straws, 3 cm above liquid nitrogen level for 20 min, with following plunging into liquid nitrogen. Straws were thawed in a water bath (6 s, 40°C). The composition of the cryoprotective medium, dilution rates, cryoprotectants used and its final concentration after dilution are presented in Table 1.

Evaluation of motility percentage and velocity

Sperm was diluted from 1 : 50 to 1 : 2000 (depending on species) with activating medium (AM) or supernatants obtained after centrifugation (10 000 ***g***, 10 min) of thawed sperm samples defined as cryosupernatant (CS). The application of CS was necessary to decrease sperm density for thawed sperm motility observation without changes of sperm environment. Composition of AM: for carp – 45 mm NaCl, 5 mm KCl, 30 mm Tris, pH 8.2; for perch – 90 mm glucose; for pike – 100 mm NaCl, 10 mm Tris, pH 9.1; for rainbow trout – 125 mm NaCl, 20 mm Tris, 30 mm Glycine, pH 9 and for sterlet –1 0 mm NaCl, 1 mm $CaCl_2$, 10 mm Tris pH 8.5.

Directly after dilution of sperm with AM or CS motility was immediately recorded until 1–2 min post-activation using a CCD video camera (Sony, SSCDC50AP,) mounted on a dark-field microscope (Olympus BX50, ×200) and illuminated with a stroboscopic lamp (Chadwick- Helmut, 9630, USA) set to a flash frequency of 50 Hz.

Video recordings were performed by video recorder (Sony SVHS, SVO-9500 MDP) Video records were analyzed to estimate spermatozoa average velocity ($\mu m\ s^{-1}$) and percentage of motile cells ⁄ motility. To compute the average sperm velocity and percentage of motile cells at different post-activation time, five successive frames were analyzed by microimage analyzer (Olympus Micro Image 4.0.1. for Windows). All spermatozoa were evaluated from each frame (20–70 cells per frame were clearly visible) and spermatozoa with velocity $< 3\ \mu m\ s^{-1}$ were considered as immotile and were omitted from calculation of motile spermatozoa (Rodina et al., 2008). Each experimental point is a result of processing the data of not < 140 cells.

Data presentation and statistical analysis

The distribution characters and homogeneity of dispersion in the groups were evaluated using Shapiro-Wilk's and Levene tests, correspondingly. Because of the presence of abnormal data distribution or the absence of homogeneity of dispersions determined, we used the non-parametrical methods for the comparison of motility percentage. For this parameter Kruskal-Wallis test was used with following consideration of the significance of the differences between the groups by means of Mann–Whitney *U*-test ($P < 0.05$ were considered to be significant). The medians with minimum and maximum values were presented in figures for motility percentage.

The values of sperm velocity before and after cryopreservation were normally distributed with similar dispersion value. For this parameter the mean values with 95% CI were presented in figures. To compare these parameters the Tukey's honest significant difference (HSD) test was applied ($P < 0.05$ were considered to be significant). To visualize sperm velocities values, individual data points were presented on scatterplots by markers in two-dimensional space, where the axes represent the post-activation time (X) and velocities of sperm before and after cryopreservation (Y) together with linear fitting lines. All analyses and plotting were performed using Statistica V8.0 (Statsoft Inc, USA).

Results

Freeze-thawing leads to spontaneous sperm activation (SSA) in carp and perch, but this phenomenon was not observed in trout, pike and sterlet.

Sperm velocities and motility percentage in species possessing spontaneous motility activation

Cryopreservation led to a decrease of motility percentage in carp and perch (Fig. 1a,b). Significant decrease of median value from 96% before freezing to 24% in condition of HSA after freeze-thawing was observed in carp. No significant difference (Mann–Whitney *U*-test, $P > 0.05$) was determined for SSA (16%) in comparison with HSA (24%) after thawing

Table 1
Composition of the cryoprotective media, dilution rates and cryoprotectant final concentration used in the experiments

Species	CM composition	Dilution rate	Cryoprotectant, final concentration	Reference
Cyprinus carpio	59 mm NaCl, 6.3 mm KCl, 0.68 mm $CaCl_2$, 2.1 mm Mg_2SO_4, 27 mm $NaHCO_3$, 3.4 mm sucrose, 69 mm D-mannitol, 118 mm Tris–HCl, pH 8.1	1 : 1	Ethylene glycol, 8%	Kopeika (1986)
Acipenser ruthenus	30 mm sucrose, 1 mm KCl, 25 mm Tris–HCl, pH 8.5	1 : 1	Methanol, 5%	Glogowski et al. (2002)
Perca fluviatilis	300 mm glucose	1 : 6	Methanol, 9%	Rodina et al. (2008)
Esox lucius	600 mm sucrose	1 : 3	DMSO, 11 255%	Babiak et al. (1999)
Oncorhynchus mykiss	300 mm glucose, 10% egg yolk	1 : 1	DMSO, 5%	Babiak et al. (2001)

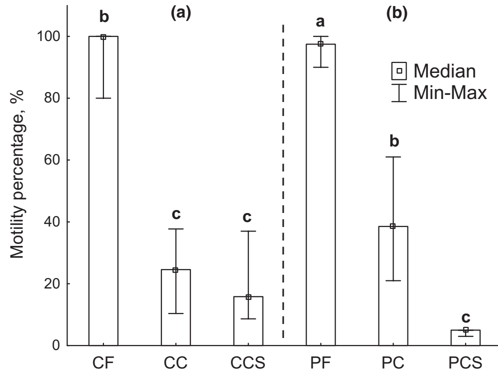


Fig. 1. Motility percentage before and after freeze-thawing in carp (a) and perch (b). CF, carp fresh sperm, hypotonic activation before freezing; CC, carp sperm hypotonic activation after cryopreservation; CCS, carp sperm spontaneous activation; PF, perch fresh sperm hypotonic activation before freezing; PC, perch sperm hypotonic activation after cryopreservation; PCS, perch sperm spontaneous activation. The values with the same superscript are not significantly different (Mann–Whitney *U*-test, P > 0.05). Median, minimal and maximal values (Min-Max) are presented

(Fig. 1a). In perch, cryopreservation resulted in a significant decrease of median value of motility percentage during HSA from 95% in fresh to 39% in thawed samples and SSA motility percentage (5%) was significantly lower than in HSA (Fig. 1b).

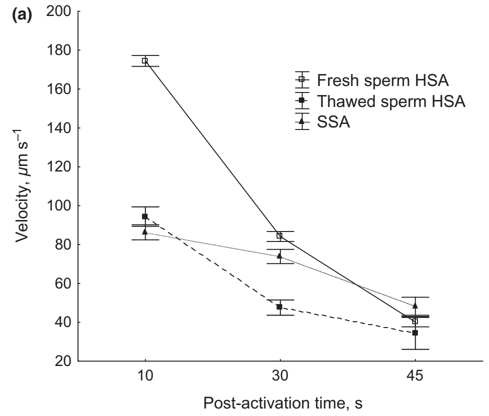
The average carp spermatozoa velocity significantly decreased (Tukey HSD test, P < 0.05) within the period of motility after both HSA and SSA (Fig. 2a,b). Cryopreservation procedure significantly decreased the mean value of velocity at 10 s post-activation for carp HSA from 175 ± 3 μm s^{-1} in fresh sperm (mean ± 95% CI) to 95 ± 5 μm s^{-1} in the samples activated after thawing. The mean velocity for SSA at 10 s of post activation was significantly lower (88 ± 6 μm s^{-1}) than in HSA of fresh sperm. No significant differences were found for sperm velocities in the case of SSA and HSA in thawed sperm at 10 s of post-activation, while at 30 and 45 s of motility the value of mean velocity in case SSA was significantly higher in comparison with HSA. The use of whiskers, representing 0.95 CI in the figure, allowed visualizing the statistical differences between the compared groups. Non-overlapping whiskers corresponded to the statistical differences at P < 0.05 and this significance was additionally proved by Tukey's honest significant difference (HSD) test.

In perch the significant reduction of mean velocity from 218 ± 6 μm s^{-1} at fresh sperm HSA down to 117 ± 4 μm s^{-1} at thawed sperm HSA and 52 ± 6 μm s^{-1} at SSA was observed at 10 s of post activation (Fig. 3).

Sperm velocities and motility percentage in species, without spontaneous motility activation

Since in pike, rainbow trout and sterlet no SSA was observed, measurements of motility percentages and sperm velocities were performed after sperm dilution using activation medium.

Sperm activation led to a significant decrease of motility percentage median values (Mann–Whitney *U*-test, P < 0.05) from 100% in fresh sperm to 25, 32 and 29% motility percentage in thawed sperm samples of pike, rainbow trout and sterlet, correspondingly (Fig. 3a–c).



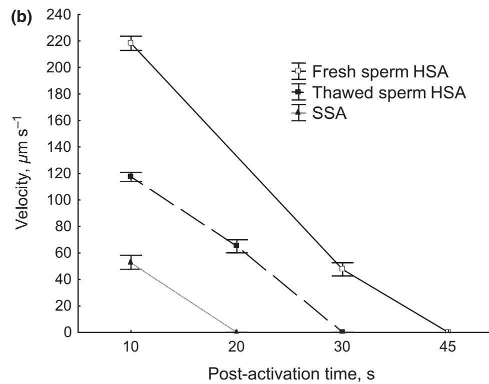


Fig. 2. Spermatozoa velocities during spontaneous and hypotonic activations in carp (a) and perch (b) before and after freeze-thawing. SSA, spontaneous sperm activation; fresh sperm HSA, hypotonic activation of sperm before freezing; thawed sperm HSA, hypotonic activation (dilution with AM) of thawed sperm. Results are presented as mean ± 0.95 CI (Values with not overleaping whiskers are significantly different, HSD-test, P < 0.05)

In pike the reduction of mean sperm velocity within motility period of fresh sperm was found and significant differences among velocities at 10, 30 and 50 s post-thaw motility were observed. After thawing mean velocity remained insignificantly changed up to 70 s of post-thaw motility (Fig. 4a).A significant reduction of velocities from 178 ± 8 μm s^{-1} in fresh sperm to 130 ± 10 μm s^{-1} at 10 s of post activation in sperm after thawing was found.

In rainbow trout the mean sperm velocity after activation of thawed sperm was significantly lower in comparison with fresh sperm. The values of mean velocities in fresh sperm were 255 ± 6, 178 ± 4 and 115 ± 3 μm s^{-1} at 5, 10 and 20 s post activation, while for thawed sperm this parameter was significantly lower (135 ± 5, 104 ± 5 and 63 ± 7 μm s^{-1}, correspondingly, Fig. 4b).

In sterlet sperm freeze-thawing leed to a significant increase of mean velocity from 162 ± 4 μm s^{-1} in fresh sperm to 190 ± 8 μm s^{-1} in thawed sperm at 10 s post activation (Fig. 4c). At 120 s post-activation, thawed sperm mean velocity value became significantly lower in comparison with fresh sperm (106 ± 7 and 123 ± 4 μm s^{-1}, correspondingly).

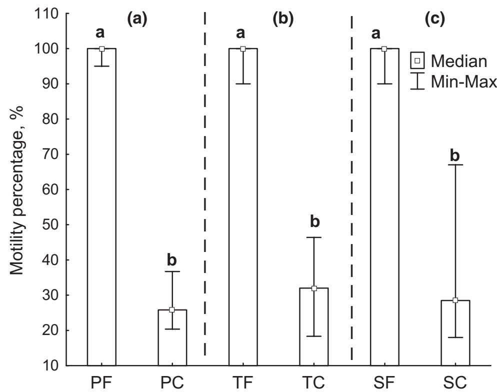


Fig. 3. Motility percentage in pike (a), rainbow trout (b) and sterlet (c) sperm before and after cryopreservation. PF,PC, pike fresh and thawed sperm, correspondingly; TF,TC, rainbow trout fresh and thawed sperm, correspondingly; SF,SC, sterlet fresh and thawed sperm, correspondingly. The values with the same superscript are not significantly different (Mann–Whitney *U*-test, P > 0.05). Median, minimal and maximal values (Min-Max) are presented

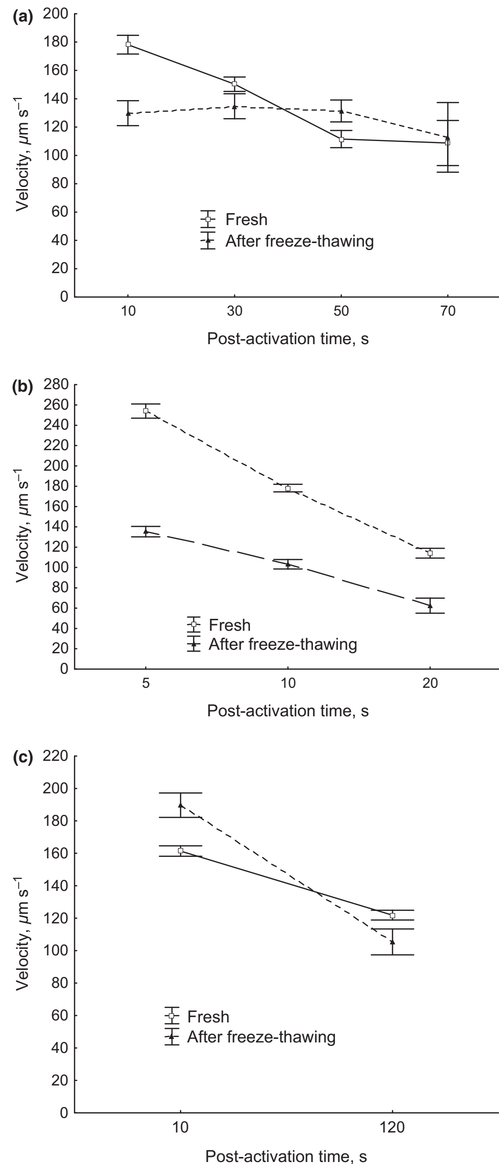


Fig. 4. Spermatozoa velocities during hypotonic activations in pike (a), rainbow trout (b) and sterlet (c) before and after freeze-thawing. Results are presented as mean ± 0.95 CI (Values with not overleaping whiskers are significantly different, HSD-test, P < 0.05)

Discussion

The findings on the existence of sperm motility activation by freeze-thawing in carp and perch showed that this phenomenon is species-specific if compared with pike, trout and sterlet, that was demonstrated by us for the first time. Nowadays the widely accepted fact is that in cyprinids the sperm motility can be achieved by osmotic pressure decrease (Perchec-Poupard et al., 1997; Cosson, 2004). The sequence of events on osmotic pressure changes could be found in model of cryodamages by 'slow' freezing regimens (for review see Muldrew et al., 2004; Petrunkina, 2007). According to this model, during cooling the cells entered hypertonic conditions, resulted from water freezing-out. During thawing the cells entered the conditions of decreasing of osmotic pressure, arising during water crystal melting. We suppose that exactly these processes are the reason of sperm motility onset in our species. Thus, the activation mechanism of spermatozoa motility by freeze-thawing could be similar to motility initiation in activation solution. In both cases the decrease of environment osmolality could be a motility initiating factor. Namely these processes could be the reason of sperm motility activation in carp, in which spermatozoa are characterized by presence of mechano-sensitive membrane channels responsible for motility initiation (Krasznai et al., 2003). Further we speculate that fish spermatozoa possess species-specific sensitivity to osmotic pressure changes. That is why the phenomenon of motility activation by freeze-thawing existing in carp and perch is not observed in pike, possessing the same mode of motility activation.

In species possessing ion-dependent mode of sperm activation (sturgeon and rainbow trout) freeze-thawing did not lead to sperm motility acquisition. In case of sterlet, possibly, this phenomenon could be suppressed by the presence of potassium ions in the cryoprotective medium under concentrations being quite high to inhibit motility (Cosson, 2004).

We studied the changes in sperm velocity after thawing in relation to the presence of spontaneous sperm activation. The absence of differences between motility percentage (Fig. 1a) and velocity (Fig. 2a) in SSA and HSA for carp thawed sperm suggested that in carp all the spermatozoa that possesss the ability for motility acquisition after cryopreservation are subjected to SSA. In perch freeze-thawing led to SSA just in part of sperm, since we were able to activate more cells under hypotonic conditions (Fig. 1b). In addition, this small part of activated sperm started motility with lower velocity (Fig. 2b).

The reduction of velocity during the spermatozoa movement period is a well known fact, as well as the decrease of

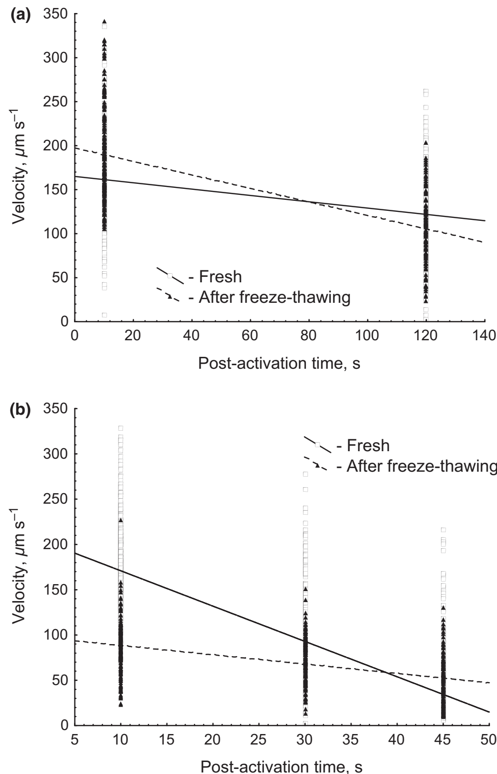


Fig. 5. Scatter plots of sperm velocities during post-activation time in sterlet (a) and carp (b) before and after cryopreservation

spermatozoa velocity and motility percentage both caused by freeze-thawing (Lahnsteiner et al., 2000; Linhart et al., 2000; Horvath et al., 2003, 2007). Therefore, our results on motility percentage and sperm velocity decrease as a consequence of freeze-thawing in carp (Figs 1a and 2a), perch (Figs 1b and 2b), pike (Figs 3a and 4a) and rainbow trout sperm (Figs 3b and 4b) are in accordance with the results described for these species previously.

However, the increase of mean velocity after freeze-thawing at initial stages of motility in sterlet (Fig. 4c) does not correspond to the data presented for this species previously (Lahnsteiner et al., 2004, for review see Billard et al., 2004). We suppose this increase is related to disappearance of slowly moving spermatozoa (observed in fresh sperm) at 10 s of thawed sperm post activation. Possibly, slowly moving spermatozoa could be more sensitive to cryopreservation damaging factors and as result in thawed samples more fast spermatozoa survived cryopreservation were observed. Scatterplots of sperm velocities in sterlet before and after cryopreservation supports this supposition. In addition, freeze-thawing resulted in more rapid decrease of velocity within post-activation time after thawing (Fig. 5a). In contrast to sterlet, in other studied species cryopreservation led to the disappearance of fast sperm and the case of sperm velocity distribution in carp is an example of this effect type (Fig. 5b).

Conclusion

Freeze-thawing procedures used in fish sperm cryopreservation practice lead to spontaneous activation of spermatozoa in some species. This phenomenon is species-specific and affects carp and perch sperm, while it was not observed in pike, rainbow trout and sterlet. In carp this activation leads to total motility initiation (all the cells survived), while in perch the spontaneous sperm activation is not so evident as in carp and led to the initiation of only one subpopulation of spermatozoa. In addition, freeze-thawing could lead to the selection of fast moving sperm in sterlet. Future studies on the effect of storage time after thawing on sperm fertilization ability focused to primary elucidation of importance of this phenomenon practical value for fish sperm cryopreservation are required.

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CHAPTER 6

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

GENERAL DISCUSSION

In the case of freshwater fish, spermatozoa possess a hypotonicity dependent mode of motility activation with rapid and short lasting motility. Motility is initiated and maintained thanks to the hydrolysis of intracellular ATP stores. Respiration rate increases together with the sperm volume during motility activation. In contrast spermatozoa can be motile in media containing no oxygen. Possibly fish spermatozoa do not need respiration during motility phase if motility is based on stored rather than newly synthesized ATP. Interesting ability of carp spermatozoa to be activated for a second time can modulate situation where spermatozoa have no ATP (it was used for the first phase of motility) and still able to be activated. Keeping initially activated fish spermatozoa in a medium with high osmolality, such as K^+-rich medium, where motility is prevented, can regenerate the ATP stores of cyprinid spermatozoa. Correlation between the respiration of spermatozoa and their motility in initially activated and reactivated spermatozoa will aid in the understanding of the metabolism of energy. The first objective of the current study was to measure respiration and motility of initially activated and reactivated spermatozoa, and to compare the above between groups of common carp males kept at 15 °C (CW for cold water) and 20 °C (WW for warm water) (Chapter 2).

The percentage of motile carp spermatozoa rapidly decreases after activation in a hypo-osmotic medium. However, addition of K^+-rich medium to the activated spermatozoa and incubation in such medium creates conditions for a second activation of sperm movement (reactivation). The reactivation of carp spermatozoa leads to changes in motility dynamics, meaning that 25–45 s post-reactivation the percentage of motility reaches the maximal value. Since the percentage of reactivated spermatozoa depends on the incubation time and individual properties of the sperm sample, the differences among males can be related to the initial ATP content of spermatozoa in each individual. Based on the literature on cellular mechanisms in fish sperm activation (Alavi and Cosson, 2006), the existence of dynamics in percentages of secondarily activated sperm could not be properly explained. It might be postulated that the observed effect is related to the inhibition effect of K+ ions on motility (Cosson, 2004; Benau and Terner, 1980; Takai and Morisawa, 1995), which is eliminated after sperm dilution and the timing reflects the activity of the cell regulatory processes.

Recovery of the initially activated spermatozoa cell volume under isotonic conditions is rapid, occurring within the first minute after the external osmotic pressure was increased (Perchec-Poupard et al., 1997; Dzuba et al., 2001). Possibly, it leads to sperm metabolic activity resulting in the ATP accumulation needed for many functions in a cell, such as compensation for membrane permeability changes, ionic readjustment, etc. Finally, spermatozoa achieve the possibility of reactivating (Perchec et al., 1995). Reactivation time is supposed to reflect the rate of ATP production available for motility purposes, whereas the maximum percentage of reactivation shows sperm integrity after hypotonic shock. It should be emphasized that sperm capable of repeated activation have fertilizing abilities, which is confirmation of their functional integrity (Linhart et al., 2008).

The study of motility and reactivation parameters from groups of fish maintained at 15 and 20 °C (CW and WW) showed that statistically significant differences were not observed in the percentage of motile spermatozoa and duration of their movement between groups. The sperm of the CW group require a significantly longer period to achieve the maximum percentage of motility; the percentage of reactivation remains statistically insignificant. A significant reduction in the sperm concentration in males of the CW group might be attributed to the slowing-down of spermiogenesis caused by a lower water temperature during pre-spawning.

The participation of oxidative phosphorylation to the movement and recovery of the potential for movement during repeated activation has been indirectly demonstrated before (Perchec et al., 1995 in carp; Christen et al., 1987 in trout). However, there are no data available on respiration parameters of

both initially activated and reactivated carp spermatozoa. An increase in the respiration rate due to the uncoupling of oxidative phosphorylation by DNP in NAM (nonactivation medium), and the absence of this effect in AM (activation medium), could be related to a maximum sperm respiration rate achieved with the activating medium.

Carp sperm gain the ability to move after passing the last stage of spermatogenesis, finishing with the yield of spermatozoa from the seminal ducts, where mature spermatozoa are mixed with seminal fluid (Morisawa et al., 1983a). This process is connected to both changes in ions and pH of the surrounding spermatozoa medium and to the possible accumulation of macroergic compounds. With the assumption that the process of gaining the potential to move is similar under *in vitro* and *in vivo* conditions, we compared the intensity of oxygen consumption in sperm samples obtained from males of two experimental groups. Differences were found due to the prespawning water temperature, which might affect the rate of spermatozoa maturation after hormonal injection and, as a consequence, spermatozoa respiration.

The absence of an increase in the respiration rates after adding DNP to AM proved that sperm respiration is at its maximum rate level during activation. The significant reduction in the sperm respiration rate in a nonactivating medium in the WW group could be related to the accumulation of an amount of ATP high enough to cause inhibition of respiration. In sperm of the CW group, ATP accumulation was probably not completed at the moment of sperm collection; therefore, there was increased consumption of oxygen in NAM. This could be attributed to a slowing down in the maturation of the spermatozoa caused by the lower water temperature during pre-spawning. An important addition to the description of the respiration process is an increase up to the maximum level of sperm respiration within the period after the first period of movement (reactivation). We assume that a reduction in ATP content at the end of movement (Perchec et al., 1995; Cosson, 2004) is the stimulus to respiration activation.

Reduced water temperature during the pre-spawning period results in a slowing-down of spermiogenesis, manifested by a decreased sperm concentration and differences in the respiration activity of spermatozoa. Therefore, sperm obtained from the males maintained at a lower temperature are characterized by lower sperm density and a higher level of endogenous respiration.

The significant dependence of fish sperm motility on hypotonic influence suggests that several sperm populations coexist within the same sample, each population requiring specific conditions for its motility activation (Alavi et al., 2007; Linhart et al., 1999, 2003; Morisawa et al., 1983b). For freshwater fish, it has been shown that during sperm movement in hypotonic conditions, a reduction of the ATP concentration (Perchec et al., 1995) and a rise in the sperm cell volume (Perchec-Poupard et al., 1997; Dzuba et al., 2001) are observed due to water penetration into a spermatozoon from the hypotonic environment, but the dynamics of ATP concentrations during the motility activation phase are not completely understood. Our second working hypothesis is that stepwise reduction of the osmotic pressure (osmolality) of the activating solution may positively affect the duration of sperm movement due to the decrease in the rate of ATP expenditure (Chapter 3).

The possibility of inducing the second motility phase *in vitro* has been shown for several fish species differing in metabolic pathways for motility activation signaling. For carp sperm, which show an osmotic pressure-dependent mode of sperm motility activation (Alavi and Cosson, 2006), the second round of motility was achieved: the spermatozoa were transferred after cessation of motility to conditions of increased up to isotonic osmolality for 10 to 20 min and then activated again in hypotonic condition (Chapter 2). Other models of multiple activation have been described for zebra fish and salmonids. In zebra fish, the activated spermatozoa were artificially stopped using higher osmolalities (at the stage prior to motility stop onset) and then the spermatozoa were again transferred to hypotonic conditions to activate spermatozoa motility, but this procedure may be repeated several times

(Takai and Morisawa, 1995). For salmonids, the second motility phase could be achieved by adding the phosphodiesterase inhibitor isobutylmethylxanthine (which inhibits the degradation of cAMP to AMP) after motility has stopped or by first activation in ovarian fluid followed by second activation in hypotonic conditions (Benau and Terner, 1980). The models of second activation described above differ from the model of multiple activation because only a stepwise decrease in osmolality was applied in our study (Chapter 3).

We developed a model of multiple activation of movement when using stepwise reduction of osmolality of activating solution. This model, due to stepwise decrease of activating media osmolality, prolong the total duration of perch sperm motility from 45 sec up to 135 sec. We consider these results as demonstrating, not the fact that the same spermatozoa have a prolonged motility period, but that the total period of motility phase (more than 10% spermatozoa are motile at the same time) became prolonged, possibly due to the activation of different fractions of spermatozoa at different time points. Increasing the period of sperm motility can also positively increase the fertilization rate (Billard et al., 1995; Kime et al., 2001).

As we have demonstrated in our model of multiple sperm motility activation, repeated activation of perch sperm movement can be achieved when the osmolality of the activating solution used for the first activation is higher than 200 mOsm/kg. Use of an activating medium with osmolality of 260 mOsm/kg rendered possible the second (at 220 mOsm/kg) and the third (at 90 mOsm/kg) activation due to decreased osmolality. We hypothesize that the observed phenomenon could be related to differences in the individual sensitivity of spermatozoa to the osmotic shock (Dzuba et al., 2001). Higher osmolality of activating medium led to activation of the motility of selected sperm populations but without affecting the functional integrity of other spermatozoa. Possibly, this level of swelling is not large enough for motility activation in another portion of the sperm population, which would require larger differences in osmotic pressure between ovarian fluid and activating media. Our current data do not lead to firm conclusions but allow us to speculate that different sperm subpopulations can be activated in conditions of different osmotic pressure. Further, the different median values of sperm velocities could be considered as characteristics of these subpopulations. In that case, the sperm subpopulation for which motility could be initiated only after osmotic pressure reduction down to 90 mOsm/kg shows significant decrease in average sperm motility at initial stages of activation, whereas for two other subpopulations, no significant differences in this parameter were found. We suppose that during the first phase of motility activation, the major part of the sperm population is activated while the rest (not activated in this condition) of the spermatozoa preserve their potential for activation in media with lower osmolality.

Our results regarding ATP content decrease in perch spermatozoa during motility phases are in good conformity with knowledge earlier described. As was shown earlier, the presence of ATP in swimming media is necessary for the motility of de-membranated carp sperm models (Cosson and Gagnon, 1988). In addition, ATP level, beat frequency, and velocity of spermatozoa are strongly and positively correlated (Perchec-Poupard et al., 1997). These findings suggest that sperm velocity could be strongly dependent on intracellular ATP content accessible to dynein ATP-ase. The total ATP content could be associated with potential for sperm motility, but the distinct meaning of this parameter is unclear because usually after cessation of motility, the level of total ATP is still high enough for dynein activity while flagellum beating becomes arrested (Cosson, 2004). This flagellum beating arrest in freshwater spawning species could be attributable to several reasons. First is the hypotonic swelling in conditions of low osmolality, which leads to flagellum morphology changes (Linhart et al., 2008; Perchec et al., 1996). Second is the decrease of cAMP levels for species demonstrating cAMP-dependent pathway responsible for flagellum beating (Benau and Terner, 1980). The third reason could be associated with changes in sperm intracellular ionic composition occurring during sperm motility and leading to

changes of membrane potential or water transmembrane balance (Krasznai et al., 2003). In addition, we can speculate that low levels of total ATP that are found in spermatozoa after motility has ceased in conditions of hypotonic activation (Perchec et al. 1995) could reflect the absence of distribution of ATP along the sperm flagellum (Brokaw, 1967). The reduction of total ATP level during motility phases is dependent on the osmolality of the activating media used (Chapter 3). Whereas the distinct metabolic pathway responsible for these changes is still not clear (for review, see Ingermann, 2008), well defined interpretation of the ATP content dynamics is complicated due to changed motility percentage and velocity, as well as the ATP consumption by immotile spermatozoa in the media with different osmolalities. That is why we could only suppose that the high ATP level in perch spermatozoa activated by 260 mOsm/kg and 220 mOsm/kg activating media could provide the ATP reserves for multiple sperm activation. We propose that the high ATP level seen after cessation of motility in spermatozoa activated by 260 mOsm/kg and 220 mOsm/kg media could be one of the reasons for the multiple sperm activation providing the ATP reserves for second and third phase of motility, respectively.

We consider that it is possible to manipulate sperm motility state by changing of the environmental osmolality. Sperm motility re-activation could be achieved after a subsequent increase of osmotic pressure of the environmental medium of activated spermatozoa up to the level of isotonicity, which would result in cessation of motility and restoration of ATP content together with the potential for a further motility phase (Chapter 2). Furthermore, the multiple motility activation could be obtained by a stepwise osmotic pressure decrease (Chapter 3). Changes in osmotic pressure outside and inside the cells following freeze-thawing processes with slow cooling rates (commonly used for fish sperm; Mazur, 1984), could as well provoke spermatozoa motility stimulation in the media used for cryopreservation. The third objective of our study was to examine spontaneous (''spontaneous'' here implies without any environmental changes made by an observer) spermatozoa activation during the freeze-thawing process and evaluate sperm motility percentage, spermatozoa velocity and ATP content changes associated with this phenomenon in comparison with hypotonic activation (Chapter 4). A fourth aim was to evaluate the influence of post-thaw storage time on fertility of thawed sperm after spontaneous activation before fertilization (Chapter 4).

The appearance of sperm motility in supernatants (CS) obtained after centrifugation of thawed sperm samples conclusively demonstrates the phenomenon of spontaneous activation of carp spermatozoa due to freeze-thawing process. According to model of cryodamage by ''slow'' freezing regimes, during cooling procedure the cells enter hypertonic conditions, which have resulted from the water freezing. During thawing the cells entered the conditions of decreasing osmotic pressure arising from the process of water crystals melting. With integrating both knowledge about carp sperm motility activation and osmotic pressure changes during freeze-thawing, we can speculate about the basis of the phenomenon of spontaneous motility activation after freeze-thawing. We postulate that the mechanism of spontaneous activation could be similar to hypotonic activation. In both situations the decrease of environment osmolality could be the motility initiating factor. However, with spontaneous activation the event of osmolality decrease is difficult to measure because it appears during thawing. We suppose that immediately after quick thawing (in the procedures used the rate of thawing was significantly higher than cooling rate) the osmotic pressure inside the sperm is higher than in the environment (CS), resulting in the activation of sperm motility. The engendered equilibration in osmotic pressure outside and inside of sperm to the level of CS (about 1800 mOsm/kg) leads to cessation of motility, but spermatozoa posses the possibility for hypotonic activation by AM. This could explain why, during postthaw storage, the spontaneously activated spermatozoa motility ceases rapidly, while saving the possibility for hypotonic activation for longer periods (at least 10 min in our experiment). The cryoprotective media and freeze-thawing regime used in our study were proposed by several authors (Kopeika, 1986; Horvath et al., 2003; Horvath et al., 2007), and provided satisfactory

results in post-thaw fertilization when hypotonic conditions for fertilization were used. Thus, it might be possible that the phenomenon of spontaneous motility activation could not be the limiting factor for use of cryopreserved sperm.

From the measurements of motility percentage in spontaneously and hypotonically activated sperm, we concluded that cryopreservation led to cryodamages in part of the spermatozoa population. Non-damaged cells were activated by freeze-thawing, and could be reactivated with activation solution with a lower osmolality. Re-activation of spermatozoa motility can be achieved by means of different experimental conditions (Perchec et al., 1995; Linhart et al., 2008; Takai and Morisawa, 1995) but hypotonic (less than 300 mOsm/kg) condition was always required for activation of carp spermatozoa motility. In our study we demonstrate, that this activation is possible in condition of extremely high osmolality of CS (1800 mOsm/kg). Moreover, such a high osmolality is not the limiting factor for saving quite long lasting spermatozoa motility as well as the potential for a second activation. The reduced velocity during the spermatozoa movement period is a well known fact (Cosson, 2008), as well as the decrease of spermatozoa velocity in AM activation solution caused by freeze-thawing (Rodina et al., 2007; Lanhsteiner et al., 2000). Therefore, our results are in good agreement with previously reported results. However it can be hypothetized that, during movement activation by freeze-thawing, suppression of movement velocity is manifested to a lesser extent when using AM solution rather distilled water. This could probably be related to different osmotic conditions for spermatozoa motility. With spermatozoa activation caused by freeze-thawing the sperm are in an environment of high osmotic pressure, even at the end of motility phase. That is why hypotonic cell swelling is impossible in condition of spontaneous activation, while activation in AM leads to hypotonic swelling, which could be the reason for the precocious ending of motility (Perchec-Poupard et al., 1997; Cosson, 2008).

In our study, we found that the freeze-thawing led to an ATP content reduction compared to sperm diluted with cryoprotective media and stored without freezing. This decrease could probable contributed to spontaneous motility activation. However, the reduction of total ATP content could be associated with ATP consumption by the processes of spermatozoa cellular volume changes or/and ATP hydrolysis in damaged cells.

We did not find a negative influence of storage within 10 min after thawing. We assume that, even after a decrease of percentage motility with activation by AM solution (on 10th minute of post-thaw storage), the motile spermatozoa possess a level of velocity high enough to allow them to fertilized (Chapter 4).

Sperm cryopreservation as a tool for enhancing the fish farming effectiveness and rare fish species conservation is an extensively developing field of biology (Billard, 2001). Water freezing occurring during cooling of cell suspension leads to changes of osmolality (Mazur, 1984) and to spontaneous activation of carp spermatozoa motility (Chapter 4). Our fifth hypothesis is that spontaneous activation could be species-specific and as a result, could be one of the factors, which influence the effectiveness of thawed sperm use (Chapter 5). To check this hypothesis we investigated the presence of motility and measured the velocity in thawed sperm before dilution with activating media in fish species possessing different sperm activation mechanisms. Perch, carp and pike were selected as models characterized by motility activation due to osmotic pressure decrease, while sterlet and rainbow trout were selected as the ones of ion dependent motility activation.

The findings on the existence of sperm motility activation by freeze-thawing in carp and perch showed that this phenomenon is species-specific as compared to pike, trout and starlet. This property was demonstrated in our study for the first time. The activation mechanism of spermatozoa motility by freeze-thawing could be similar to motility initiation in activation solution (Chapter 4). In both cases the decrease of environment osmolality could be a motility initiating factor. Further we speculate that fish spermatozoa possess species-specific sensitivity to osmotic pressure changes. That is why the

phenomenon of motility activation by freezethawing existing in carp and perch is not observed in pike, possessing the same mode of motility activation. In species possessing ion-dependent mode of sperm activation (sturgeon and rainbow trout) freeze-thawing did not lead to sperm motility acquisition. In case of sterlet, possibly, this phenomenon could be suppressed by the presence of potassium ions in the cryoprotective medium under concentrations high enough to inhibit motility (Cosson, 2004).

We studied the changes in sperm velocity after thawing in relation to the presence of spontaneous sperm activation. The absence of differences between motility percentage and velocity in spontaneous and hypotonic motility for carp thawed sperm suggested that in carp all the spermatozoa that possess the ability for motility acquisition after cryopreservation are subjected to spontaneous activation. In perch freeze-thawing led to spontaneous activation just in part of sperm population, since we were able to activate more cells under hypotonic conditions. In addition, this small part of activated sperm started their motility with lower velocity.

As a conclusion to present study: the physiological backgrounds for sperm motility multiple activation in fish spermatozoa and appearance of spontaneous activations during cryopreservation have been described in details. Spontaneous motility activation by freezing-thawing and motility activation by stepwise decrease of osmotic pressure of activation media were describe for the first time. The ability for multiple sperm activation could be the base for the elaboration of the most optimal sperm use because the prolongation of total sperm motility duration potentially and more likely makes the fertilization rate higher. Spontaneous sperm motility activation during freeze-thawing should be taken into account during cryopreserved sperm use. Finally, conjunction of the ability of sperm for multiple activation and its cryopreservation ability could be at the base of multiple sperm use for fertilization if there is a stringent limitation of valuable individuals' sperm.

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ENGLISH SUMMARY

Energetic and motility of fish spermatozoa

Sergii Boryshpolets

A spermatozoon has the crucial function to transport the male haploid chromosomes set into the oocyte. In freshwater fish sperm motility activation is related to low osmolality of the fresh water environment where spawning occur, duration lasts for short periods and the lost motility is associated with disappearance of fertilizing ability. Motility of fish spermatozoa is initiated and maintained by the hydrolysis of ATP stores, which were produced in the sperm prior to activation. During motility activation, rate of fish sperm respiration is increasing together with a sperm volume. Incubation of sperm under anaerobic condition results in reversible decrease of both ATP level and further motility. We suppose that the respiration of fish spermatozoa is involved in producing and maintaining high enough intracellular ATP store. To study the respiration rate of fish spermatozoa, ability of carp sperm to be activated for a second time was used as a model where spermatozoa have no ATP (it was exhausted during the first phase of motility) and still retain ability to be activated.

The first objective of the current study was to measure respiration and motility of initially activated and secondly reactivated spermatozoa, and to compare the above between groups of common carp males kept at 15 °C (CW for cold water) and 20 °C (WW for warm water). Reactivation (second activation) was achieved after incubation of initially activated sperm in media with osmotic pressure adjusted up to 300 $mOsm.kg^{-1}$ by adding of K^+ rich medium.

Significant differences between experimental groups were found in concentration of spermatozoa (46.0 ± 12.5 for CW and 59.3 ± 7 10^9 spermatozoa/ml for WW). In addition sperm of the CW group required a significantly longer incubation time (37 min) under isotonic conditions to achieve a maximum percentage of potent motility at repeated activation than the WW group (23 min).

An increased respiration rate during motility was at a maximum level and this level was statistically not different from respiration rate under conditions of recovering the potential for repeated activation. Respiration rate of activated spermatozoa in the CW group (6.1 nmol O_2/min/10^9spermatozoa) and the WW group (3.9 nmol O_2/min/10^9spermatozoa) were significantly higher than the nonactivated sperm (2.4 nmol O_2/min/10^9spermatozoa for CW and 1.1 nmol O_2/min/10^9spermatozoa for WW). Decreasing of water temperature down to 15 °C for 7 days increased the respiration rate of the sperm.

Ability of fish spermatozoa to activate their motility for a second time and dependence of this motility on hypotonic influence constitutes the background of our second study aim, in order study the dynamics of ATP content and sperm movement parameters in conditions of stepwise reduction of osmotic pressure of the activating solution.

The first phase of sperm motility activation was initiated by dilution in a 260 mM glucose solution (75% motility). The second phase of motility was achieved by adding water to previously activated sperm, so that the glucose concentration dropped down to 220 mM (24% motility). Finally, the third phase was obtained by further addition of water (down to 90 mM glucose) to the activated sperm suspension (15% motility). ATP concentration in spermatozoa before motility activation was 43.9 nmol ATP/10^9 spermatozoa. After motility activation under different experimental conditions the ATP concentration significantly decreased from 35 to 7 nmol ATP/10^9 spermatozoa. Value of sperm velocity ranged in value from 25 to 330 $\mu m\ s^{-1}$ at 10 s. postactivation, from 10 to 290 $\mu m.s^{-1}$ at 30 s, and from 0 to 200 $\mu m.s^{-1}$ at 45 s.

It is possible to manipulate sperm motility state by changing the environmental osmolality (sperm motility re-activation and multiple motility activation). Changes in osmotic pressure outside and inside

the cells following freeze-thawing processes with slow cooling rates (commonly used for fish sperm), could as well provoke spermatozoa motility stimulation in the media used for cryopreservation. The third objective of our study was to examine spontaneous (''spontaneous" here implies without any environmental changes made by an observer) spermatozoa activation during the freeze-thawing process and evaluate sperm motility percentage, spermatozoa velocity and ATP content changes associated with this phenomenon in comparison with hypotonic activation. A fourth aim was to evaluate the influence of post-thaw storage time on fertility of thawed sperm after spontaneous activation before fertilization.

Percentage and velocity of spontaneously activated during the freeze-thawing spermatozoa were 16% and 98 μm.s^{-1} respectively. The fresh sperm samples were characterized by median value of 100% for sperm motility and 175 lm s^{-1} for sperm velocity. Five minutes post-thaw storage resulted in absences of sperm spontaneous motility activation. Freeze-thawing leads to significantly decreasing of the ATP level with post-thaw time (46 nmol ATP/10^9 and 10 nmol ATP/10^9 at 25 s and 10 min after thawing, respectively). Fertility of spermatozoa was not significantly affected within 10 min post-thaw. Freeze-thawing procedure spontaneously activated spermatozoa motility in common carp. However, this activation did not negatively affect the fertility of frozen-thawed sperm.

The fifth aim was to investigate the presence of spontaneous motility activation in fish species presenting variant forms in their activation mechanisms. We investigated the possibility of spermatozoa motility activation during freeze-thawing in common carp (*Cyprinus carpio* L.), Eurasian perch (*Perca fluviatilis* L.), rainbow trout (*Oncorhynchus mykiss*), Northern pike (*Esox lucius* L.) and sterlet (*Acipenser ruthenus* L.) using cryopreservation methods described previously. The phenomenon of spontaneous motility activation was found in carp and perch, but not in pike, rainbow trout and sterlet. The motility and velocity of spontaneously activated carp spermatozoa were characterized by 16% and velocity 98 ± 2 μm.s^{-1}, for perch 5% and 53 ± 5 μm.s^{-1}, correspondingly. We believe that the phenomena of spontaneous activation by freeze-thawing processes are species-specific.

In the present study we summarized our results on studying the physiological backgrounds for sperm motility multiple activation in fish spermatozoa and appearance of spontaneous activations during cryopreservation.

CZECH SUMMARY

Energetika a motilita rybích spermií

Sergii Boryshpolets

Spermie mají klíčovou úlohu, a sice přenos samčí haploidní sady chromozomů do oocytu. U sladkovodních druhů ryb je aktivace motility spermií způsobena nízkou osmolalitou sladkovodního prostředí, motilita trvá pro krátkou dobu a ztráta motility je spojena se ztrátou schopnosti oplození. Motilita rybích spermií je iniciována a ovládána hydrolýzou zásob ATP, které byly vyprodukovány spermiemi před aktivací. Během aktivace motility se zvyšuje intenzita respirace a také roste objem spermie. Inkubace spermií v anaerobních podmínkách má za následek reverzibilní snížení zásob ATP, a následně i motility. Předpokládáme, že respirace rybích spermí je začleněna v systému produkce a ovládání dostatečných zásob ATP. Pro studium respirace rybích spermií jsme použili spermie kapra pro jejich schopnost druhé aktivace poté, co spermie spotřebují veškeré zásby ATP (během první fáze motility) a stále si zachovávají schopnost být re-aktivovány.

Prvním cílem studia bylo změřit intenzitu respirace a motilitu poprvé aktivovaných a re-aktivovaných spermií a porovnat obojí mezi skupinami kapra obecného drženými při 15 °C (CW – cold water) a 20 °C (WW – warm water). Re-aktivace (druhá aktivace) byla dosažena po inkubaci spermií v mediu s osmotickým tlakem vyšším než 300 mOsm.kg^{-1} s přidáním media bohatého na K^+ ionty. Statisticky významné rozdíly byly nalezeny v koncentraci spermií (46,0 ± 12,5 pro skupinu CW a 59,3 ± 7 10^9 spermií/ml pro skupinu WW). Spermie ryb ze skupiny CW navíc vyžadovaly signifikantně delší dobu inkubace (37 minut) v isotonických podmínkách k dosažení maximální možné motility při re-aktivaci než skupina WW (23 minut). Zvýšená respirace během motility byla na maximální úrovni a tato úroveň nebyla statisticky odlišná od respirace. Zvýšení intenzity respirace během pohybu spermií bylo až na maximální možnou úroveň a tato se statisticky nelišila od respirace v podmínkách inkubace pro opakovanou aktivaci.

Respirace aktivovaných spermií ryb ze skupiny CW (6,1 nmol O_2/min/10^9spermií) a skupiny WW (3,9 nmol O_2/min/10^9spermií) byla výrazně vyšší než respirace neaktivovaných spermií (2,4 nmol O_2/min/10^9spermií pro skupinu CW a 1,1 nmol O_2/min/10^9spermií pro skupinu WW). Snížení teploty na 15 °C po dobu 7 dnů zvýšilo intenzitu respirace spermií.

Schopnost rybích spermií aktivovat jejich motilitu podruhé a odeznění této druhé fáze motility je námětem další části studie, zabývající se dynamikou obsahu ATP a parametry pohybu spermií v podmínkách postupně snižovaného osmotického tlaku aktivačního roztoku.

První fáze motility spermií byla iniciována zředěním v 260mM roztoku glukosy (75% motility). Druhá fáze motility byla dosažena přidáním vody do předchozího ředění spermatu tak, aby koncentrace glukosy klesla na 220mM (24% motility). Nakonec byla třetí fáze motility iniciována dalším přidáním vody (koncentrace glukosy klesla na 90mM) do suspenze spermatu (15% motility). Zásoby ATP ve spermiích před aktivací byly 43,9 nmol ATP/109 spermií. Po aktivaci spermií v různých experimentálních podmínkách klesla koncentrace ATP na 35 až 7 nmol ATP/10^9 spermií. Hodnoty rychlosti pohybu spermií byly v rozmezí 25 až 330 μm.s^{-1} v čase 10 s po aktivaci, od 10 do 290 μm.s^{-1} v čase 30 s a od 0 do 200 μm.s^{-1} v čase 45 s po aktivaci.

Změnou osmolality prostředí je možné manipulovat s motilitou spermií (re-aktivace motility spermií, několikanásobná aktivace). Změny v osmotickém tlaku vně a uvnitř buněk jsou podobné změnám při zmrazování a následném rozmrazování spermií při nízkých rychlostech zmrazování (běžně používaných pro rybí spermie) a stejně tak mohou způsobovat motilitu spermií v roztoku pro kryoprezervaci.

Třetím z cílů studia bylo objasnit spontánní aktivaci spermií během procesu rozmrazování a

vyhodnotit procentuální zastoupení pohyblivých spermií, rychlost pohybu spermií a změny obsahu ATP spojené s tímto fenoménem, vše ve srovnání s hypotonickou aktivací. Čtvrtým cílem bylo vyhodnotit vliv skladování spermatu po rozmrazení na schopnost oplození rozmrazených spermií.

Zastoupení spermií aktivovaných procesem rozmrazení a rychlost pohybu spontánně aktivovaných spermií byly 16% a 98 $\mu m.s^{-1}$ Vzorky čerstvého spermatu vykazovaly motilitu 100% a rychlost pohybu spermií 175 $\mu m.s^{-1}$. Zmrazení a rozmrazení vede k signifikantnímu snížení úrovně ATP společně s časem (46 nmol ATP/10^9 a 10 nmol ATP/10^9 v čase 25 s a 10 minut po rozmrazení). Schopnost oplození však nebyla signifikantně snížena v rámci 10 minut po rozmrazení. Zmrazení a rozmrazení dávky spermatu spontálně aktivovalo motilitu spermií u kapra obecného. Nicméně, tato aktivace neovlivnila negativně schopnost oplození rozmrazovaných spermií.

Pátým z cílů bylo zjistit, jestli se tento fenomén spontánní aktivace spermií procesem zmrazení a rozmrazení dávky spermatu vyskytuje i u jiných rybích druhů. Možnost spontánní aktivace byla studována u kapra obecného (*Cyprinus carpio* L.), okouna říčního (*Perca fluviatilis* L.), pstruha duhového (*Oncorhynchus mykiss* L.), štiky obecné (*Esox lucius* L.) a jesetera malého (*Acipenser ruthenus* L.) v rámci protokolů kryokonzervace popsaných dříve. Fenomén spontánní aktivace motility byl nalezen u spermií kapra a okouna, ne však u štiky, pstruha nebo jesetera malého. Motilita a rychlost pohybu spontánně aktivovaných spermií byly 16% a 98 ± 2 $\mu m.s^{-1}$ pro kapra a 5% a 53 ± 5 $\mu m.s^{-1}$ pro okouna. Zdá se, že tato spontánní aktivace spermií je druhově specifická.

V této práci byly shrnuty výsledky studia několikanásobné aktivace motility rybích spermií a spontánní aktivace spermií procesem kryokonzervace.

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LIST OF PUBLICATIONS

PEER-REVIEWED JOURNALS WITH IF

Pšenička, M., Kašpar, V., Alavi, S.M.H., Rodina, M., Gela, D., Li, P., **Boryshpolets, S.**, Cosson, J., Linhart, O., Ciereszko, A., 2011. Potential role of the acrosome of sturgeon spermatozoa in the fertilization process. Journal of Applied Ichthyology 27 (2), 678–682.

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Boryshpolets, S., Dzyuba, B., Stejskal, V., Linhart, O., 2009. Dynamics of ATP and movement in Eurasian perch *Perca fluviatilis* L. sperm in conditions of multiple activation. In: Asturiano, J.F. (Eds), Second International Workshop on Biology of Fish Gametes, Valencia (Spain), September 9–11, 2009, (Oral presentation).	2009
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Boryshpolets, S., Dzyuba, B., Rodina, M., Li, P., Hulák, M., Gela, D., Linhart, O., 2010. Can we avoid the spontaneous sperm motility activation arisen from freeze-thawing in carp? Book of abstracts of 47 Annual Meeting of Society for Cryobiology CRYO-2010, July 17–20, Bristol, UK, (Oral presentation).	2010
Boryshpolets, S., Dzyuba, B., Shalutina, A., Rodina, M., Gela, D., Yamaner, G., Linhart, O., 2010. Sperm multiple collection from sterlet (*Acipenser ruthenus*) males with following cryopreservation and fertilization. “Seafarming Tomorrow”, International conference of European Aquaculture Society. Porto, Portugal October 5–8, 2010, (Oral presentation).	2010

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