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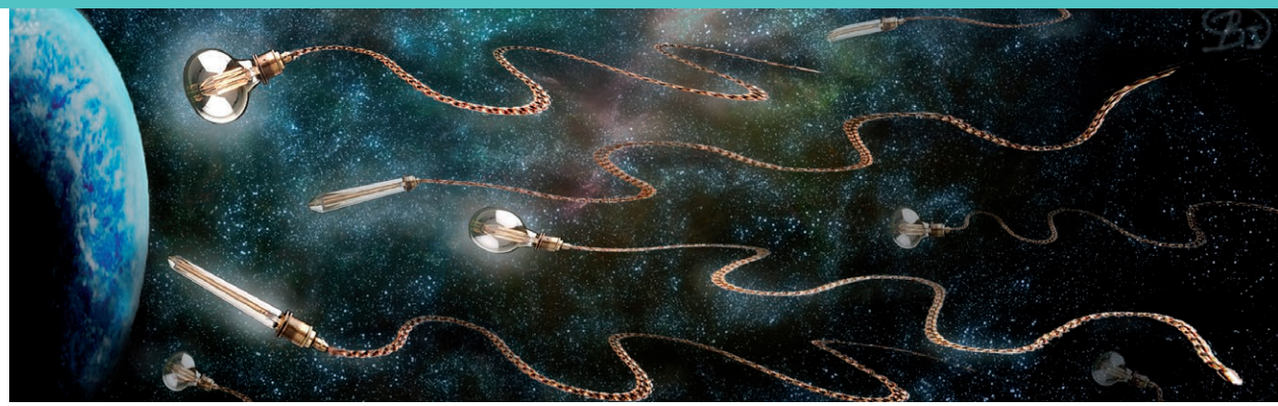
# The role of osmotic and ionic agents in fish sperm physiology

## Úloha osmotických a iontových činidel ve fyziologii rybích spermií



Olga Bondarenko

Czech Republic, Vodňany, 2015

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**Supervisor**
**Borys Dzyuba, Ph.D.**
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

**Consultant**
**Prof. Jacky Cosson, Dr. h.c**
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

**Head of Laboratory of Reproductive Physiology**
**Prof. Otomar Linhart**

**Dean of Faculty of Fisheries and Protection of Waters**
**Prof. Otomar Linhart**

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## CONTENT

# CHAPTER 1

## GENERAL INTRODUCTION

## GENERAL INTRODUCTION

According to the FAO, over one billion people worldwide rely on fish as their primary source of animal protein food. Fish and fishery products represent an important source of protein and essential micronutrients for balanced nutrition and good health of humans. The fishery sector contributes to development and growth in many countries, playing an important role for food security and nutrition, poverty reduction, employment and trade. The production of high-value, high trophic food fish continues to receive the greatest support politically and economically.

Control of reproduction is a key issue in aquaculture, and one of the limiting factors of the reproductive success is the quality of male and female gametes. The use of high quality gametes from captive fish brood-stock is of great importance for ensuring the production of valuable offspring for aquaculture (Kjorsvik et al., 1990). The issue of fish eggs (Kjorsvik et al., 1990; Brooks et al., 1997) and sperm (Billard et al., 1995; Fauvel et al., 2010) quality has previously been reviewed. But why does one need a good quality sperm? In commercial hatcheries, milt is occasionally inadequate, both in terms of quantity and quality, and does not always give successful fertilization rate in the artificial insemination procedures commonly used for aquaculture species. Thus, a better knowledge of the sperm biochemistry, physiology, and the mechanisms of reproduction are essential to improve artificial fertilization procedures for fish. Among others, sperm motility is an important characteristic for efficient fertilization of eggs (Morisawa and Suzuki, 1980).

In most fish species, spermatozoa are immotile in the testis and in their seminal plasma (Morisawa et al., 1990). Motility is induced right after spermatozoa are released and are exposed to the aqueous environment during natural reproduction or with the diluent during artificial propagation. Several models of sperm activation in different fish species are presented in the literature. Sperm motility is initiated because of an abrupt change in the surrounding environment i.e. osmolality, ion composition (concentration of $K^+$, $Na^+$ and $Ca^{2+}$), or pH (Alavi and Cosson, 2005, 2006). However, different components of the surrounding fluids control sperm motility in fishes. In that way, a cascade of motility activation can be distinguished according to two modes: osmotic or ionic.

For spermatozoa with osmotic mode of motility activation, the ionic composition in the surrounding fluid is not the most important factor. For example, carp sperm motility could be activated in hypotonic media of different $Na^+$, $K^+$ or $Ca^{2+}$ concentrations, or in media completely devoid of any ions (Mannitol or Sucrose) (Morisawa and Suzuki, 1980; Perchec et al., 1995). Moreover, carp sperm motility cannot be activated under isotonic or hypertonic conditions whatever surrounding ionic composition (Perchec et al., 1995).

Sperm motility of sturgeon, in contrast to carp, is quite strictly under control of environmental ions and can be activated in solutions presenting a wide range of osmolalities (from hypotonicity to hypertonicity) (Alavi and Cosson, 2006), presenting sturgeons as a perfect model of fish with ionic mode of sperm activation.

However, some fish species cannot be classified as having only osmotic or ionic modes of motility activation. In freshwater salmonids for example, sperm motility activation is controlled by environmental ions, but only in isotonic or hypotonic conditions. Whereas, hypertonicity leads to completely suppressed sperm motility independently from surrounding ion composition and concentration (Ingermann et al., 2008).

Despite its participation in motility activation, osmotic stress has another influence on fish sperm. As a live cell with a membrane permeable to water, spermatozoa should react on environmental osmotic changes by alteration of its volume. Remarkably, sperm with different modes of motility activation do not show the same reaction to volume changes (Dzuba and

Kopeika, 2002). Carp spermatozoa significantly increase their volume during the motility period and the swelling rate is dependent on the tonicity of the activating medium (Dzuba et al., 2001; Dzuba and Kopeika, 2002), whereas there are no volume changes in salmonid spermatozoa during motility period were observed under different osmotic conditions (Cabrita et al., 1999). Osmotic shock that occurs during release of sperm into environment during spawning does play a physiologically different role regarding spermatozoa activation. A better understanding of the processes that control spermatozoa motility in fishes will be helpful for improving artificial reproduction methods and provide information for developing better short- and long-term storage (cryopreservation) conditions for sperm.

## 1.1. Mechanisms of water transport

Since rapid changes of environmental osmolality always accompany, and in some species are involved in fish spermatozoa motility activation, more precise definitions should be given.

**Osmosis** is a process of water movement across a semi-permeable membrane due to the difference between intra- and extra- cellular soluble components (ions as well as small and large molecules). Because of this difference in concentration, water moves according to a concentration gradient so as to equalize solute concentration on both sides of the membrane. The physical pressure that a cell membrane sustains when experiencing osmosis, is called **osmotic pressure**. Since the earliest study on osmotic pressures in 1780 (Moore, 1972), many examples of the osmotic-pressure phenomenon have been described in chemical and biological systems. Osmosis is of prime importance in living organisms, because of the influence of the distribution of nutrients and the release of metabolic waste products. Living cells of plants and animals as well as prokaryotes are enclosed by a semipermeable membrane which regulates the flow of liquids and dissolved solids and gases into and out of the cell (Lodish et al., 2000).

**Osmolality** is an experimental measurement of solute concentration, defined as the number of osmoles of solute per kilogram of solvent ($Osmol.kg^{-1}$ or $Osm.kg^{-1}$) at a certain temperature.

**Osmolarity** is the osmotic concentration of a solution expressed as osmoles of solute per liter of solution. ($Osmol.L^{-1}$ or $Osm.L^{-1}$) at a certain temperature. Osmolarity is based on calculated molar composition of all components in solution. Osmolarity is a theoretical value while osmolality is experimental one which can be detected by osmometer. In chemistry, the osmole (Osm or Osmol) is a non-SI unit of measurement that defines the number of moles of a chemical compound that contributes to a solution's osmotic pressure. The term comes from the phenomenon of osmosis, and is typically used for osmotically-active solutions. For example, the NaCl salt particle dissociates fully in water to become two separate entities: a $Na^+$ ion and a $Cl^-$ ion. Thus, a solution of 1 $mol.L^{-1}$ NaCl corresponds to an osmolarity of 2 Osmol/L.

It should be mentioned that dissociation into ions is not usually total but equilibrium remains such that NaCl: $Na^+ + Cl^-$, which favors ionization to the right of this equilibration. Measurement of osmolality experimentally is slightly lower than calculated one.

Osmotic pressure on a natural cell membrane is closely related to the solute, or ionic concentration in solutions on either side of the membrane. Thus, media surrounding a cell could be:

**Isotonic** – solution in which concentrations of solute inside and outside of the cell are the same.

**Hypotonic** – solutions in which concentration of solute is lower than its concentration inside of cell.

**Hypertonic** – solutions in which concentration of solute is higher than its concentration inside of cell.

**Osmotic shock or osmotic stress** is a sudden change in the solute concentration around a cell, causing a rapid change in the movement of water across its cell membrane: obviously, the direction and intensity of this water movement is also dependent on the internal solute concentration in the cytoplasm. Under conditions of high concentrations of either salts, substrates or any solute in the cytoplasm, (water is) drawn into the cells through osmosis. Sudden change of environment osmolality leads to cell volume alteration. When a cell is placed in a hypotonic medium surrounding this cell, the water molecules will move into the cell causing the cell to swell. If osmosis continues and becomes excessive the cell will eventually burst (Lang et al., 2005) depending on its membrane resistance.

Cellular water content is one of the major variables controlled physiologically by homeostasis. To accomplish osmotic homeostasis, concentration of solutes must be rapidly equilibrated across cell membranes. There are two ways by which water can go through membrane: by passive and active transport.

**Passive water transport**

By definition, **passive transport** means movement of biochemical substances (usually of small size) across membranes. This process does not involve chemical energy and is dependent on the permeability of the cell membrane, determined by its lipid and protein composition. In passive transport, water and solute molecules move freely across the cell membrane due to hydrostatic pressure, which appeared because of difference in external and internal osmolalities (Merdaw et al., 2010).

**Active water transport**

Water active transport transfer through a membrane implies cell energy is expended, usually in the form of ATP. In contrast to passive, active transport can develop, both via and against concentration gradient, since water molecules go through specific water channels (aquaporines) (Verkman, 1999), or accompany ion transport thorough ion channels, co-transporters, exchangers etc. (Macaulay et al., 2004).

Water movement across membranes are regulated by **aquaporins (AQPs)**, a family of water-specific channel proteins that allow acceleration of water and other solutes transport such as glycerol and/or urea, in the presence of osmotic or concentration gradients (Borgnia et al., 1999). Several authors have reported the relevance of AQPs to sperm physiology in mouse (Chen and Duan 2011) and human sperm (Saito et al., 2004; Yeung C. H. et al., 2010). It has been reported that AQPs in mammalian (mouse and human) spermatozoa are involved in volume regulation (Chen and Duan 2011; Yeung C. H. et al., 2010). In fish, it was demonstrated that aquaporins are involved in the motility activation process of sea bream (*Sparus auratus*) spermatozoa and that in this fish motility initiation occurs via a cAMP-dependent protein phosphorylation/dephosphorylation (Zilli et al., 2009). $HgCl_2$, the well-known specific AQP inhibitor (Verkman, 2005) was investigated as an effecting agent on the phosphorylation/dephosphorylation state of spermatozoa head proteins. Aqp1a and Aqp10b were previously detected in sea bream spermatozoa head and flagella plasma membranes (Cosson et al., 2008). Additionally, incubation of sperm with low concentration of $HgCl_2$ leads to complete suppression of motility activation in sea bream (Zilli et al., 2009). The inhibitory effect of $HgCl_2$ on sperm motility activation also has been demonstrated in North African catfish (Rurangwa et al., 1998), sea bass (Abascal et al., 2007) and turbot (Cosson et al., 1999). Recently, the involvement of AQPs in trout sperm motility was discussed. It was shown that AQP 1a and AQP 10 but not AQP 3 are expressed in trout testes. Treatment with $HgCl_2$, an inhibitor of

aquaporin, reduced the duration of motility in intact salmonid sperm in a dose-dependent manner and suppressed hypotonic shock-induced expansion of cellular volume (Takei et al., 2015). Further search for AQPs in fish sperm and the elucidation of its spatial and temporal distribution would help to explain the physiology role of AQPs in fish spermatozoa of different activation mode.

Movement of water molecules can be coupled with ion and substrate transport. It was demonstrated that this coupling can take place in a specific type of membrane protein, called cotransporters: ions and water interact inside the protein in such a way that ion fluxes give rise to a co-flux of water and vice versa, even though water is moving against concentration gradient (Goodman, 2002; Pappenheimer, 1998).

It was observed that the activity of some **cotransporters and uniporters** induce water fluxes but how these fluxes are generated is still an object of debate (MacAulay et al., 2002). Two mechanisms have been suggested: cotransport (secondary active transport) of water along with the non-aqueous substrates, and osmosis, due to the local osmotic gradients generated by intracellular substrate accumulation, where the protein acts also as a water pathway. Recently, a third mechanism has been proposed (Naftalin et al., 2004). Simulated $Na^+$/glucose cotransport showed that active glucose accumulation within a vestibule of the protein can generate a water flow before the formation of any intracellular hypertonicity (Naftalin, 2008). Besides, water movements occur through uniporters and cotransporters in the presence of imposed osmotic gradients and in the absence of cotransported substrates (MacAulay et al., 2001) which means that these proteins can act as water pathways. Possibly, different water transport modalities occur simultaneously in a given protein (Santacroce et al., 2010).

There is a fixed ratio between the number of water molecules transported per ion, and ion fluxes can even lead to an up-stream flux of water. The number of water molecules transported per turnover is specific for a given cotransporter and ranges from 50 to 500 intracellular substrate accumulation, where the protein acts also as a water pathway Involvement of cotransporters activity in regulation of cell volume was recently described in *Xenopus* oocytes (MacAulay and Zeuthen, 2010). It was shown, that ion transport via $Na^+$-$K^+$-$2Cl^-$ (NKCC) cotransporter accompanies active water influx. Moreover, inhibition if NKCC activity leads to suppressed *Xenopus* oocyte volume changes in respond to altering environmental osmolality (Zeuthen and MacAulay, 2012).

Altogether, water transport, occurring in live cells could not be completely separated from transport of ions and its interactions require more studies.

## 1.2. Influence of osmotic stress on sperm

Adaptation to the changes in environmental osmolality is a physiological feature for spermatozoa of evolutionarily distant species. In mammals, spermatozoa need to perform volume regulation because the fluids along the male and female tracts possess different osmolality. In the testis, seminal fluid osmolality is approximately 290 $mOsmol.kg^{-1}$ (Yeung et al., 2006). During spermatozoa migration through the epididymis, osmolality of fluid surrounding spermatozoa is increasing and can reach values higher than 400 $mOsmol.kg^{-1}$ in the hamster and mouse (Cooper and Yeung, 2003). The spermatozoa are held at this high osmolality condition in the cauda epididymidis until ejaculation. In murines, the difference in extracellular osmolality at ejaculation is approximately 100 $mOsmol.kg^{-1}$, as the female post-coital uterine fluid osmolality values are approximately 300 $mOsmol.kg^{-1}$ (Yeung et al., 2006). Human spermatozoa osmotic environment also changes, starting from the vas deferens (342 $mOsmol.kg^{-1}$) (Hinton et al., 1981), through cervical mucus (287 $mOsmol.kg^{-1}$) (Rossato et

al., 1996), to uterine fluid (284 mOsmol.kg$^{-1}$) (Casslen and Nilsson, 1984), and into oviductal fluid (274 mOsmol.kg$^{-1}$) (Menezo et al., 1982). The physiological role of the higher osmolality of semen is currently poorly understood. It was predicted that that hyper-osmotic conditions may promote sperm fertilizing ability and prevent a premature acrosome reaction (Liu et al., 2010). However, this prediction requires further studies.

Fish spermatozoa are subjected to osmotic stress when isotonic seminal fluid is diluted by natural external media at spawning. In these conditions, motility can be activated because of two main reasons. As a result of alteration of environmental osmolality, water will move through cell membrane to equalize concentration of solute in solvent (water) from both sides. In case of elastic cell membrane, osmosis will lead to alterations of cell volume. In cyprinids, for example, it is exactly such alteration of cell volume that activates biochemical cascade leading to spermatozoa motility (Krasznai et al., 2003b). Alternatively, osmotic pressure appears because of rapid and significant decrease (in case of freshwater fish) or increase (for marine fish species) of surrounding ions concentration. It is known that for some fish species, it is a certain environmental ionic composition that is crucial for sperm motility activation (Alavi and Cosson, 2006). For these species, osmotic pressure might not be so crucial. Although, a hypo-osmotic shock naturally accompanies and in some cases is essential for spermatozoa activation and for fertilization (Linhart et al., 2003), under these conditions partial membrane lysis could occur, both before initiation of motility and after its termination (Cabrita et al., 1999). Nevertheless, it was shown, in several fish species, that the increased cell volume stress caused by exposure to drastic osmotic condition could be recovered at the end of the motility period. Provided such cells are transferred transiently into an iso-osmotic medium, they can recover a normal cell shape and sustain a second round of motility (Cosson, 2004) as well as competence for fertilization (Linhart et al., 2003).

### 1.2.1. Osmotic stress and fish spermatozoa volume

Reduction in ambient osmolality leads to osmotic re-equilibration and water transport across the spermatozoa membrane, which should be accompanied by volume changes, since a sperm cell is presumed to react as a 'true' osmometer (Du et al., 1994). Considering that cell volume changes in response to environmental osmolality occur due to trans-membrane water influx, our observations of differences among freshwater fish species could be due to differences in water influx rates. As explained above in this text, water influx can take place either by passive diffusion, possibly facilitated through water channel proteins (aquaporins), or actively through ion co-transporters (Goodman, 2002). Water transport processes have not been sufficiently investigated to provide a precise description of their role in fish spermatozoa motility.

However, the reaction of sperm cells to osmotic shock in terms of cell volume alteration is species-specific. Alteration of environmental osmolality could differentially affect fish sperm, depending on species, because of differences in their osmotic- or ionic-dependent spermatozoa motility activation mode. Swelling was detected in rainbow trout (*Oncorhynchus mykiss*) spermatozoa after 0.5, 2, 5, 10, 20, and 30 min incubation in hypotonic non-activating solution (Cabrita et al., 1999). It was shown that, in this species, two sperm cell populations could be distinguished. One population (approximately 20% of total spermatozoa) showed no swelling, while, in the remaining 80%, average spermatozoa volume was shown to increase. Dependency of average sperm volume on either environmental osmolality or time of incubation in a hypotonic environment was not observed. In contrast to salmonids, the volume of carp spermatozoa is dependent on environmental osmolality (Dzuba and Kopeika, 2002; Dzuba et al., 2001; Perchec et al., 1995).

Some published reports point to activation of stretch-dependent channels as a prerequisite for motility in fish sperm with the osmotic mode of activation (Krasznai et al., 2003b). However, water diffusion appears not to be sufficiently rapid to induce membrane stretch immediately upon sperm contact with a hypotonic media. Alternatively, much more rapid water transport could occur due to water influx through aquaporins (Goodman, 2002). Hypothetically, in carp spermatozoa, water transport through aquaporins could lead to sperm swelling with further activation of stretch-dependent channels and motility activation. Salmonid spermatozoa show no volume changes during motility, suggesting that ion channel signaling is not dependent upon membrane stretching, and that ion transfer occurs only because of differences between internal and external ion concentration. However, the involvement of ion co-transporters cannot be excluded. It has been shown that during ion transfer the amount of co-transported water is significant, ranging from 35 to 500 water molecules per single act of ions transport, and that water transport proceeds, along with substrates, independent of the osmotic gradient (Goodman, 2002). Therefore, during motility, ion transport through the membrane would occur via co-transporters and be accompanied by water transport, thus affecting the volume of spermatozoa of fish such as trout and sturgeon, which possess an ionic mode of motility activation.

### 1.2.2. Osmotic regulation of fish sperm motility

Spermatozoa of evolutionally distant fish species require quite different environmental conditions for activation of their mobility. In many fish species, whatever their spawning environment (either freshwater or seawater), sperm motility is associated with an osmotic shock when sperm cells are ejaculated into the surrounding fluid. Generally, osmolality of seminal fluid is around 300 $mOsmol.kg^{-1}$ and thus, is referred to isotonic conditions. Thus, freshwater fish spermatozoa motility activates in hypotonic water (<300 $mOsmol.kg^{-1}$) and in marine in hypertonic water (>300 $mOsmol.kg^{-1}$), and show the highest motility at approximately 1000–1100 $mOsmol.kg^{-1}$, which is almost equivalent to that of seawater (Morisawa and Suzuki, 1980; Oda and Morisawa 1993). For fishes with ionic mode of sperm motility activation, alteration of osmolality in physiological values does not seem to play a major role in motility activation or in duration of movement, nor in fertilization ability (Alavi and Cosson, 2006). In contrast for fishes with osmotic mode of sperm activation, it is precisely the osmotic shock that provide the key role for triggering spermatozoa motility (Morisawa M. and Suzuki 1980). Carp sperm motility can be fully initiated in media of osmotic pressure below 150–200 $mOsmol.kg^{-1}$ (Menezo et al., 1983; Perchec et al., 1995; Perchec et al., 1996). However, spermatozoa activation under natural osmotic conditions (15–30 $mOsm.kg^{-1}$), distilled water (below 10 $mOsm.kg^{-1}$) or urine, leads to changes in morphology and capacity for movement (Perchec et al., 1995; Perchec et al., 1996). Environmental hypotonicity leads to significant carp spermatozoa swelling during motility (Dzuba and Kopeika, 2002; Dzuba et al., 2001; Perchec et al., 1996). The water flux might lead to decreasing the intracellular ion concentration, consequently altering the membrane potential. It was shown that hypo-osmotic shock leads to membrane potential alteration with subsequent opening of the voltage-gated potassium channels of carp spermatozoa (Krasznai et al., 1995). Further decrease of intracellular $K^+$ leads to membrane potential changes that provoke an activation of stretch-dependent $Ca^{2+}$ channels (Krasznai et al., 1995; Krasznai et al., 2003b). However, it was previously reported that intracellular $Ca^{2+}$ as a second messengers is not crucial for initiation of carp sperm motility (Perchec Poupard et al., 1997). This set of results allows us to suggest that carp sperm motility is under control of membrane potential alteration which appears because of water influx with further dilution of intracellular ions concentration from

one side and alteration of cell volume (and activation of stretch-dependent ion channels) from another.

It was shown in case of some freshwater fish species, that hypo-osmotic induction of sperm motility is accompanied by a reorganization of the membrane structure and by hyperpolarization of the cell membrane (Krasznai et al., 2003a). We predict that sperm swelling at the moment of activation could result from water transport through aquaporins because water diffusion, without intervention of the latter, is a much slower process (Verkman et al., 1993). Thus, activation of stretch-dependent channels would constitute a second step in motility activation of carp spermatozoa that would follow the water transport step through aquaporins. A similar measure of spermatozoa activation was proposed for turbot (Cosson et al., 2008).

Anadromous fish species (fishes that are migrates form marine to fresh water for spawning) such as salmonids and sturgeons, possess ionic mode of sperm motility activation (Alavi and Cosson, 2006). It was previously discussed, that spermatozoa of these species are incapable of volume changes during their motility period (see 1.3.1). Thus, activation of stretch-dependent channels seems to be impossible because of the absence of detected stretch during motility activation and whole period of motility. Sperm capacity to maintain a constant volume under hypotonic conditions remains unclear. It was previously described, that AQP are involved in motility of sea bream and trout sperm motility (Takei et al., 2015; Zilli et al., 2011). However, it is still unknown if AQPs transport water via or against the concentration gradient. Additionally, water influx, as a result of osmosis, could be partially overcomed by activity of co-transporters.

Euryhaline fishes like some tilapias (fish that can acclimate to wide range of salinities, from freshwater to seawater or even higher salinity) possess unique sperm osmotic sensitivity. It was shown that tilapia sperm could modulate their regulatory mechanism to suit fertilization in either high or low salinity (Legendre et al., 2008). As a result of tilapia acclimation to environmental salinity, spermatozoa sensitivity to osmotic stress and external ion composition are altered. Thus, seawater acclimated tilapia sperm requires $Ca^{2+}$ for motility activation in hypertonic media (seawater), whereas in freshwater acclimation, tilapia sperm could not swim in hypertonic conditions even in the presence of $Ca^{2+}$ (Morita et al., 2003). Linhart et al. (1999) reported that during acclimation of the fish from freshwater to seawater, tilapia sperm adapt to conditions of high salinity by losing their $Ca^{2+}$ independent pathways of sperm motility activation (Linhart et al., 1999).

Altogether, the osmotic shock, that is always accompanying spermatozoa activation in fishes with external fertilization, also involves ionic components in the activation cascade. Moreover, impact of ions in spermatozoa motility is species-specific and requires further studies.

### 1.2.3. Sperm volume changes in the course of cryopreservation

Storage of fish sperm at low temperatures in a frozen state (cryopreservation), for *in vitro* gamete manipulation procedure has high potential in aquaculture and biodiversity conservation measures (Cabrita et al., 2010). In the course of freezing and thawing (required for application of cryopreserved sperm) spermatozoa are exposed to an extreme range of fluctuating osmotic conditions which influences the success (Watson and Fuller, 2001). Cryopreservation processes lead to extracellular ice crystals formation with sudden increase in solute concentration within unfrozen pockets of liquid and consequently form hypertonic micro-environments. This in turn leads to cell shrinkage (Gao et al., 1993) and distortion of the molecular and cytoplasmic organization of organelles. The thawing process and removal of the cryoprotective agent cause cell swelling since the osmotic environment is restored, and

an influx of water across the sperm plasma membrane leads to further damage of an already compromised cell, causing loss of membrane integrity and motility (Curry and Watson, 1994, Gao et al., 1993; Gao et al., 1995). Several studies have indicated that harmful the osmotic injury occurs during warming and rehydration rather than cooling (Watson and Fuller, 2001). This may be the result of the inability of the cell membrane to cope with rapid changes in volume which disrupt cytoskeletal elements (Watson and Fuller, 2001). Volume regulation must be functional during the challenges to maintain membrane integrity. However, the resistance of cells to rapid alteration of environmental temperature and osmolality during cryopreservation is species-specific. Particularly important are differences in plasma membrane composition that can affect membrane permeability to water, ions and solutes and the lipid phase transition temperature (Holt, 2000). Since each step of the cryopreservation process forces spermatozoa to cope with drastic osmotic challenges and potentially large changes in cell volume, understanding of mechanisms controlling sperm shrinkage-swelling regulation in general could prospectively help to improve post-thaw motility and spermatozoa viability.

Negative effects of hypotonic treatment in mammalian cells before freezing have been shown to affect cryoresistance (Pegg and Diaper, 1991). It was previously mentioned, that physiological activity of freshwater fish sperm took place in conditions of hypotonic environment contrary to mammals spermatozoa. Moreover, rapid increase in membrane fluidity in response to hypotonicity has been observed in carp sperm (Krasznai et al., 2003b), which could be favorable for sperm surviving water/cryoprotectant fluxes occurring during freeze-thawing and following fertilization in hypotonic condition. This speculation is also supported by observation made in rainbow trout, in which increased membrane fluidity was correlated with higher post-thaw sperm fertilizing ability (Ciereszko et al., 2011). We propose that carp sperm volume increase, which leads to sperm membrane modification (increased fluidity), could lead to improve the spermatozoa ability to survive freeze-thawing processes.

## 1.3. Mechanisms of ion transport

In all cells, ion transport occurs via specific ion-permeable protein pores (ion channels) in the lipid membranes. Like other signaling proteins, ion channels are flexible molecules that undergo conformational changes between open (active) and closed (inactive) states. Channels are opened chemically by specific agents such as neurotransmitters, $Ca^{2+}$ ions, and cAMP, mechanically by stretching the membrane, or electrically by changing the transmembrane voltage. However, the detailed molecular mechanisms of opening/closing processes, as well as transport or exchange of ions, are poorly understood.

Ion channels are incredible catalyzers of ion transport through the nonconducting lipid bilayer. Each ion channel allows the flow of millions of ions per second suggesting that just a few of them can induce substantial electric and concentration changes in a small cell, such as the sperm (Hille, 1992). Ionic gradients across cells not only determine membrane potential through ion selective channels, but permeant ions can modulate enzymes and further influence channel activity, causing additional membrane potential changes and ion flow (Hille 1992).

The largest superfamily of ion channels consists of tetrameric voltage-gated cation channels and their relatives (Armstrong and Hille, 1998; Hille et al., 1999). Voltage-gated channels are exclusively sensitive to small changes in membrane potential. Thus, alteration of membrane potential results in activation of voltage-gated channels leading to transport of ions. It has been shown, that opening of $Na^+$ voltage-dependent channel lead to $Na^+$ influx with further polarization of membrane. After inactivation of $Na^+$ channel, opening of $K^+$ voltage-dependent channel occurs following $K^+$ efflux, driving the membrane voltage back (Armstrong, 2003).

Voltage-gated $Ca^{2+}$channels are responsible for $[Ca^{2+}]_i$ increases induced by membrane potential changes with further $Ca^{2+}$ influx from environment or efflux from the intracellular stores.

Except channels activated by alteration of membrane potential, mechanically sensitive (stretch activated) channels are ubiquitous in plant and animal cells (Sachs and Sokabe, 1990, Yang and Sachs, 1993). These channels are implicated in a wide range of responses to mechanical perturbations, including cell volume regulation, increase in intracellular $Ca^{2+}$, cell proliferation, and others (Caldwell et al., 1998). These channels are highly sensitive to membrane tension; they modify the activity of certain membrane proteins (Vandorpe et al., 1994). Mechanically sensitive channels are in most cases action selective, targeted to calcium as well as monovalent cations (Yang and Sachs, 1993).

Prospectively, ion transport can be divided to:

- **Mono-transport.** Transport of one ion or cation via highly selective channel or pump. A mono-transport of $Ca^{2+}$ ions in sperm cells, for example, occurs via voltage-dependent $Ca^{2+}$ channels or Catspers (Darszon et al., 2011); $K^+$ and $Na^+$ mono-transport can also occur via voltage-dependent $K^+$ and $Na^+$ channels respectively (Armstrong and Hille, 1998) that were also detected in sperm (Darszon et al., 1999; Darszon et al., 2011; Darszon et al., 2001).
- **Cotransport.** Transport of at least two different ions or substances in one direction. As an example, $Na^+$–$K^+$–2Cl, $K^+$–$Cl^-$, $Na^+$–glucose could be highlighted (MacAulay and Zeuthen, 2010; Zeuthen, 2010).
- **Ionic exchange.** During ionic exchange transport of influx of one ion accompanied by efflux of another. It was shown that activity of such an exchanger as $Na^+/K^+$ pump, $Na^+/Ca^{2+}$ or $Na^+/H^+$ regulate various processes in sperm physiology of different species (Gatti and Christen, 1985; Jimenez et al., 2010; Vines et al., 2002).

Ion channels are known to be important in sperm physiology (Darszon et al., 2011). Thus, learning how these integral membrane proteins operate and are regulated in sperm is essential to understand fertilization.

## 1.4. The role of ion transport in regulation sperm motility of different fish species

As was previously discussed, sperm motility is initiated because of rapid alteration of environment surrounding i.e. osmolality, ion composition (concentration of $K^+$, $Na^+$ and $Ca^{2+}$), or pH (Alavi and Cosson 2005, 2006). Rapid alteration of environmental ionic composition prospectively results in activation of ion channels and re-equilibration of intracellular ions concentrations. It is well known that transport of ions ($Na^+$, $K^+$ or $Ca^{2+}$) or changes in pH are in a closely related membrane potential alteration (Armstrong and Hille, 1998; Hille, 1967). Thus, description of the ionic regulatory role in fish sperm motility could not be separated from studies of membrane potential alteration.

Previous studies suggest that the involvement of ion transport in fish sperm motility is species specific. Membrane hyperpolarization triggers the activation of ascidian sperm motility through hyperpolarization-induced direct activation of adenylyl cyclase to synthesize cAMP (Izumi et al., 1999). Changes in the concentrations of specific ions such as $Ca^{2+}$, $K^+$, and possibly $Na^+$ and $Cl^-$ also have been linked to motility initiation in Atlantic croaker sperm (Detweiler and Thomas, 1998; Thomas et al., 1998). In herrings an efflux of $Na^+$ and an influx of $Ca^{2+}$ partially controls the motility initiation process and occurs via reverse-$Na^+/Ca^{2+}$ exchanger present on the sperm surface (Vines et al., 2002).

In cyprinids, triggering mechano-sensitive ion channels activation is caused by changes of environmental osmolality, leading to membrane polarization and induction of $Ca^{2+}$ release from intracellular stores and initiation of sperm motility through a calmodulin cascade signaling (Krasznai et al., 2000; Krasznai et al., 2003a, b).. In salmonids, membrane hyperpolarization due to transmembrane $K^+$ efflux results to a membrane hyperpolarization and an increase in intracellular calcium ($[Ca^{2+}]_i$) because of $Ca^{2+}$ influx through dihydropyridine-sensitive $Ca^{2+}$ channels (Boitano and Omoto, 1991; Darszon et al., 1999; Gatti et al., 1990; Kho et al., 2001). It is well known that extracellular potassium ($[K^+]_{ext}$) has inhibitory effect on sturgeon and salmonid sperm motility activation by suppressing $K^+$ efflux (Alavi and Cosson, 2006). Since the inhibition of sperm motility by $K^+$ can be overcome by increased external $Na^+$ concentration in Acipenseridae (Li et al., 2012) and Salmonidae (Baynes et al., 1981) sodium transport seems to have a crucial but still unclear role in fish sperm motility. As a prediction, efflux of $K^+$ leads to hyperpolarization of the plasma membrane, but, because of the absence of external $Ca^{2+}$ and $Na^+$, a breakdown of the membrane potential could appear. These results, as well as evidence from previous research on sperm and other cell types (Armstrong and Hille, 1998; Baynes et al., 1981; Darszon et al., 1999; Li et al., 2012), allows prediction that the membrane potential alteration would not be ion specific. Alternatively, alteration of membrane potential required for sperm motility activation could be reached by competitive ion transport. However, the interaction between ions transport on the one hand and membrane potential alteration on the other hand remains to be better understood, knowing that membrane potential represent a key factor controlling sperm motility.

## 1.5. Osmotic and ionic agents in fish sperm maturation

Development of spermatozoa in fishes from spermatogonia through spermiogenesis, occurs through a series of cytological stages (Hoar, 1969). During this process, spermatogonia proliferate by repeated mitoses, and then once meiosis is initiated spermatocytes, the cells grow and develop into secondary spermatocytes. As a result of the secondary spermatocyte division, spermatids are produced and metamorphose into potentially functional spermatozoa.

During spawning, spermatozoa in the testis are morphologically developed, but, in some species they are still lacking the capability for motility activation and fertilization. The development from non-functional gametes to mature spermatozoa capable of motility and fertilization constitutes sperm maturation, a process of physiological but not morphological alteration. In salmonids, spermatozoa acquire motility during passage from the testes along the sperm duct (Morisawa and Morisawa, 1986) and high concentrations of bicarbonate ions and a higher pH (7.8–8.15) are important factors that promote the acquisition of motility (Morisawa and Morisawa, 1988). Acquisition of sperm motility in response to an increase in pH was also confirmed by studies *in vitro* in chum salmon, rainbow trout (Morisawa and Morisawa, 1988), and masu salmon (Miura et al., 1995).These results strongly suggest that increases of environmental pH are essential for acquisition of motility by spermatozoa. Acquisition of motility of Japanese eel spermatozoa requires $K^+$ and $HCO_3$ ions or $K^+$ ions and a high pH (Ohta et al., 1997). An increase in the concentration of $HCO_3$ ions an essential factor for the acquisition of motility under physiological conditions. These reports suggest that the ionic constituents of testicular fluid might be involved in the acquisition of motility of teleostean sperm, in addition to pH.

In the sturgeon, the efferent ducts coming from the testis directly contact the kidneys. Thus, testicular sperm present in Wolffian seems to be diluted by urine. Such an arrangement might lead to decreased semen osmolality and low concentration of ions and proteins in

seminal fluid additionally to the low sperm concentration – the characteristics specific for sturgeons (Piros et al., 2002). However, dilution of testicular sperm by urine and its role in spermatozoa maturation remains to be speculative and needs to be investigated.

Altogether, ionic and osmotic regulation of sperm maturation process in fishes is still poorly investigated and requires further in depth studies.

## 1.6. Aims of the thesis

The main aims of present study were:

1. To investigate the interspecific differences in spermatozoa respond to the osmotic stress in fishes with osmotic and ionic mode of sperm motility activation.
2. To use hypotonic treatment prior to freezing for improvement of spermatozoa survival and motility rate together with fertilizing ability after fish sperm cryopreservation.
3. To study the role of environmental osmolality and ion composition in sperm motility.
4. To investigate the role of ions in spermatozoa maturation and seasonal changes

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## CHAPTER 2

### VOLUME CHANGES DURING THE MOTILITY PERIOD OF FISH SPERMATOZOA: INTERSPECIES DIFFERENCES

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# Volume changes during the motility period of fish spermatozoa: Interspecies differences

Olga Bondarenko [a,*], Borys Dzyuba [a], Jacky Cosson [a], Gunes Yamaner [b], Galina Prokopchuk [a], Martin Psenicka [a], Otomar Linhart [a]

[a] *Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic*
[b] *Istanbul University, Faculty of Fisheries, Aquaculture Department, Beyazit/Eminonu, Istanbul, Turkey*

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ABSTRACT

The aim of this study was to describe spermatozoa volume changes during the motility period of fish species with either osmotic (common carp *Cyprinus carpio*) or with ionic (sterlet *Acipenseri ruthenus* and brook trout *Salvelinus fontinalis*) modes of motility activation. Nephelometry, light microscopy, and spermatocrit methods were used for quantitative assessment of cell volume changes in media of different osmolalities. Significant correlation ($R^2 = 0.7341$; $P < 0.001$) between parameter of volume changes measured using nephelometry and light microscopy methods confirmed nephelometry as a sufficiently sensitive method to detect changes of spermatozoa volume. The spermatocrit alteration method resulted in a large proportion of damaged and potentially immotile spermatozoa in media of osmolality less than 150 mOsm/kg in carp and osmolalities from 10 to 300 mOsm/kg in sterlet and brook trout. Therefore, this method is not reliable for assessing spermatozoa swelling in hypotonic solutions, because the integrity of the cells is not fully preserved. Increase in carp spermatozoa (osmotic activation mode) volume occurred during the motility period in hypotonic conditions, but no indications of volume changes were found in sterlet and brook trout spermatozoa (ionic activation mode) associated with environmental osmolality alteration. Accordingly, we conclude that sperm volume changes are differentially involved in the motility activation process. Species-specific differences in spermatozoa volume changes as a response to a hypotonic environment during the motility period are discussed in relation to their potent physiological role.



## 1. Introduction

Decreasing osmolality and changes in ion concentrations of the surrounding media are triggers for activation of freshwater fish spermatozoa [1–4]. Under natural conditions, a hypoosmotic environment is essential for spermatozoa activation and for fertilization [5], and partial sperm membrane lysis could occur at initiation of motility and up to its arrest [6] because of the large amplitude of the osmotic stress.

The reaction of spermatozoa to osmotic shock varies among fish species, primarily depending on their marine or freshwater environment. In freshwater species, very low ambient water osmolality is directly involved in spermatozoa motility activation in carp (*Cyprinus carpio*) [7,8], and in sturgeon and salmonids, activation can be achieved without osmolality alteration (as compared with seminal fluid) through changes in ionic composition of the surrounding medium [3]. Therefore, in general, spermatozoa motility activation mechanisms in freshwater fish can be

* Corresponding author. Tel.: +420 773 559 118.
*E-mail address:* toolgab@gmail.com (O. Bondarenko).

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classified either as the osmotic mode or, if osmolality independent, as ionic mode.

The mechanism by which spermatozoa motility activates through osmotic pressure change is not fully understood. Osmolality-mediated mechanosensitive calcium channels have been shown to be involved in some fish species [9]. Environmental osmolality reduction has also been demonstrated to lead to cell volume increase [7]. We suggest that an increase of sperm cell volume might generate a signal responsible for alteration of mechanosensitive channel activity [9]. Spermatozoa swelling in hypotonic conditions could play an unequal role depending on the fish species sperm motility signaling mode. If swelling of spermatozoa is assumed to play a key role in motility activation, it should occur very quickly. Motility in freshwater fish spermatozoa should be initiated in less than 1 second after transfer of sperm cells into a hypotonic environment as previously predicted [10].

Information about spermatozoa swelling in fish is scarce, and only a few attempts to evaluate volume changes as a response to environmental osmolality have been conducted, using methods such as spermatocrit [7], light microscopy [7], spectrophotometry [11,12], electron paramagnetic resonance (EPR) [13], coulter counting [14], and resistance impulse spectroscopy [6].

Use of any of these methods allows detection of increases in spermatozoa volume during their motility period in carp (osmotic mode of activation). The level of swelling is dependent on the ambient aquatic osmolality and occurs not only at activation but also progressively throughout the motility period [11,12].

In species with an ionic mode of activation, spermatozoa swelling has been detected using the resistance impulse spectroscopy method. Cabrita et al. [6] demonstrated that rainbow trout (*Oncorhynchus mykiss*) spermatozoa swelled immediately after transfer into a hypotonic nonactivating environment; however, the level of volume increase was independent of the surrounding osmolality and incubation period.

Because of the brief period of fish spermatozoa motility, and studies of changes in their volume are technically complicated, a combination of techniques is recommended for volume change detection at activation and during the motility period.

The goal of the present study was to comparatively describe sperm cell volume changes during motility in fish species with ether osmotic or ionic modes of motility activation.

## 2. Materials and methods

### 2.1. Broodfish and sperm collection

Fish were maintained in facilities of the Genetic Fisheries Center, and experiments were carried out in the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic.

Spermiation in common carp (*Cyprinus carpio*) and sterlet (*Acipenseri ruthenus*) was stimulated by temperature manipulation followed by hormone treatment. Sperm from brook trout (*Salvelinus fontinalis*) was obtained during the natural reproduction season without hormone intervention.

Five mature sterlet males (1.2–1.8 kg) were kept in a 400 L indoor tank with water temperature gradually increasing from 7 °C to 15 °C. After a 14-day acclimation period, the fish were intramuscularly injected with carp pituitary suspension at 4 mg/kg of body weight. Milt was collected from the urogenital tract 36 hours after injection using a catheter, and samples were kept on ice during the experiments for 2 hours or less.

Five mature common carp males (2.5–3 kg) were maintained in 1000 L tanks at a temperature of 18 °C for 14 days before injection with carp pituitary extract at a dose of 1 mg/kg of body weight. Sperm collection was carried out 24 hours after injection. Milt was obtained using abdominal massage directly into 20-mL plastic syringes.

Five mature brook trout males (300–500 g) were maintained in a 10,000 L outdoor hatchery tank. Water temperature ranged from 4 °C to 8 °C. Milt was obtained by abdominal massage directly into 10-mL plastic syringes. Special care was taken to avoid contamination by urine, mucus, feces, or water during carp and brook trout sperm collection [15].

Only the samples meeting the following criteria were used: motility 90% to 100% initiated using water from tanks and estimated using routine methods [16] and osmolality of seminal fluid for carp 275 to 290 mOsm/kg, for sterlet 35 to 65 mOsm/kg, and for brook trout 280 to 295 mOsm/kg.

### 2.2. Media used for experiments

For evaluation of sperm cell volume changes, solutions with osmolality of 300, 250, 200, 150, 100, 50, and 10 mOsm/kg were prepared including 10 mM TRIS-HCl, pH 8.0, and NaCl. Osmolality of each solution was monitored using a vapor pressure osmometer (Wescor).

### 2.3. Light microscopy

Sperm was added to a drop of the experimental medium (300, 250, 200, 150, 100, 50, and 10 mOsm/kg were prepared including 10 mM TRIS-HCl, pH 8.0, and NaCl) on a microscope slide using the tip of a dissecting needle with which the sperm suspension was thoroughly mixed for 2 seconds. Immediately after dilution, the cell suspension was video-recorded using a negative phase contrast microscope (Olympus BX50, Olympus Plan 40× lens) equipped with CCD video camera (Sony, SSCDC50AP) until cessation of motility. Twenty to 30 spermatozoa were evaluated per each frame. Measurements were conducted in triplicate.

### 2.4. Scanning electron microscopy

Sperm of five sterlet males were prepared for observation in a cryo-field emission scanning electron microscope (FESEM, CryoSEMs) JSM 7401F (Jeol Ltd., Tokyo, Japan). Milt was prediluted (1:10) with 10 mM TRIS-HCl or 100 mOsm/kg NaCl. After a 60-second incubation period, 10 μL of diluted sperm was placed on the aluminum target and excess liquid drained. The specimen was then frozen by

plunging into liquid nitrogen and immediately transferred in a vacuum to the chamber of the cryo-attachment CryoALTO 2500 (Gatan). Vacuum sublimation was performed at −90 °C for 10 minutes to evaporate liquid from the surface of the specimen, and the specimen was stained with platinum/palladium at −140 °C for 2 minutes. The frozen specimen was observed in an FESEM JEOL 7401F operated at 1.5 kV with a working distance of approximately 6 mm and a stage temperature of −135 °C.

Sperm samples from five male brook trout were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 48 hours at 4 °C, washed repeatedly, postfixed in 4% osmium tetroxide for 2 hours at 4 °C, washed repeatedly, and dehydrated through an acetone series (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 15 minutes at each step [14].

Dehydrated samples for scanning electron microscopy were dried in a critical point dryer (Pelco CPD 2; Ted Pella, Inc., Redding, CA, USA), coated with gold in a vacuum with a scanning electron microscope coating unit (E5100; Polaron Equipment Ltd.), and observed using the same electron microscope [17,18].

### 2.5. Spermatozoa volume measurements

#### 2.5.1. Sperm volume calculation from scanning electron and light microscopy images

Video frames from light microscopy and scanning electron micrographs were analyzed using Olympus MicroImage 4.0.1. for Windows to estimate spermatozoa head volume in all solutions studied.

Mathematical calculations of spermatozoa head volume were conducted based on species-specific head shape. Volume of carp spermatozoon, with a spherical head, was measured using the formula:

$V = R3 \times (4 \times \pi)/3$ where R is the radius of the head image.

Sterlet and brook trout spermatozoa have a frustum-shaped head and were measured using the formula:

$V = \pi \times L \times (r_{12} + r_1 \times r_2 + r_{22})/3$ where L is the length of the frustum, and $r_1$ and $r_2$ are the radii of the upper and lower bases of the frustum. Details for obtaining R, L, $r_1$, and $r_2$ measurements are presented in Figure 1.

In carp and brook trout, the relative spermatozoa head volume change was calculated from light microscopy observations ($V/V_{0(micro)}$) using the formula:

$$V/V_{0(micro)} = V_m/V_{300}$$

where $V_m$ is the volume of the spermatozoa head at 60-second incubation in the studied media for carp and after cessation of motility for brook trout; $V_{300}$ is the volume of the head in a 300 mOsm/kg solution.

For sterlet, the $V/V_{0(micro)}$ was calculated using the formula:

$$V/V_{0(micro)} = V_m/V_{100}$$

where $V_m$ is the volume of the head after cessation of motility in the studied media and $V_{100}$ is the volume of the head in 100 mOsm/kg solution.

Scanning electron microscopy images were used to calculate the volume of spermatozoa using the same method of evaluation in two critical situations: isotonic solution (300 NaCl + 10 mM Tris-HCl, pH 8.0) for carp and brook trout spermatozoa, light hypertonic (100 NaCl + 10 mM Tris-HCl, pH 8.0) for sterlet, and hypotonic conditions (10 mM Tris-HCl, pH 8.0) for all studied fish species.

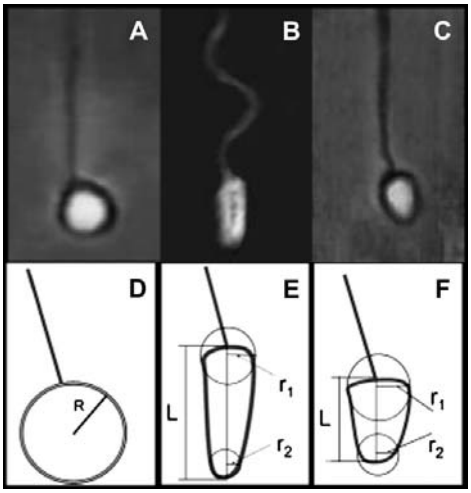


**Fig. 1.** Light microscopy images of spermatozoa (A–C) and graphic depictions of spermatozoa head parameter calculation (D–F): (A) and (D), carp; (B) and (E), sterlet; (C) and (F), brook trout. The head contour is shown as a thick line in each image. The limit of the head was considered to be the center of the thick line. L, $r_1$, and $r_2$ are geometric parameters measured for head volume calculations.

#### 2.5.2. Nephelometry

Light absorbance measurements of cell suspension were conducted using a spectrophotometer (SPECORD 210; Analytic Jena AG) equipped with a thermostat-controlled cell chamber in a 1-cm light path cuvette. A wavelength of 500 nm was chosen because of the absence of stain in spermatozoa at this wavelength. The absorbance values present inaccuracy of less than 0.015% at a constant temperature of 20 °C. The dilution rate of the sperm suspension was varied according to the species and was adjusted to 0.5 initial absorbance in all cases. Immediately after adding sperm to the study solution, the cuvette was placed in the spectrophotometer. This allows accurate recording of the dynamics of optical density changes as a function of time. For each osmotic condition, three repetitions of optical density change records were run for each of the sperm samples. Variation in absorbance (or light diffusion) value linearly correlates with variation in optical density of the sample; therefore, with nephelometry, alteration in optical density reflects the volume change of cells [11].

The value of relative change in absorbance ($A_m$) was considered indicative of volume changes [11,12] and calculated according to the equation:

$$A_m = (A_0 - A_1)/A_0$$

where $A_0$ is the value of initial absorbance immediately after adding sperm to the solution, and $A_1$ is the value of absorbance after the time period needed to reach a plateau (approximately 60 seconds) for carp spermatozoa and at the cessation of motility for sterlet (120 seconds) and brook trout (40 seconds).

*2.5.3. Spermatocrit alteration measurements*

Sperm of sterlet was concentrated using centrifugation for 30 minutes at 300× *g* followed by a 1:1 dilution of the sperm pellet in its own supernatant. The obtained suspension was diluted 1:3 with each of the studied media. Sperm of carp and brook trout was diluted 1:1 with the studied medium. The diluted sperm was centrifuged for 10 minutes at 1500× *g* in a hematocrit tube [7]. Relative sperm volume evaluated using the spermatocrit alteration method was calculated using the formulas:

$$V/V_{0(SpC)} = SpC_m/SpC_{300}(\text{for carp and brook trout}); \text{ and}$$

$$V/V_{0(SpC)} = SpC_m/SpC_{100}(\text{for sterlet})$$

where $SpC_m$ is spermatocrit in the studied media and $SpC_{100}$ and $SpC_{300}$ are spermatocrit in 100 and 300 mOsm/kg solutions, respectively.

*2.6. Statistical analysis*

Sperm from five carp, five sterlet, and five brook trout were analyzed. At least three repetitions of optical density changes (nephelometry), video records (microscopy), and spermatocrit alteration were made for sperm in solutions of differing osmolalities. Morphological parameters of 30 images of sterlet and brook trout spermatozoa heads were measured in each experimental medium. All analyses and plotting were conducted using Statistica 10 computer program (Statsoft Inc.). All values are presented as the mean values ± SD. The values of parameters were checked for distribution characteristics and homogeneity of dispersion using the Shapiro–Wilk and Levene tests, respectively. Sperm head volume changes, measured using video microscopy, showed normal distribution with similar dispersion values. Parametric ANOVA was applied, and Tukey honestly significant difference test was used to identify differences among subgroups. Nonparametric statistics using the Kruskal–Wallis test followed by the Mann–Whitney *U* test were conducted for comparison among groups for results obtained using nephelometry, spermatocrit, and cryo-field emission scanning electron microscope (CryoSEM) methods because of nonnormal distribution of values. Correlation analysis with calculation of parameters $R^2$ and P for $R^2$ were performed on data from carp spermatozoa using Pearson analysis for normally distributed (sperm head volume changes, measured by video microscopy) and Spearman analysis for nonnormally distributed data (results obtained using nephelometry, spermatocrit, and CryoSEM methods).

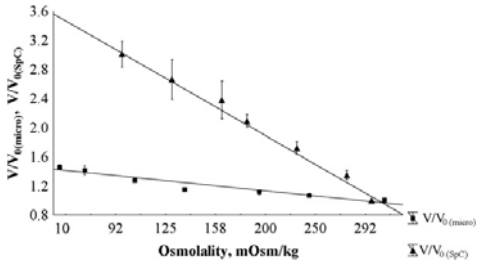


**Fig. 2.** Relative volume of carp spermatozoa measured using spermatocrit and relative spermatozoa head volume measured using microscopy in media of differing osmolalities. Values are presented as mean ± SD. $V/V_{0(SpC)}$: $R^2 = 0.9974$; $P < 0.001$; $V/V_{0(micro)}$: $R^2 = 0.9605$; $P < 0.001$. $V/V_{0(micro)}$, relative spermatozoa head volume measured using microscopy; $V/V_{0(SpC)}$, relative volume of carp spermatozoa measured using spermatocrit.

## 3. Results

*3.1. Relative spermatozoa head volume calculation using light microscopy and spermatocrit alteration*

The spermatocrit and head volume of carp spermatozoa increased with decreasing osmolality of the solution.

A significant linear relationship of environmental osmolality with cell volume calculated using spermatocrit ($V/V_{0(SpC)}$: $R^2 = 0.9974$; $P < 0.001$) and microscopy ($V/V_{0(micro)}$: $R^2 = 0.9605$; $P < 0.001$) was determined (Fig. 2).

Therefore, a strong correlation ($R^2 = 0.9475$; $P < 0.001$) of spermatozoa volume, as measured using spermatocrit alteration, with spermatozoa head volume, as measured using microscopy, was observed (Fig. 3).

Changes in spermatocrit were investigated in sterlet sperm in NaCl solutions of osmolalities of 100, 50, and 0 mOsm/kg and in brook trout sperm in 300, 100, and 0 mOsm/kg NaCl. Significant differences in spermatocrit in solutions with different osmolalities (data not shown) were

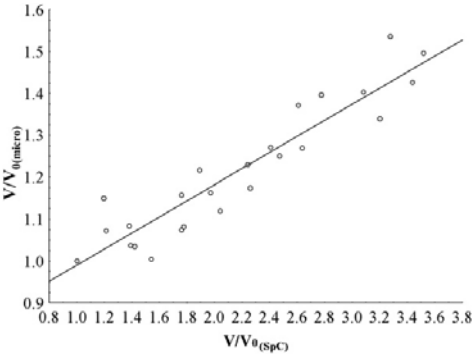


**Fig. 3.** Correlation of carp spermatozoa volume changes measured using microscopy and spermatocrit alteration methods. $R^2 = 0.9475$; $P < 0.001$. $V/V_{0(micro)}$, relative spermatozoa head volume measured using micrroscopy; $V/V_{0(SpC)}$, relative volume of carp spermatozoa measured using spermatocrit.

not detected in either sterlet ($P = 0.7788$, Kruskal–Wallis test) or brook trout ($P = 0.2786$, Kruskal–Wallis test).

More detailed investigation of the spermatocrit alteration method demonstrated that after 5 minutes of incubation in the studied low osmolality solutions, a large proportion of sperm cells collapsed. After centrifugation in capillaries with solutions of osmolality less than 150 mOsm/kg, the carp sperm pellet was not homogeneous and split into two clearly visible layers. In sterlet and brook trout sperm, heterogeneous pellets were obtained in all studied solutions.

Light microscopy showed all layers of the pellet to comprise a large proportion of damaged cells whatever the species (Fig. 4).

### *3.2. Relative sperm volume measured using light microscopy and nephelometry*

Curves of relative absorbance changes and spermatozoa head volume changes were obtained for carp (Fig. 5), sterlet (Fig. 7), and brook trout (Fig. 10).

Significant changes ($P < 0.001$) were seen in carp spermatozoa head volume and absorbance in solutions of differing osmolalities. Linear relationship between environmental osmolality and carp spermatozoa head volume measured using microscopy ($V/V_{0(micro)}$: $R^2 = 0.835$; $P < 0.001$) and linear relationship between environmental osmolality and absorbance ($A_m$: $R^2 = 0.7084$; $P < 0.001$) were observed (Fig. 5B).

For better understanding of the correlation between alteration in absorbance parameter and spermatozoa head volume changes, a correlation curve of $A_m$ versus $V/V_{0(micro)}$ was plotted (Fig. 6).

The high coefficient of linear regression ($R^2 = 0.7341$) and low P-value ($P < 0.001$) suggests a strong correlation between light absorbance alteration and carp spermatozoa head volume change.

In contrast, significant differences in relative absorbance $A_m$ ($P = 0.3679$) and sterlet spermatozoa heads $V/V_{0(micro)}$ ($P = 0.1153$) in solutions of differing osmolalities were not detected (Fig. 7).

To confirm the absence of spermatozoa head volume changes for sterlet in solutions of differing osmolality, $V/V_{0(micro)}$ was calculated from CryoSEM micrographs (Fig. 8). Again, no significant differences ($P = 0.3946$) between $V/V_{0(micro)}$ in hypotonic (10 mM TRIS-HCl) and

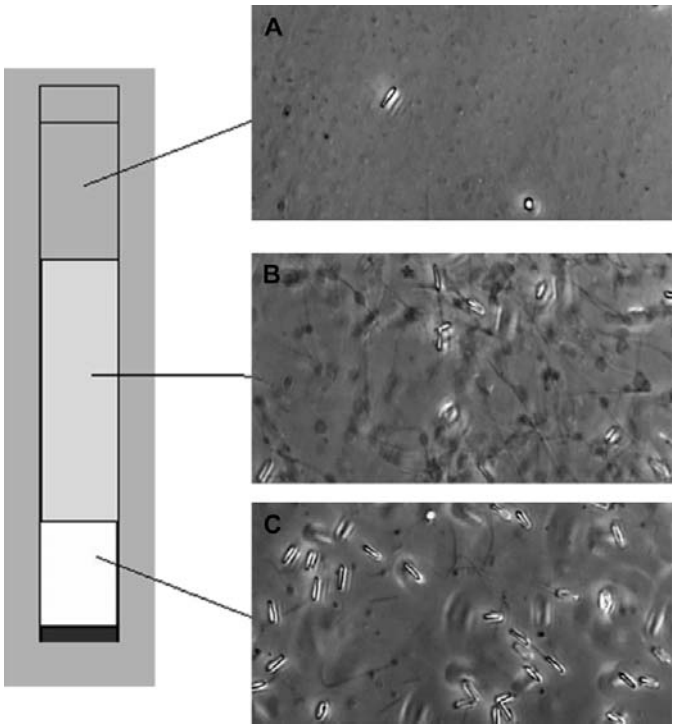


**Fig. 4.** Typical view of the layers in the hematocrit capillary after spermatocrit alteration measurements in sterlet sperm: (A), (B), and (C) represent supernatant, upper fraction of the pellet, and lower fraction of the pellet, respectively. (A) Fluid containing low numbers of heads, flagella, and whole spermatozoa. (B) Upper layer of the pellet containing mainly damaged spermatozoa and intact spermatozoa that show no motility when transferred to the activation media. (C) Lower layer of the pellet containing mainly heads of damaged spermatozoa devoid of flagella and some intact, but immotile spermatozoa.

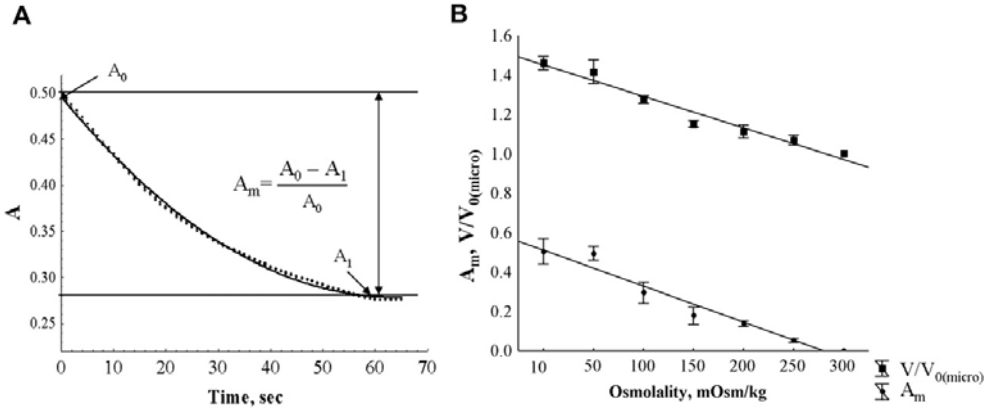


**Fig. 5.** (A) Schematic of dynamics of optical density in carp sperm suspended in hypotonic solution. Values $A_0$, $A_1$, and the formula for calculation of relative change in absorbance ($A_m$) are presented. (B) Relative volume of carp spermatozoa measured using microscopy and $A_m$ in media of different osmolalities. Values are presented as mean ± SD. $A_m$: $R^2 = 0.7084$; $P < 0.001$. $V/V_{0(micro)}$: $R^2 = 0.835$; $P < 0.001$. $V/V_{0(micro)}$, relative carp spermatozoa measured using microscopy.

low hypertonic (100 mOsm NaCl) solutions were found (data are not shown).

Absorbance changes and spermatozoa head volume changes were obtained for brook trout spermatozoa. No significant differences in $V/V_{0(micro)}$ ($P = 0.1566$) and $A_m$ ($P = 0.6205$) in solutions of different osmolalities were observed (Fig. 9).

Light microscopy observations during the motility period showed flagella loops and/or bubbles and blebs in carp, sterlet, and brook trout spermatozoa (Fig. 10).

To understand the nature of these loops and/or bubbles and their influence on alteration of light absorbance of sperm in hypotonic conditions, scanning electron microscopy was used in the case of brook trout spermatozoa (Fig. 11).

Figure 11 clearly shows flagella structures already detected using light microscopy corresponding to curled and looped flagella appearance during the motility period in hypotonic conditions.

More details of the brook trout spermatozoa head and flagellum structure were obtained using scanning electron microscopy (Fig. 12). However, the specific shape of trout spermatozoa heads could not be described using a simple geometric formula.

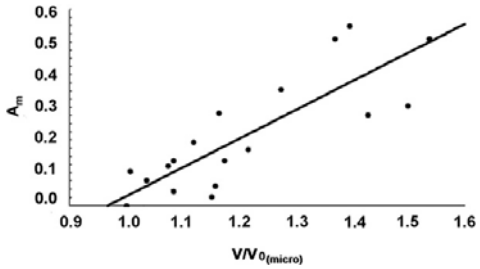


**Fig. 6.** Correlation between relative change in absorbance ($A_m$) and $V/V_{0(micro)}$. $R^2 = 0.7341$; $P < 0.001$. $V/V_{0(micro)}$, relative carp spermatozoa head volume measured using microscopy.

## 4. Discussion

### 4.1. Methods of cell volume change detection

We used three previously described methods to measure cell volume change. The results obtained using nephelometry in combination with those using spermatocrit alteration and light microscopy produced a satisfactory explanation of the fish sperm swelling process.

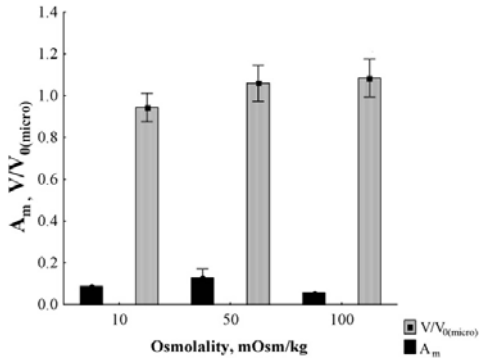


**Fig. 7.** Relative volume of sterlet spermatozoa measured using microscopy, and relative absorbance alteration (with respect to initial absorbance) methods in media of different osmolalities. Average values ± SD are presented; no significant difference between values of $A_m$ ($P = 0.3679$) and $V/V_{0(micro)}$ ($P = 0.1153$) are shown. $A_m$, relative change in absorbance; $V/V_{0(micro)}$, relative volume of sterlet spermatozoa measured using microscopy.

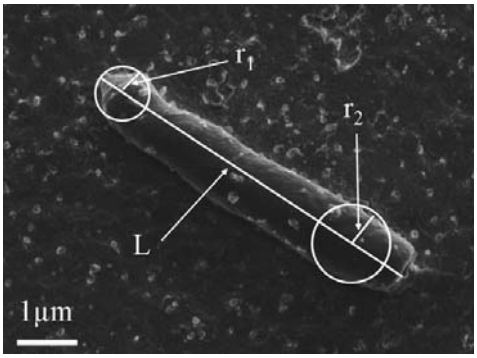


**Fig. 8.** Scanning electron micrograph (CryoSEM) of the sterlet spermatozoa head observed in a cryo-field emission microscope. L, $r_1$, and $r_2$ are geometric parameters measured for head volume calculations. For more detailed description see 2. Materials and methods.

As previously described, the spermatocrit alteration technique gives complementary information on total sperm volume change in solutions of different osmolalities [7]. We found a high coefficient of correlation ($R^2 = 0.9475$; Fig. 3) between volume changes measured using spermatocrit and that measured using morphology under microscopy in carp. However, the spermatocrit alteration method leads to generation of a large proportion of damaged cells and immotile spermatozoa at osmolalities less than 150 mOsm/kg in carp and at those from 10 to 300 mOsm/kg in sterlet and brook trout spermatozoa. Hence, despite previous reports [7] suggesting that spermatocrit alteration measurements could be used to analyze sperm volume changes, this method appears not reliable for describing spermatozoa swelling in hypotonic solutions, because integrity of cells is not preserved. We suggest that long-term (5-minute) incubation of sperm in a hypotonic solution can induce membrane damage, and that further centrifugation leads to partial sperm cell collapse.

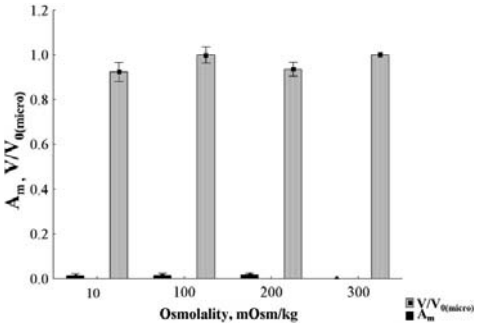


**Fig. 9.** Relative volume of brook trout spermatozoa measured by microscopy and $A_m$ alteration in media of different osmolalities. Average values ± SD are presented. No significant difference between values of $A_m$ ($P = 0.6205$) and $V/V_{0(micro)}$ ($P = 0.1566$) are shown. $A_m$, relative absorbance; $V/V_{0(micro)}$, relative volume of brook trout spermatozoa measured by microscopy.

Light microscopy can be used for cell volume evaluation, although the complex shape of sperm cells, especially the small diameter of flagella, means that only volume of heads can be reliably calculated.

The shape of carp spermatozoa heads can be simply approximated to a sphere. Thus, volume change of carp spermatozoa heads could be calculated using analysis of video records from light microscopy and measurements of the head radius. In contrast to carp, heads of sterlet and brook trout spermatozoa present a more complex nonspherical shape (Figs. 9 and 12). The results obtained from light microscopy indicate that carp spermatozoa heads gradually alter in volume according to environmental osmolality changes (Figs. 2 and 5). Heads of spermatozoa of sterlet and brook trout maintain a constant volume in differing osmotic conditions (Figs. 7 and 9). Therefore CryoSEM micrographs of sterlet spermatozoa heads were used to corroborate the calculations obtained from light microscopy images.

The production of good quality photographs with high resolution is the primary advantage of electron microscopy. However, it presents several disadvantages. Glutaraldehyde fixation, acetone dehydration, and critical point drying [17] are not suitable for evaluation of cell volume change, because they potentially alter spermatozoa volume. Thus, alternative means of spermatozoa fixation should be used for measures of head volume. In the present study, cryofixation was used, because it has been shown to allow cell fixation without affecting volume [17]. This cryofixation method was used for control of results obtained using light microscopy in sterlet spermatozoa.

CryoSEM images could not be used for head volume measurements of brook trout spermatozoa because of their complex ellipsoid shape (Fig. 12), mostly because of the thickness of the spermatozoa head rendering this determination difficult (with light microscopy this was assimilated to a frustum shape; Fig. 1). Thus, volume changes in brook trout spermatozoa were not analyzed using the CryoSEM method but only using the light microscopy method. For measures of global volume change of spermatozoa, other methods should be chosen.

Nephelometry allows determination of changes in sperm suspension light absorbance in solutions of any osmolality and has been reported to be associated with cell volume change [11,12]. High correlation between $A_m$ and $V/V_{0(micro)}$ suggests that $A_m$ can quantitatively describe spermatozoa head volume changes (Fig. 6).

The shape of the spermatozoa flagellum of all three of the species studied was modified during the motility period in hypotonic solutions (Fig. 11). Light microscopy showed changes in flagellum shape with apparent bubbles or loops at their distal tip. Occurrence of these loops was previously described in carp [7]. However, because nephelometry describes volume changes of the total cell and cannot be employed for investigation of specific cell compartments (head or tail), the significance of loops appearing at the tip of a flagellum remains unclear. Bubbles observed using light microscopy correspond to the occurrence of curled loops on flagella during the motility period

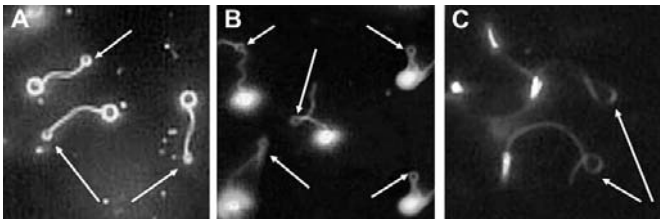


**Fig. 10.** Images of spermatozoa exposed for 40 seconds to hypotonic solution activating motility: (A) carp sperm; (B) trout sperm; and (C) sterlet sperm. Loops and/or bubbles are indicated by arrows.

in hypotonic conditions (Fig. 12). Such loops are, however, reversible using reverse change of osmolality [19] and such revived sperm cells can be sustained in a second round of motility (in trout [20], carp [19,21], and sterlet [22]). Thus we expect that such loops do not affect cell volume changes measured using nephelometry because of their negligible volume. Moreover, no changes of absorbance in brook trout and sterlet sperm occurs when exposed to hypotonic conditions, although these conditions do generate flagellum loops in these species.

The chief advantage of nephelometry is that it allows assessment of dynamic volume changes of cells exposed to a wide range of osmolalities. For this reason we propose the use of nephelometry as a rapid method for determining spermatozoa osmotic resistance.

Several other methods for sperm volume changes measurement have been described, for example EPR [13] and resistance impulse spectroscopy [7,11]. The main advantage of the resistance impulse spectroscopy method is that it can be used to obtain the volume of individual spermatozoa in a suspension. Thus, a large number of cells can be analyzed in a short period of time. A disadvantage of this method is that calibration employs solutions containing spheres of predetermined volume. Spermatozoa are usually not perfectly spherical, and results obtained from resistance impulse spectroscopy correspond to volume of the spermatozoa, but do not take into account the shape of the cell.

The EPR method allows measures of cell volume changes indirectly by measuring alterations in the intracellular quantity of free water [13]. However, several reports give evidence that free water quantity can change without affecting cell volume [23,24]. Change in free water quantity might be reflected in cell volume or be related to modification of aqueous–protein interactions in the cytosol and cytoskeleton [23,24]. Therefore, the EPR method does not allow a cell volume description with enough precision.

Thus, we propose nephelometry as the simplest and most rapid method for investigation of spermatozoa volume changes in solutions of different osmolality. A significant correlation coefficient ($R^2 = 0.7341$) between volume changes measured using nephelometry and light microscopy (Fig. 6) confirmed that nephelometry is adequate to reveal changes of spermatozoa volume. In this study, a volume change as low as approximately 10% was detected.

### *4.2. Differences in sperm cell volume alteration among species*

Rapid and acute changes of environmental osmotic pressure might be differentially involved in mechanisms

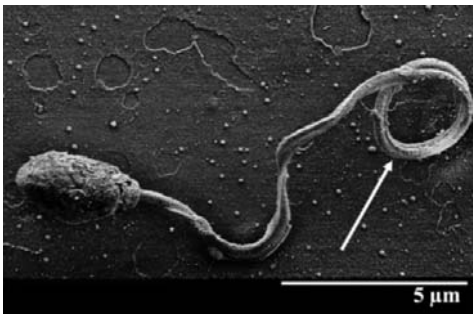


**Fig. 11.** Scanning electron micrograph of brook trout spermatozoa in hypotonic conditions fixed by glutaraldehyde solution. Arrow points to a flagellum loop.

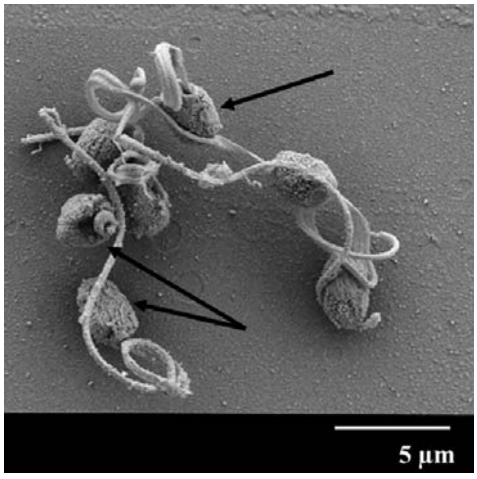


**Fig. 12.** Scanning electron micrograph of brook trout spermatozoa fixed in glutaraldehyde solution. Disc shape spermatozoa head is indicated by arrows. Loops and blebs are visible on flagella.

of fish sperm motility activation. Under natural spawning conditions, rapid alteration of osmolality of the surrounding medium exists as one of the triggers for fish sperm motility activation. For example, a decrease of osmolality to less than 200 mOsm/kg is sufficient to activate motility in carp spermatozoa [3]. Low osmotic pressure, which results in a high dilution rate of the seminal fluid by the hypotonic surrounding media, is the main factor in sperm motility activation in carp [25]. However, in some freshwater fish species, a hypotonic environment seems less critical to sperm activation. Isotonic osmolality of activation media (300 mOsm/kg) does not prevent motility in spermatozoa of steelhead trout [26]. In sturgeon, sperm motility has been shown to be activated in hypotonic, isotonic, or even slightly hypertonic conditions [3], leading to the conclusion that alteration of surrounding osmolality does not play a major role in sperm motility activation in some fish species.

Nevertheless, reduction in osmolality of surrounding medium leads to osmotic re-equilibration and water transport across the spermatozoa membrane, which should be accompanied by volume changes, because a sperm cell was shown to react as an osmometer [27].

We demonstrate that increase of carp spermatozoa volume occurs during motility in hypotonic conditions, and propose several methods to characterize this process. Our findings agree with previous reports [7,11,12] describing carp spermatozoa swelling dependent on ambient aquatic osmolality. In contrast to carp, we did not find indications of spermatozoa volume changes in sterlet (Figs. 4 and 8) or brook trout (Fig. 10) spermatozoa associated with environmental osmolality. To our knowledge, data on sterlet spermatozoa volume during the motility period have not been previously reported.

Swelling was detected in rainbow trout (*Oncorhynchus mykiss*) spermatozoa after 0.5-, 2-, 5-, 10-, 20-, and 30-minute incubation in hypotonic nonactivating solution [6]. It was shown that, in this species, two sperm cell populations could be distinguished. One population (approximately 20% of total spermatozoa) showed no swelling, and, in the remaining 80%, average spermatozoa volume was shown to increase. Dependency of average sperm volume on either environmental osmolality or time of incubation in a hypotonic environment was not observed. Discrepancy between our results and those of others [6] could be related to species differences. Our results, together with data reported by other authors [6,7,12], indicate that alteration of environmental osmolality could differentially affect fish sperm, depending on species, because of differences in their osmotic- or ionic-dependent spermatozoa motility activation mode.

Considering that cell volume changes in response to environmental osmolality occur because of transmembrane water influx, our observations of differences among species could be because of differences in water influx rates. Water influx can take place either by passive diffusion, possibly facilitated through water channel proteins (aquaporines), or actively through ion cotransporters [27,28]. It was hypotized by Zilli et al. that membrane water transport and further alteration of internal ion concentration could lead to the activation of adenylyl cyclase that, in turn, triggers the cAMP signaling pathway, which determines the phosphorylation and/or dephosphorylation of proteins and then the initiation of sperm motility [29]. However, water transport processes have not been sufficiently investigated to provide a precise description of their role in fish spermatozoa motility.

Some published reports point to activation of stretch-dependent channels as a prerequisite for motility in fish sperm with the osmotic mode of activation [9,30]. However, water diffusion appears not to be sufficiently rapid to induce membrane stretch immediately on sperm contact with a hypotonic media. Alternatively, much more rapid water transport could occur because of water influx through aquaporins [27]. Hypothetically, in carp spermatozoa, water transport through aquaporins could lead to sperm swelling with further activation of stretch-dependent channels and motility activation. Sturgeon and salmonid spermatozoa show no volume changes during motility, suggesting that these species might lack aquaporins, and water slowly penetrates membranes by diffusion only. Thus, we assume that in sturgeon and salmonids, ion channel signaling is not dependent on membrane stretching, and that ion transfer occurs only because of differences between internal and external ion concentration. However, the involvement of ion cotransporters cannot be excluded. It has been shown that during ion transfer the amount of cotransported water is significant, ranging from 35 to 500 water molecules, and that water transport proceeds, along with substrates, independently of the osmotic gradient [31]. Cotransport of water could be independent of the osmotic gradient across membranes for transport of substances such as gamma-aminobutyric acid. Gamma-aminobutyric acid has been found in sperm and testes of mammals [31] and in *Xenopus laevis* oocytes [32]. Gamma-aminobutyric acid and other osmotically independent cotransporters might be present in fish sperm. Therefore, during motility, ion transport through the membrane would occur via cotransporters and be accompanied by water transport, thus affecting the volume of spermatozoa of fish such as trout and sturgeon, which possess an ionic mode of motility activation.

### 4.3. Conclusions

Our results, in conjunction with data reported by other authors, suggest species-specific sperm volume changes in a hypotonic environment during spermatozoa motility. Spermatozoa swelling in hypotonic solutions was detected using several methods, of which nephelometry could be a preferred technique because of its simplicity and rapidity. Further research is required to better understand how volume changes are associated with motility activation.

**Acknowledgments**

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## CHAPTER 3

### HYPOTONIC TREATMENT PRIOR TO FREEZING IMPROVES CRYORESISTANCE OF COMMON CARP (*CYPRINUS CARPIO* L.) SPERMATOZOA

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Brief Communication

# Hypotonic treatment prior to freezing improves cryoresistance of common carp (*Cyprinus carpio* L.) spermatozoa

Borys Dzyuba [a,*], Jacky Cosson [a], Gunes Yamaner [b], Olga Bondarenko [a], Marek Rodina [a], David Gela [a], Volodymir Bondarenko [a], Anna Shaliutina [a], Otomar Linhart [a]

[a] *University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic*
[b] *Istanbul University, Faculty of Fisheries, Beyazit/Eminonu, 34452 Istanbul, Turkey*

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ABSTRACT

Post-thaw motility rate, curvilinear velocity (VCL), and fertilizing ability of carp spermatozoa can be improved by short-term treatment with moderately hypotonic media prior to freezing. Before cryopreservation, carp sperm samples were treated with NaCl solutions of differing osmolalities, ranging from 100 to 300 mOsm $kg^{-1}$ for 10 s, after which final osmolality was adjusted to 300 mOsm $kg^{-1}$. The resulting sperm suspension was diluted 1:1 with cryoprotective medium and frozen using conventional techniques. Control samples were treated in the same way, without the pre-dilution step. Post-thaw motility rate in samples pretreated with 200 mOsm $kg^{1}$ NaCl was significantly higher (44 ± 10%) than in controls (21 ± 15%) and samples pretreated with 100 mOsm $kg^{-1}$ (25 ± 15%) and 300 mOsm $kg^{-1}$ (25 ± 12%) NaCl. Significantly higher mean VCL were observed in samples pretreated with 100, 150, and 200 mOsm $kg^{-1}$ (119 ± 24, 118 ± 22, and 115 ± 32 μm $s^{-1}$, respectively) compared to controls (92 ± 27 μm $s^{-1}$). Fertilization rate of frozen-thawed sperm treated with 200 mOsm $kg^{-1}$ solution of 2 M NaCl was significantly higher (25 ± 18%) than that of sperm treated with 300 mOsm $kg^{-1}$ NaCl solutions (12 ± 7%) and the control (9 ± 6%).



Common carp, *Cyprinus carpio*, sperm cryopreservation is considered a potentially powerful tool in aquaculture and conservation programs [6]. Many cryobiological studies have been conducted on carp sperm, leading to the development of cryoprotective media, cryoprotectants, and freeze/thaw protocols boosting spermatozoa post-thaw motility characteristics and fertilization rate [5,8]. The selection of optimal methods from among available protocols, and sometimes their modification, is necessary for some breeders because of a wide diversity of spermatozoa cryoresistance, most likely arising from maintenance conditions of males prior to stripping [6]. Carp, which exhibit a hypotonic mode of spermatozoa motility activation, provide several advantages for cryobiological studies. Carp spermatozoa respond within 1 s to environmental hypotonicity with activation of motility characterized by rearrangement of intracellular ion concentration, changes in membrane structure [7], and spermatozoa volume increase [3]. These changes can be reversed by increasing osmolality, leading to immediate cessation of motility. Therefore, carp sperm can be considered an appropriate model for studies of the relationship between cell membrane changes prior to freezing and cell cryoresistance. We predict that outcomes of carp sperm cryopreservation can be improved by hypotonic treatment of sperm prior to freezing. In the present study we investigated whether sperm cryoresistance can be manipulated by exposure of spermatozoa to hypotonic NaCl solutions. As carp spermatozoa are activated in hypotonic solutions, which leads to a dramatic decrease in ATP content, we treated sperm samples for only 10 s before readjusting the osmolality to isotonicity.

Twenty-four hours after injection of common carp with carp pituitary extract (1 mg $kg^{-1}$ of body weight), sperm was stripped by abdominal massage and collected in plastic syringes. Sperm samples from 5 males, showing 95 ± 3% motility rate and 276 ± 6 mOsm $kg^{-1}$ seminal fluid (SF) osmolality, were selected for experiments. Seminal fluid was obtained through sperm centrifugation at 10 000*g* for 10 min. NaCl solutions in which osmolality was adjusted to 100, 150, 200, 250, and 300 mOsm $kg^{-1}$, and which were buffered with 10 mM Tris–HCl buffer to pH 8.0, were used as pre-dilution media. Prior to cryopreservation, sperm samples were mixed with the pre-dilution media at a dilution rate of 1:4. The resultant cell suspension osmolalities were within the ranges of

* Corresponding author. Address: University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 389 25 Vodňany, Czech Republic. Fax: +420 387 774 634.
*E-mail address:* bdzyuba@frov.jcu.cz (B. Dzyuba).

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132–137, 174–177, 214–217, 254–257, and 294–298 mOsm $kg^{-1}$ (min–max values of the sperm samples, lowest to highest osmolalities). Ten seconds after dilution, osmolality was increased to 300 mOsm $kg^{-1}$ by addition of 2 M KCl or 2 M NaCl to inhibit spermatozoa motility. This osmolality value was the minimum required for immediate spermatozoa motility cessation for a sample pre-diluted with media used in the experiments, regardless of its osmolality. The resultant sperm suspension was diluted 1:1 with cryoprotective medium consisting of 59 mM NaCl, 6.3 mM KCl, 0.68 mM $CaCl_2$, 2.1 mM $MgCl_2$, 27 mM $NaHCO_3$, 3.4 mM sucrose, 69 mM manitol, 118 mM Tris pH 8.2, and 16% ethylene glycol [5]. Final sperm dilution ratio was 1:9 (1 volume sperm +4 volumes pre-dilution medium, 5 volumes of cryoprotective medium). According to Lahnsteiner et al. [8], the protective effect of seminal plasma should not be appreciably affected at this dilution rate. Ten min after dilution, sperm samples were frozen in 0.5 ml straws, 3 cm above liquid nitrogen for 20 min followed by plunging the straws into liquid nitrogen. Time between sperm sample treatment and freezing onset was approximately 15 min. Sperm samples that were not pre-diluted with NaCl before freezing and were frozen after 1:1 dilution in cryoprotective medium were used as controls. All pre-dilution procedures were carried out at 4 °C. Frozen samples were thawed at 40 °C for 6 s and immediately used for motility and fertility tests. Post-thaw motility rate and VCL were assessed for sperm samples pretreated with 100, 150, 200, 250, and 300 mOsm $kg^{-1}$ NaCl and for control samples. For motility parameters assessment, osmolality of the pre-diluted sperm samples was readjusted with 2 M KCl. Motility was analyzed at room temperature (18–20 °C). Osmolalities of media used before and after KCl addition, and of SF, were evaluated using a Vapor Pressure Osmometer 5520 (Wescor, USA) and expressed in mOsm $kg^{-1}$.

For fertilization tests, the osmolality of the pre-diluted sperm samples was readjusted with KCl or NaCl solution to investigate if $Na^+$ or $K^+$ ions influenced the fertilization process.

For motility activation, a saline activating medium (AM) consisting of 45 mM NaCl, 5 mM KCl, and 30 mM Tris–HCl, pH 8.2 (147 mOsm $kg^{-1}$) was used. Sperm was added to the AM using the tip of a dissecting needle, and the sperm suspensions were thoroughly mixed for 2 s. Motility was recorded for 1–2 min post-activation using video microscopy combined with stroboscopic illumination (ExposureScope®). Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL) and percent motile cells (motility rate) by micro-image analyzer (Olympus Micro-Image 4.0.1. for Windows). The micro-image analyzer allows measurement of VCL and motility with five successive overlapping video frames. Overlapping tracks of spermatozoa heads became visible, permitting calculation of VCL (defined as total point-to-point distance traveled by the sperm over the time period between the first and fifth frames) and motility rate [9]. To assess sperm post-thaw fertility, the pooled ova from three females were used. Egg samples (2 g) were inseminated in a dish with either fresh or frozen-thawed sperm immediately after thawing. Eggs were fertilized at a sperm to ova ratio of $10^5$:1. Comparison to the ratio previously proposed as optimal [8], the lower ratio avoids excessive sperm which could lead to maximal fertilization rate in all trials, obscuring differences among samples. Spermatozoa concentration of each sample was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on an Olympus BX 50 phase contrast microscope (Olympus, Japan). 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, ova were transferred to three Petri dishes. 100–200 eggs were placed into each dish and incubated with aerated, dechlorinated, and UV-sterilized tap water. Living (transparent, with visible embryo inside) and dead (not transparent, whitish) eggs were counted in each Petri dish during incubation and dead eggs were removed. Live embryos were counted at the eyed stage after 2 days incubation at 20–21 °C, and fertilization rate was expressed as the percent of live embryos from the initial number of eggs incubated.

The values of variables were checked for distribution characteristics and homogeneity of dispersion using the Shapiro–Wilkes and Levene's tests, respectively. For normally distributed sperm velocity values before and after cryopreservation, parametric ANOVA was applied, and the Fisher LSD test was used as the criterion for differences among subgroups. Nonparametric statistics using the Kruskal–Wallis test followed by the Mann–Whitney U-test were conducted for comparison of motility rate, motility duration, and fertilization rate values because of the small number of values obtained in the study ($n = 5$). Statistical significance was considered at $P < 0.05$. Mean values and standard error of the mean (SEM) are presented in the figures, and mean ± standard deviation values are mentioned in the text. All analyses and graph plotting were conducted using Statistica V9.1 computer program (Statsoft Inc, USA).

Higher post-thaw motility rate, spermatozoa velocity, and fertilization rate were observed in sperm pretreated with hypotonic solutions (with osmolality readjusted with 2 M KCl) compared to non-treated sperm or sperm treated with the isotonic solution. Post-thaw motility rate was significantly higher in samples pretreated with 200 and 150 mOsm $kg^{-1}$ NaCl solutions compared to those pretreated with 100 and 300 mOsm $kg^{-1}$ NaCl solutions

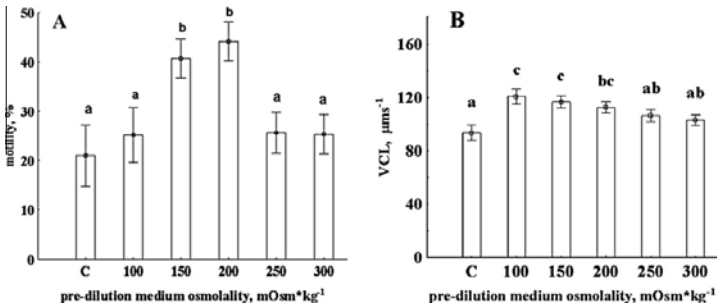


**Fig. 1.** Post-thaw motility rate (A) and curvilinear velocity (B) of *Cyprinus carpio* spermatozoa pretreated with NaCl solutions of different osmolalities before cryopreservation. Sperm sample osmolality after 10 s in pre-dilution medium was adjusted to 300 mOsm $kg^{-1}$ by addition of 2 M KCl solution. Values with different letters are significantly different ($P < 0.05$). (C) Control sperm samples, which were not pretreated before freezing.

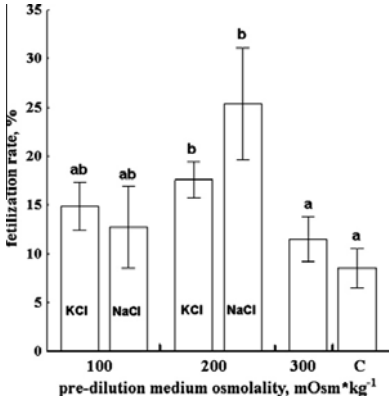


**Fig. 2.** Post-thaw fertilization rate for *Cyprinus carpio* sperm pretreated with NaCl solutions of different osmolalities before cryopreservation. Values with different letters are significantly different ($P < 0.05$). Osmolality was readjusted to isotonic values by addition of concentrated KCl or NaCl as indicated in the bars. For sperm pre-diluted with 300 mOsm $kg^{-1}$ NaCl, no re-adjustment of osmolality was necessary.

and the controls (Fig. 1A). Significantly higher spermatozoa velocities were observed in samples pretreated with 100, 150, and 200 mOsm $kg^{-1}$ NaCl solutions than in controls (Fig. 1B).

Fresh sperm fertilization rate was 87 ± 6%. There was no significant difference in sperm post-thaw fertilization rate between samples with osmolality readjusted with 2 M KCl and that with 2 M NaCl (Fig. 2). Sperm pretreated with 200 mOsm $kg^{-1}$ NaCl solution produced significantly higher post-thaw fertility rates than sperm pretreated with 100 or 300 mOsm $kg^{-1}$ NaCl solutions or the untreated control sperm (Fig. 2). These results indicate that post-thaw motility rate, spermatozoa velocity, and fertilization capability of carp sperm may be improved by short-term pretreatment with 200 mOsm $kg^{-1}$ NaCl solution.

Spermatozoa post-thaw motility rate, velocity, and fertilizing ability found here are comparable to results obtained by Boryshpolets et al. [1]. Motility and fertilization rates obtained in the present study are lower than some previously reported [4]. This discrepancy in results could arise from within-species differences in sperm cryoresistance [6].

In our study, a low sperm to egg ratio was used to avoid masking the effect of the experiments which may have reduced the fertilization rate. However, these results represent the first report of the potential for improvement of post-thaw spermatozoa motility parameters through hypotonic treatment of sperm prior to freezing. We have found no previously published information regarding the influence of hypotonic treatment before freezing on fish sperm cryoresistance. Hypotonic treatment before freezing is known to have a negative effect on human red blood cells cryoresistance, as was shown by Pegg and Diaper [10]. Discrepancies between our data and results of Pegg and Diaper could arise from possible intra-specific variation in membrane response to hypotonicity, as, in contrast to mammals, evolution of the physiological activity of fresh water fish sperm took place in a hypotonic environment. Apart from motility activation, little is known about the consequences of fish spermatozoa transfer from the isotonic seminal fluid to a hypotonic environment. The rapid increase in carp spermatozoa membrane fluidity in response to hypotonicity [7] could facilitate sperm survival of water/cryoprotectant fluxes occurring during the freeze–thaw and following fertilization under hypotonic conditions. This speculation is also supported by observations of rainbow trout, in which increased membrane fluidity was correlated with higher sperm post-thaw fertilizing ability (for review, see [2]).

We suggest that the phenomenon observed in the present study may arise from spermatozoa volume changes, which lead to spermatozoa membrane modification, enhancing the ability of spermatozoa to survive the freeze–thaw processes. We consider our results to be preliminary and needing further study for application of this methodology to become practical for fish sperm cryopreservation.

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## CHAPTER 4

### CALCIUM ION SUPPLEMENTATION INCREASES BROOK TROUT *SALVELINUS FONTINALIS* SPERMATOZOA ACTIVATION AT THE END OF THE SPAWNING SEASON

Bondarenko, O., Dzyuba, B., Cosson, J., Rodina, M., Linhart, O., 2014. Calcium ion supplementation increases brook trout *Salvelinus fontinalis* spermatozoa activation at the end of the spawning season. Journal of Fish Biology 85 (3), 933–937.

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# Calcium ion supplementation increases brook trout *Salvelinus fontinalis* spermatozoa activation at the end of the spawning season

O. Bondarenko*, B. Dzyuba, J. Cosson, M. Rodina and O. Linhart

*Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic*

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The role of environmental ion composition and osmolality in calcium ion ($Ca^{2+}$) signalling of spermatozoa activation over the course of the spawning period of brook trout *Salvelinus fontinalis* was investigated. Motility at the end of spawning was low (mean ± s.d. of 5 ± 2% motile spermatozoa with curvilinear velocity of 25 ± 8 μm $s^{-1}$). Addition of 10 mM $Ca^{2+}$ to the activation medium resulted in values similar to those recorded during the middle of the spawning period (mean ± s.d. of 95 ± 6% motile spermatozoa with curvilinear velocity of 130 ± 15 μm $s^{-1}$).



Key words: salmonid; semen quality; sperm motility.

The brook trout *Salvelinus fontinalis* (Mitchill 1814) is an important aquaculture species, and evaluation of semen quality may contribute to increased efficacy of artificial fertilization. Salmonid semen characteristics such as osmolality, ion composition, protein concentration, volume and spermatocrit vary over the course of the spawning period, which is often associated with a reduction in motility rate, velocity and duration (Munkittrick & Moccia, 1987; Koldras *et al.*, 1996; Lahnsteiner *et al.*, 1998; Nynca *et al.*, 2012). It is unclear whether diminished spermatozoa motility can be prevented by *in vitro* semen treatment.

Fish spermatozoa activation is under the control of ion concentration, osmolality and pH. While activation of salmonid spermatozoa occurs over a wide range of osmolalities (Alavi & Cosson, 2006; Cosson, 2010), specific ion concentrations and pH levels are critical under all osmotic conditions (Morisawa & Suzuki, 1980; Morisawa *et al.*, 1983; Boitano & Omoto, 1991; Cosson *et al.*, 1991; Dziewulska & Domaga, 2013). High potassium ion ($K^+$) is a major inhibitor of sperm motility (Morisawa *et al.*, 1983; Billard *et al.*, 1987; Cosson *et al.*, 1989; Kho *et al.*, 2001), but inhibitory effects of $K^+$ can be overcome by increasing external calcium ion ($Ca^{2+}$).

*Author to whom correspondence should be addressed. Tel.: +420773559118; email: toolgab@gmail.com

It has therefore been suggested that intracellular free $Ca^{2+}$, produced by a flux of external $Ca^{2+}$, plays a significant role in activation of salmonid spermatozoa (Cosson *et al.*, 1989).

This study investigated whether adequate spermatozoa motility can be maintained throughout the spawning period by manipulation of the activating medium. *Salvelinus fontinalis* were used as a model salmonid species.

Twenty mature *S. fontinalis* males (300–500 g) were maintained in a 10 000 l outdoor hatchery tank at the Genetic Fisheries Center, and experiments were carried out at the Faculty of Fisheries and Protection of Waters. Water temperature ranged from 2 to 8° C, depending on season. Semen was obtained by abdominal massage directly into 5 ml plastic syringes from 10 fish from 24 November to 4 December and from the remaining 10 fish from 8 to 19 January, periods that correspond to the middle and the end of the *S. fontinalis* spawning season in Europe (Cucherousset *et al.*, 2008). Care was taken to avoid contamination by urine, mucus, faeces or water during semen collection.

Motility characteristics of sperm obtained at the middle and end of the spawning season were assessed in hypotonic (10 mM Tris–HCl, pH 8·5) and isotonic (300 mOsm $kg^{-1}$ NaCl, buffered by 10 mM Tris–HCl, pH 8·5) solutions to determine the effects of environmental osmolality. At the end of spawning, motility was also observed in 10 mM Tris–HCl and 300 mOsm $kg^{-1}$ NaCl, pH 8·5, in the presence of 10 mM $CaCl_2$, as previous reports suggest that this calcium concentration improves low rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) spermatozoa motility observed at the end of spawning (Billard & Cosson, 1992). In the mid-spawning season, high values of motility were sustained without $Ca^{2+}$ supplementation.

Sperm suspensions were thoroughly mixed for 2 s and, immediately after dilution in the test media, spermatozoa activity was recorded for 1 min using a charge-couple device video camera (Sony, SSCDC50AP; www.sony.com) mounted on a dark-field microscope (Olympus BX41, ×200; www.olympus.com) and illuminated with an ExposureScope (www.frov.jcu.cz/en/aktuality-2014/new-promotional-videos) set to a flash frequency of 50 Hz. Video recordings (Sony SVHS, SVO-9500 MDP) of the period from 5 to 8 s post-activation were analysed to estimate curvilinear velocity of spermatozoa ($V_{CL}$, μm $s^{-1}$) and percentage of motile spermatozoa (motility rate). Motility characteristics were calculated according to Linhart *et al.* (2000) and Rodina *et al.* (2008).

Semen samples were centrifuged at 300 *g* for 30 min at 4° C, and the supernatant was collected. Seminal fluid (SF) osmolality as well as osmolality of all studied media were measured using a vapour pressure osmometer (Wescor, Logan; www.wescor.com/biomedical/osmometer/vapro.html) and expressed in mOsm $kg^{-1}$. SF osmolality and ion concentrations [sodium ion ($Na^+$), $K^+$ and $Ca^{2+}$] were measured in triplicate.

Concentrations of $Na^+$ and $K^+$ were measured in triplicate for each fish by potentiometry using ion-selective electrodes (Bayer HealthCare; healthcare.bayer.com/). $Ca^{2+}$ concentration was measured in triplicate for each fish by absorption photometry using the *o*-cresolphthalein complexone method.

Measures of SF osmolality and ion concentrations, along with spermatozoa motility rate and $V_{CL}$, were conducted in triplicate for each fish. Data were analysed using Statistica 10 software (www.statsoft.com). Results are shown as mean ± S.D. for each group. The values were checked for distribution characteristics and homogeneity of dispersion using the Shapiro–Wilk and Levene tests, respectively. To compare

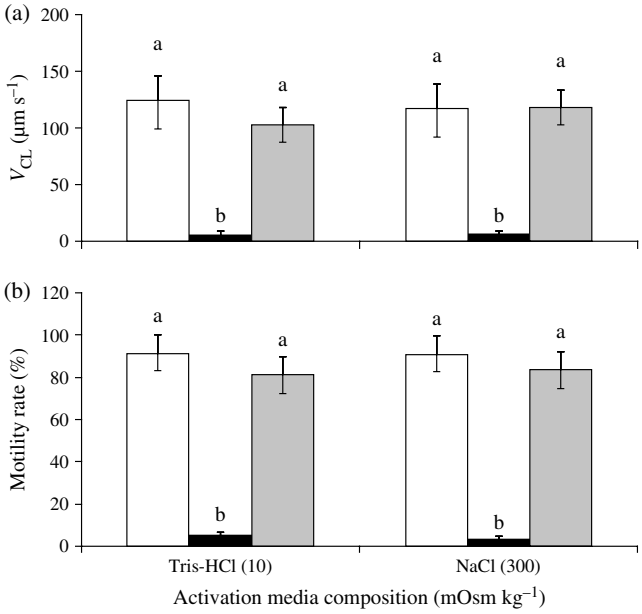


FIG. 1. *Salvelinus fontinalis* spermatozoa (a) curvilinear velocity ($V_{CL}$) and (b) motility rate in solutions of differing osmolalities. Motility variables were evaluated at the middle (□) and end (■) of the spawning season ($n = 10$, for each group). Spermatozoa motility was initiated in 10 mM Tris–HCl, pH buffer [Tris–HCl (10)] and 300 mOsm kg$^{-1}$ NaCl buffered by 10 mM Tris–HCl [NaCl (300)]. At the end of the spawning season, 10 mM $CaCl_2$ was added to the studied media (▤). Values are presented as mean ± S.D. Values with the same lower case letter are not significantly different ($P > 0{\cdot}05$).

spermatozoa $V_{CL}$ among test groups, parametric ANOVA was applied, and Tukey's honest significant difference (HSD) test was used to identify differences among groups. Motility rate, ion content and osmolality of SF in the middle and end of spawning were compared using the Kruskal–Wallis test, followed by the multiple comparison of mean ranks of all test groups (Siegel & Castellan, 1988). Significance was considered at $P < 0{\cdot}05$.

Seasonal variations in sperm reaction to osmolality were determined in isotonic (300 mOsm kg$^{-1}$) and hypotonic (10 mM Tris–HCl) media (Fig. 1). Significant decreases were observed in motility at the end of spawning (motility rate 5 ± 2%, $V_{CL}$ 25 ± 8 μm s$^{-1}$) compared with the mid-spawning period (motility rate 95 ± 3%, $V_{CL}$ 130 ± 12 μm s$^{-1}$) in both hypotonic and isotonic environments. Addition of 10 mM $Ca^{2+}$ to the activation medium was associated with a significant ($P < 0{\cdot}05$) increase in motility rate from 5 ± 2% to 95 ± 6% and in $V_{CL}$ from 25 ± 8 to 130 ± 15 μm s$^{-1}$ at the end of the spawning period. Characteristics of spermatozoa motility in 10 mM $Ca^{2+}$ at the end of spawning did not differ from those observed in mid-spawning period ($P > 0{\cdot}05$) (Fig. 1).

No significant differences in $Na^+$ and $Ca^{2+}$ concentrations ($P > 0{\cdot}05$) or SF osmolality ($P > 0{\cdot}05$) were detected in samples from the middle and end of the spawning season (Table I). A significant decrease was observed in SF $K^+$ concentration ($P < 0{\cdot}05$), from 24 mM in the middle to 11 mM at the end of the spawning season.

 

TABLE I. Ion composition and osmolality of seminal fluid in the middle and end of the spawning period *Salvelinus fontinalis*

| Spawning season | $Na^+$ (mM) | $K^+$ (mM) | $Ca^{2+}$ (mM) | Seminal fluid osmolality (mOsm $kg^{-1}$) |
|---|---|---|---|---|
| Middle | 130 ± 4 | 24·2 ± 3·1* | 1·0 ± 0·1 | 280 ± 10 |
| End | 130 ± 2 | 11·0 ± 0·2 | 1·0 ± 0·1 | 272 ± 10 |

*Significant difference between variables measured at the middle ($n = 10$) and end ($n = 10$) of the spawning season (Mann–Whitney $U$-test, $P < 0{\cdot}05$). Values are presented as mean ± S.D.

The decline in sperm quality over the course of the spawning season is reflected in a decrease in the per cent of motile spermatozoa and their velocity, leading to reduced fertilization (Lahnsteiner *et al.*, 1998; Linhart *et al.*, 2000). Under experimental conditions, freshwater salmonid spermatozoa can be activated at a wide range of osmolalities, from hypotonic (10–30 mOsm $kg^{-1}$) to slightly hypertonic (300–310 mOsm $kg^{-1}$). More hypertonic conditions (400 mOsm $kg^{-1}$) are reported to suppress spermatozoa activation (Alavi & Cosson, 2006). The absence of observed differences in characteristics of spermatozoa motility in activation media of different osmolalities indicates that osmolality does not play a critical regulatory role in *S. fontinalis* spermatozoa activation. *Salvelinus fontinalis* spermatozoa motility was enhanced by the presence of high (10 mM) $Ca^{2+}$ concentration in activation media at the end of the spawning season (Fig. 1). In contrast, in mid-spawning, addition of $Ca^{2+}$ was not required to obtain a high motility rate (95 ± 3%) and $V_{CL}$ (130 ± 12 μm $s^{-1}$). Hence, spermatozoa sensitivity to $Ca^{2+}$ appears to be altered during late spawning, associated with changes in semen quality.

No seasonal change in SF $Ca^{2+}$ and $Na^+$ concentrations or osmolality was detected. A significant decrease in $K^+$ concentration was detected, however, at the end of spawning (Table I). Reduced $K^+$ concentration could be involved in the observed decrease in motility at the end of the spawning season. Reduced $K^+$ concentration may result from an influx of $K^+$ from SF into spermatozoa. As a higher concentration of $K^+$ requires an increase in $Ca^{2+}$ concentration in the activation medium (Morisawa & Suzuki, 1980), the same phenomenon could exist within the cell. Increase of intracellular $K^+$ concentration suppresses activation under natural conditions, but increase of extracellular $Ca^{2+}$ concentration, up to 10 mM, with further influx into the spermatozoon, could trigger motility. The role of SF $K^+$ concentration decrease in motility triggering by calcium at the end of the spawning season requires further study.

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## CHAPTER 5

### THE ROLE OF $CA^{2+}$ AND $NA^{+}$ MEMBRANE TRANSPORT IN BROOK TROUT (*SALVELINUS FONTINALIS*) SPERMATOZOA MOTILITY

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Fish Physiol Biochem (2014) 40:1417–1421
DOI 10.1007/s10695-014-9936-5

# The role of $Ca^{2+}$ and $Na^{+}$ membrane transport in brook trout (*Salvelinus fontinalis*) spermatozoa motility

**Olga Bondarenko · Borys Dzyuba · Jacky Cosson · Marek Rodina · Otomar Linhart**

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**Abstract** The role of environmental ion composition and osmolality in $Ca^{2+}$ signaled activation was assessed in spermatozoa of brook trout *Salvelinus fontinalis*. Milt from ten mature males was obtained by abdominal massage. Spermatozoa motility was evaluated in 0, 100, and 300 mOsm/kg NaCl or sucrose solutions, buffered by 10 mM Tris–HCl pH 8.5. For investigation of spermatozoa reaction to external $Ca^{2+}$ concentration, 2 mM ethylene glycol tetraacetic acid (EGTA) was added to the activation media as a calcium ions chelator. For investigation of the effect of external $Na^{+}$ concentration in conditions of low external $Ca^{2+}$, 100 μM amiloride was added to the EGTA-containing solutions as a $Na^{+}$ transport blocker. Low motility was observed in sucrose ($Na^{+}$ free) solutions containing 2 mM EGTA but not in $Na^{+}$ solutions containing 2 mM EGTA. Addition of amiloride led to significantly increased motility ($P < 0.05$) compared with sucrose ($Na^{+}$ free) solutions containing 2 mM EGTA. We conclude that $Na^{+}$ transport in $Ca^{2+}$-free solutions plays a regulatory role in brook trout spermatozoa activation. The influence of competitive $Na^{+}$ and $Ca^{2+}$ transport on the control of spermatozoa activation requires further study with respect to its application for improvement of artificial activation and storage media.

**Keywords** Sperm motility activation · Osmolality · Ion transport · Amiloride · Salmonid fish

O. Bondarenko (✉) · B. Dzyuba · J. Cosson · M. Rodina · O. Linhart
Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in Ceske Budejovice, Vodnany, Czech Republic
e-mail: toolgab@gmail.com

## Introduction

Activation of fish spermatozoa motility is controlled by specific ion concentrations, osmolality, and environmental pH. In salmonids, osmolality seems to play a minor regulatory role in spermatozoa activation as the later occurs over a wide range of environmental osmolalities (Morisawa and Suzuki 1980; Alavi and Cosson 2006; Cosson 2010). In contrast, concentration of some ions as well as pH is critical under any osmotic conditions (Morisawa and Suzuki 1980; 1983; Cosson et al. 1989; Boitano and Omoto 1991). Transport through ion channels, co-transporters, anti-porters (integral membrane protein involved in active transport of two or more different molecules or ions), and voltage-dependent gates regulates membrane potential, eventually leading to spermatozoa motility (Morisawa and Suzuki 1980; Krasznai et al. 1999, 2003; Kho et al. 2001; Cosson et al. 1989; Padan et al. 2001). Spermatozoa motility in salmonids is suppressed by extracellular $K^{+}$ concentration in seminal plasma, and decreased $K^{+}$ concentration upon spawning initiates motility (Morisawa and Suzuki 1980; Baynes et al. 1981).

Prevention of motility by $K^+$ can be reversed by increasing $Na^+$, $Ca^{2+}$, or $Mg^{2+}$ concentration in activation media (Alavi and Cosson 2006; Cosson et al. 1999; Li et al. 2012) or by application of a hyperosmotic treatment immediately followed by hypoosmotic shock (Morita et al. 2005; Takei et al. 2011), indicating that the reaction of trout spermatozoa to ionic composition of the environment is somehow dependent on environmental osmotic pressure. Nevertheless, there is no evidence for alteration of spermatozoa ion sensitivity due to a change in osmotic conditions.

The goal of the present study was to investigate the role of $Ca^{2+}$ and $Na^+$ membrane transport in stimulating brook trout (*S. fontinalis*) spermatozoa motility under differing osmotic conditions.

## Materials and methods

### Milt sampling

Ten mature brook trout males (300–500 g) were maintained in a 10,000-L outdoor hatchery tank at the Genetic Fisheries Center, and experiments were carried out at the Faculty of Fisheries and Protection of Waters. Water temperature ranged from 2 to 8 °C. Sperm from each male was collected in the middle of spawning season (14.11–4.12.2011) once by abdominal massage directly into the plastic syringe. Special care was taken to avoid contamination by urine, mucus, feces, and water during sperm collection.

### Spermatozoa motility

Immediately after dilution of milt in the test media (using the tip of a dissecting needle, the sperm suspensions were thoroughly mixed for 2 s), motility was recorded for 1 min postactivation using a CCD video camera (Sony, SSCDC50AP) mounted on a dark-field microscope (Olympus BX41, 200X) and illuminated with an ExposureScope® (Vodnany, Czech Republic) set to a flash frequency of 50 Hz. Recordings were made using a video recorder (Sony SVHS, SVO-9500 MDP, Japan) and analyzed to estimate curvilinear velocity (VCL) of spermatozoa (VCL, μm/s), percentage of motile spermatozoa after activation (motility %), and motility duration (s) in triplicate for each fish. Motility parameters were calculated according to Linhart et al. (2000).

### The role of external $Na^+$ and $Ca^{2+}$ in brook trout spermatozoa motility

For evaluation of spermatozoa reaction to external sodium concentration as indicated by motility activation in solutions of varying ion composition, NaCl or sucrose solutions of differing osmolalities (0, 100, and 300 mOsm/kg) were used as control. All solutions contained 10 mM Tris–HCl, pH 8.5. A wide range of osmolalities was chosen to investigate the interaction between osmotic pressure and reaction of spermatozoa to external $Ca^{2+}$ and $Na^+$. For evaluation of spermatozoa sensitivity to external $Ca^{2+}$, 2 mM ethylene glycol tetraacetic acid (EGTA) as $Ca^{2+}$ chelating agent was added to all studied media to remove free external calcium. For investigation of the role of external $Na^+$ in conditions of low external $Ca^{2+}$ concentration, 100 μM amiloride hydrochloride hydrate [inhibitor of $Na^+$–$H^+$ antiporter that plays a major role in pH homeostasis (Padan et al. 2001)] was added to 0, 100, and 300 mOsm/kg NaCl and to sucrose solutions containing 2 mM EGTA.

### Statistical analysis

Assessment of motility parameters was conducted in triplicate for each fish. Data were analyzed with Statistica 10 software (Statsoft Inc, USA). Results are shown as mean ± SD for each group. The values were checked for distribution characteristics and homogeneity of dispersion using the Shapiro–Wilk's and Levene's tests, respectively. To compare spermatozoa velocity (VCL), parametric ANOVA was applied, and Tukey's honest significant difference (HSD) test was used to identify differences among subgroups. Motility percentage and duration were calculated by the Kruskal–Wallis test, followed by the multiply comparison of mean ranks of all groups test (Siegel and Castellan 1988). Statistical significance was considered at $P < 0.05$.

## Results

No significant differences in VCL ($P > 0.05$) were observed between the control media (0, 100,

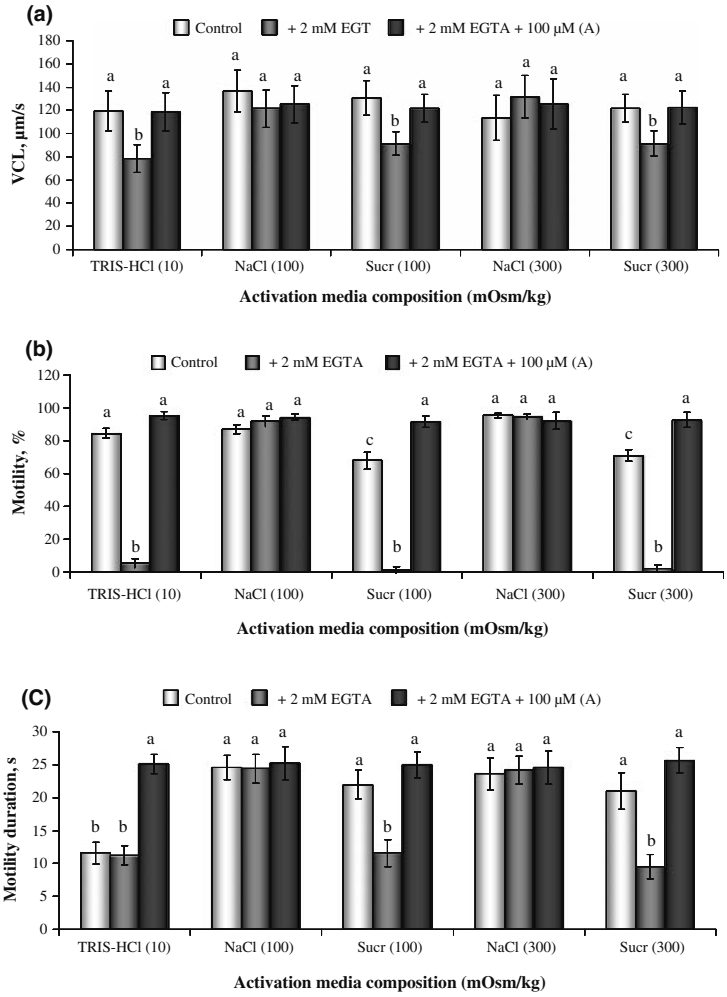


**Fig. 1** Brook trout sperm motility values **a** curvilinear velocity (VCL), **b** motility percentage, and **c** motility duration in solutions differing in osmolality and ion content ($n = 10$). Motility parameters were investigated in: control—isotonic (300 mOsm/kg NaCl or 300 mOsm/kg sucrose) and hypotonic (10 mOsm/kg Tris–HCl, 100 mOsm/kg NaCl or 100 mOsm/kg NaCl sucrose) media; +2 mM EGTA—after addition of 2 mM EGTA to each medium; +2 mM EGTA + 100 μM (**a**)—after addition of 2 mM EGTA and 100 μM amiloride to each medium. Values are presented as mean ± SD. Values with the *same superscript* are not significantly different ($P > 0.05$)

300 mOsm/kg NaCl, and the corresponding sucrose solutions buffered by 10 mM Tris–HCl, pH 8.5) (Fig. 1a). Motility in sucrose solutions (70 ± 4.6 %) was significantly ($P < 0.05$) lower than that in sodium solutions (90 ± 2.7 %) of different osmolalities (Fig. 1b). A significantly shorter duration of motility ($P < 0.05$) was found in 10 mM Tris–HCl (12 ± 1.7 s) as compared to 100 and 300 mOsm/kg

NaCl and 100 and 300 mOsm/kg sucrose solutions (25 ± 2.2 s) (Fig. 1c).

To investigate the role of external $Ca^{2+}$ ions in the presence or absence of external $Na^+$ under different osmotic conditions, 2 mM EGTA was added to all media (Fig. 1). Comparatively reduced motility parameters, such as VCL (90 ± 15.2 μm/s, $P < 0.05$), motility (5 ± 1.2 %, $P < 0.05$), and motility duration (12 ± 2.2 s), were observed in sucrose ($Na^+$ free) solutions containing 2 mM EGTA. No significant differences in motility, motility duration, or VCL were observed in 2 mM EGTA $Na^+$-containing solutions of differing osmolalities ($P > 0.05$) (Fig. 1).

For determination of the role of external $Na^+$ in the absence of external $Ca^{2+}$, 100 μM amiloride (inhibitor of $Na^+$–$H^+$ antiporter) was added to all solutions containing 2 mM EGTA. The amiloride-containing solutions showed significantly higher VCL (130 ± 16.1 μm/s, $P < 0.05$), motility (95 ± 3.4 %, $P < 0.05$), and motility duration (25 ± 1.9 s, $P < 0.05$) compared with those observed in sucrose ($Na^+$ free) solutions containing 2 mM EGTA (Fig. 1). Spermatozoa motility parameters in solutions containing 2 mM EGTA and 100 μM amiloride did not differ from the control ($P > 0.05$). No differences in motility rate, VCL, and motility duration were found in sodium solutions of different osmolalities containing EGTA and amiloride compared with control and solutions containing 2 mM EGTA and 100 and 300 mOsm/kg NaCl ($P > 0.05$) (Fig. 1).

## Discussion

The addition of 2 mM EGTA decreased sperm motility in $Na^+$-free solutions of different osmolalities but not in solutions where $Na^+$ was present (Fig. 1). Thus, this leads to emphasize a regulatory role for $Na^+$ in spermatozoa activation in $Ca^{2+}$-free solutions under a wide range of environmental osmolalities. It has been reported that $Na^+$ as well as $Ca^{2+}$ ions can counteract the inhibition by $K^+$ in salmonids (Baynes et al. 1981) and acipencerids (Alavi and Cosson 2006; Li et al. 2012). The major mechanism sustaining $K^+$ membrane transport is the $Na^+$–$K^+$–ATP pump (Mogas et al. 2010), through which efflux of 3 $K^+$ ions is accompanied by influx of 2 $Na^+$. Conversely, efflux of 2 $Na^+$ is also accompanied by influx of 3 $H^+$ through the $Na^+$–$H^+$ exchanger (Padan et al. 2001). Thus, $Na^+$ membrane transport has a major role in the membrane potential generation. The role of membrane potential alteration in fish sperm motility is known to be controlled by transport of ions such as $K^+$, $Ca^{2+}$, $Na^+$, etc., was widely discussed in the literature (Krasznai et al. 2003; Morisawa 2008). Several models of fish sperm motility activation suggest $K^+$ transport to be a main actor in membrane hyperpolarization, which is an essential step in sperm motility (Krasznai et al. 1999; Cosson 2004; Alavi et al. 2011). In addition, it has been demonstrated that the influx of $Na^+$ as well as $Ca^{2+}$ ions participates actively to the membrane depolarization of the sea urchin spermatozoa (Darszon et al. 2001). In salmonids, sperm motility is known to be prevented by high extracellular $K^+$ concentration, but this inhibitory effect can be eliminated by an increase in extracellular $Ca^{2+}$ concentration (Cosson et al. 1989). It was shown that during trout sperm motility, internal concentration of calcium increases (Cosson et al. 1989) and correlates with membrane potential alteration (Boitano and Omoto 1991). Evidence from previous research as well as the present study allows prediction that the membrane potential is not ion specific, and from our present results, $Na^+$ ions contribute to membrane potential generation in calcium-free solutions. In sucrose solutions containing EGTA, efflux of $K^+$ led to hyperpolarization of the plasma membrane, but, because of the absence of external $Ca^{2+}$ and $Na^+$, a breakdown of the membrane potential would occur, resulting in sperm motility suppression. Under the same conditions, spermatozoa were activated by the addition of 100 μM amiloride (Fig. 1). We state that inhibition of $Na^+$–$H^+$ antiporter allows sperm motility activation in $Ca^{2+}$/$Na^+$-free solutions. From the other hand, blocking of $Na^+$–$H^+$ antiporter could result to alteration of internal pH, as $H^+$ transport control this process. It was shown that external pH could control sperm motility activation in different fish species (Alavi and Cosson 2005). However, it was also shown that alteration of internal pH has no regulatory role in salmonids sperm motility activation (Boitano and Omoto 1991).

To conclude, spermatozoa motility is not dependent on external $Ca^{2+}$ concentration in the presence of external $Na^+$ and 100 μM amiloride. However, spermatozoa could not be activated in $Ca^{2+}$-and $Na^+$-free solutions. We conclude that $Na^+$ transport in $Ca^{2+}$-free solutions plays a crucial role in the

regulation of brook trout spermatozoa activation. Osmotic pressure (in media with 10–300 mOsm/kg osmolality) was shown to have no effect on spermatozoa ion sensitivity. Therefore, alteration of osmolality does not seem to play a major regulatory role in trout spermatozoa activation.

Study of the combined effects of $K^+$, $Na^+$, and $Ca^{2+}$, through modification of ion composition and concentration of spermatozoa activation media, will help to clarify the role of ion transport at the sperm activation step. This will be useful for improvement and elaboration of activation media for artificial fish reproduction as well as for development of cryopreservation techniques and reactivation media for fish gamete cryobiology.

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## CHAPTER 6

### *IN VITRO* SPERM MATURATION IN STERLET, *ACIPENSER RUTHENUS*

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Short Communication

# *In vitro* sperm maturation in sterlet, *Acipenser ruthenus*

*Borys Dzyuba* [a,*], *Jacky Cosson* [a], *Sergii Boryshpolets* [a], *Olga Bondarenko* [a], *Viktoriya Dzyuba* [a,b], *Galina Prokopchuk* [a], *Ievgeniia Gazo* [a], *Marek Rodina* [a], *Otomar Linhart* [a]

[a] *Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, 38925 Vodnany, Czech Republic*
[b] *Institute of Biology, V.N. Karazin Kharkiv National University, Kharkiv, Ukraine*

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ABSTRACT

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



## 1. Introduction

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

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

* *Corresponding author*. Tel.: +420 38 777 4614; fax: +420 387 774634.
E-mail address: bdzyuba@frov.jcu.cz (B. Dzyuba).
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examined whether sperm maturation in the sturgeon is under control of: (1) external enzymatic activity, as it was found in mammals [1], or (2) the ions as it was shown for other fish species [4].

## 2. Materials and methods

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

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

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

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

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

## 3. Results and discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

## Conflict of interest

None declared.

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## CHAPTER 7

### THE ROLE OF $CA^{2+}$ IONS IN STERLET *ACIPENCER RUTHENUS* SPERMATOZOA MATURATION *IN VITRO*

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# THE ROLE OF $CA^{2+}$ IONS IN STERLET *ACIPENCER RUTHENUS* SPERMATOZOA MATURATION *IN VITRO*

***Olga Bondarenko*, Borys Dzyuba, Marek Rodina, Jacky Cosson***

*Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodňany, Czech Republic; *corresponding author: obondarenko@frov.jcu.cz, toolgab@gmail.com*

## ABSTRACT

The role of $Ca^{2+}$ in sturgeon spermatozoa maturation and motility was investigated. Sperm from mature male sterlet *Acipenser ruthenus* was collected from the Wolffian duct (WS) and testis (TS) 24 h after hormone inducement. Testicular sperm was incubated in WS seminal fluid for 5 min at 20 °C and was designated testicular sperm after *in vitro* maturation (TSM). Spermatozoa motility was stimulated in media of differing ionic compositions, and the motility parameters were analyzed from standard video microscopy. For investigation of the role of calcium transport, *in vitro* maturation was performed in the presence of EGTA, verapamil, or tetracaine. No motility was observed in TS. Both addition of $Ca^{2+}$ to the activation medium and incubation of TS in seminal fluid from WS led to motility. Addition of a $Ca^{2+}$ channel inhibitor (verapamil) or EGTA to the incubation medium during *in vitro* maturation reduced or completely suppressed motility, implying $Ca^{2+}$ involvement in sturgeon sperm maturation. The influence of tetracaine provided first evidence of a role for intracellular $Ca^{2+}$ stores in sturgeon spermatozoa function. Further investigation of the roles of $Ca^{2+}$ transport and $Ca^{2+}$ stores in sturgeon spermatozoon maturation and motility are required.

***Keywords:*** *sturgeon, sperm maturation, ion transport, $Ca^{2+}$, $Na^{+}$, spermatozoa motility*

## 1. Introduction

Development of spermatozoa from spermatogonia in fish occurs through a series of cytological stages during spermiogenesis (Hoar, 1969). During this process, spermatogonia form primary spermatocytes through repeated mitotic divisions and cell growth, followed by formation of secondary spermatocytes. As a result of the secondary spermatocyte division, spermatids are produced and metamorphose into potentially functional spermatozoa.

During spawning, spermatozoa in the testis are morphologically developed, but, in some species they are still lack capability for motility activation and fertilization. Although sperm collected from the salmonid sperm duct can be activated by contact with fresh water, testicular sperm remains immotile after dilution (Morisawa and Morisawa, 1986), demonstrating that salmonid spermatozoa obtain the potential for motility during their passage through the sperm duct (Morisawa and Morisawa, 1986). The development from non-functional gametes to mature spermatozoa capable of motility and fertilization constitutes sperm maturation, a process of physiological but not morphological alteration. In salmonids and eels, high concentrations of $HCO_3$ ions increase the pH of the seminal plasma in the sperm duct to approximately 8.0, allowing maturation of sperm (Morisawa and Morisawa, 1988; Miura et al., 1995; Ohta et al., 1997).

In sturgeons (Chondrostei), the reproductive tract presents a specific and unique anatomy, with efferent ducts coming into direct contact with the kidney. During spawning, sperm

is released from the testis into the kidney where it combines with urine. This process is accompanied by a significant decrease in ambient osmolality and ion concentration, and brings about testicular spermatozoa maturation (Dzyuba et al., 2014 a, b). The higher pH alters the spermatozoa membrane potential with respect to activation of voltage-dependent ion channels, similar to the situation previously described in other species during spermatozoa motility activation (Darszon et al., 2001; Krasznai et al., 2003). After testicular spermatozoa traverse through the kidney, they are released into the mesonephric (Wolffian) duct where their maturation is completed. Our previous research demonstrated that maturation can be induced outside the testes through dilution of testicular sperm in urine, and that it involves the participation of substances of molecular weight greater than 10 kDa along with $Ca^{2+}$ ions (Dzyuba et al., 2014 a). It is known that $Ca^{2+}$ ions are involved in regulation of various sperm functions. Calcium ions are involved in spermatozoa hyper-activation and capacitation in mammals (Huang et al., 2009; Harayama and Kato, 2001; Fraser, 1998; Xia et al., 2007; Rahman et al., 2014; Tash and Means, 1987), chemotaxis in ascidians (Yoshida et al., 2003; Krasznai et al., 2000; Ishikawa et al., 2004), and acrosome reaction in various species (Breitbart, 2002; Breitbart et al., 2002; Rossato et al., 2001). $Ca^{2+}$ plays an important role in activation of spermatozoa of freshwater and marine fishes (Alavi and Cosson, 2006; Cosson, 2004; Vines et al., 2002; Bondarenko et al., 2014), including sturgeon (Dzyuba et al., 2014 a; Alavi et al., 2011). The participation of $Ca^{2+}$ in motility suggests its involvement in the maturation process as well. The goal of the present study was to investigate the role of calcium ions in sturgeon sperm maturation.

## 2. Materials and methods

### 2.1. Sampling

Ten mature male sterlet *Acipenser ruthenus* were held in a 0.8 $m^3$ closed water recirculation system located at the hatchery of the Research Institute of Fish Culture, FROV JCU during the natural spawning season. Water temperature was gradually increased from 7 to 15 °C. Following a 14-day acclimation period, spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w ⁄ v) NaCl solution at 4 mg/kg body weight.

Wolffian duct sperm (WS) was collected from the urogenital tract 24 h post-injection using a catheter, and samples were kept on ice for 2 h or less during experimentation. Immediately after WS collection, testicular sperm (TS) was collected from efferent ducts according to (Dzyuba et al., 2014 b). The WS was considered a control, while TS was used in the experimental assays.

Seminal fluid from WS was obtained by centrifugation at 300 g for 30 min at 4 °C. Testicular sperm was incubated in WS seminal fluid for 5 min at 20 °C and was designated testicular sperm after *in vitro* maturation (TSM).

### 2.2. Spermatozoa motility

Immediately after dilution of sperm (dilution rate, WS 1:100, TS 1:1000, TSM 1:100) in the studied media, motility was recorded for 3 min using a CCD video camera (Sony, SSCDC50AP) mounted on a dark-field microscope (Olympus BX41, 200X) and illuminated with a ExposureScope® (Vodnany, Czech Republic) set to a flash frequency of 50 Hz, the same frequency as the video images. Spermatozoa curvilinear velocity of (VCL, μm/s) and percentage of motile spermatozoa (Motility rate, %) were evaluated at 8 s post-activation

by analyzing five successive video frames using a micro-image analyzer (Olympus Micro-Image 4.0.1. for Windows). Motility duration (Mot.Dur, s) was defined as the period of time from spermatozoa activation to cessation of motility (VCL less than 20 μm/s). Ten to 50 spermatozoa were evaluated per frame. Spermatozoa exhibiting velocity lower than 10 μm/s were considered immotile and excluded from further analysis.

### 2.3. The role of external $Ca^{2+}$ in motility activation at different spermatozoa maturation stages

Motility of WS, TS, and TSM was determined in hypotonic (10 mM TRIS-HCl, pH 8.5) and isotonic (25 mM NaCl or 50 mM sucrose (Sucr) added to the 10 mM TRIS-HCl, pH 8.5) medium. Sucrose solutions were used to detect the effect of $[Na^+]_e$ deficiency, and EGTA was added to investigate the role of $[Ca^{2+}]_e$, in activation of spermatozoa motility. Media of different tonicity were used to investigate the role of osmotic pressure in spermatozoa sensitivity to $Na^+$ and $Ca^{2+}$ ions with respect to motility.

For evaluation of the role of extracellular $Ca^{2+}$, 2 mM ethylene glycol tetraacetic acid (EGTA) as a $Ca^{2+}$ chelating agent was added to all studied media to decrease free calcium to virtually zero. Subsequently, calcium titration was performed in presence or absence of 10 μM calcium ionophore A23187 (Io) to determine the difference between external and intracellular $Ca^{2+}$ concentration required for activation of spermatozoa at different maturation stages. Free $Ca^{2+}$ concentration was calculated with the CABUF program (Ishii et al., 1997). For investigation of the role of calcium transport in spermatozoa *in vitro* maturation, TS was incubated in WS seminal fluid supplemented with 100 μM verapamil (Ver inhibitor of L-type $Ca^{2+}$ channel (Kumar and Hall, 2003), 1mM EGTA, or 100 μM tetracaine (an inhibitor of ryanodine receptors), which induces $Ca^{2+}$ release from intracellular stores (Watanabe et al., 2009; Laver et al., 2011).

### 2.4. Statistical analysis

Measurements of spermatozoa curvilinear velocity (VCL) and motility rate and duration were conducted in triplicate for each of the ten male sterlet. Data were analyzed with Statistica 10 software (Statsoft Inc, USA). Results are shown as mean ± standard deviation for each group. The data were checked for distribution characteristics and homogeneity of dispersion using the Shapiro–Wilk's and Levene's tests, respectively. To compare spermatozoa velocities, parametric ANOVA was applied, and Tukey's honest significant difference (HSD) test was used to identify differences among subgroups. Differences in motility rate and duration were calculated by the Kruskal–Wallis test, followed by the multiply comparison of mean ranks of all groups test (Siegel and Castellan, 1988) [30]. Statistical significance was considered at $P < 0.05$.

## 3. Results

Since sturgeon spermatozoa have an ionic mode of activation (Breitbart, 2002), motility parameters were observed in media of differing ionic composition.

No significant differences ($P < 0.05$) were observed in motility parameters in hypotonic (10 mM Tris-HCl) and isotonic (25 mM NaCl or 50 mM Sucr) media in WS (Fig. 1).

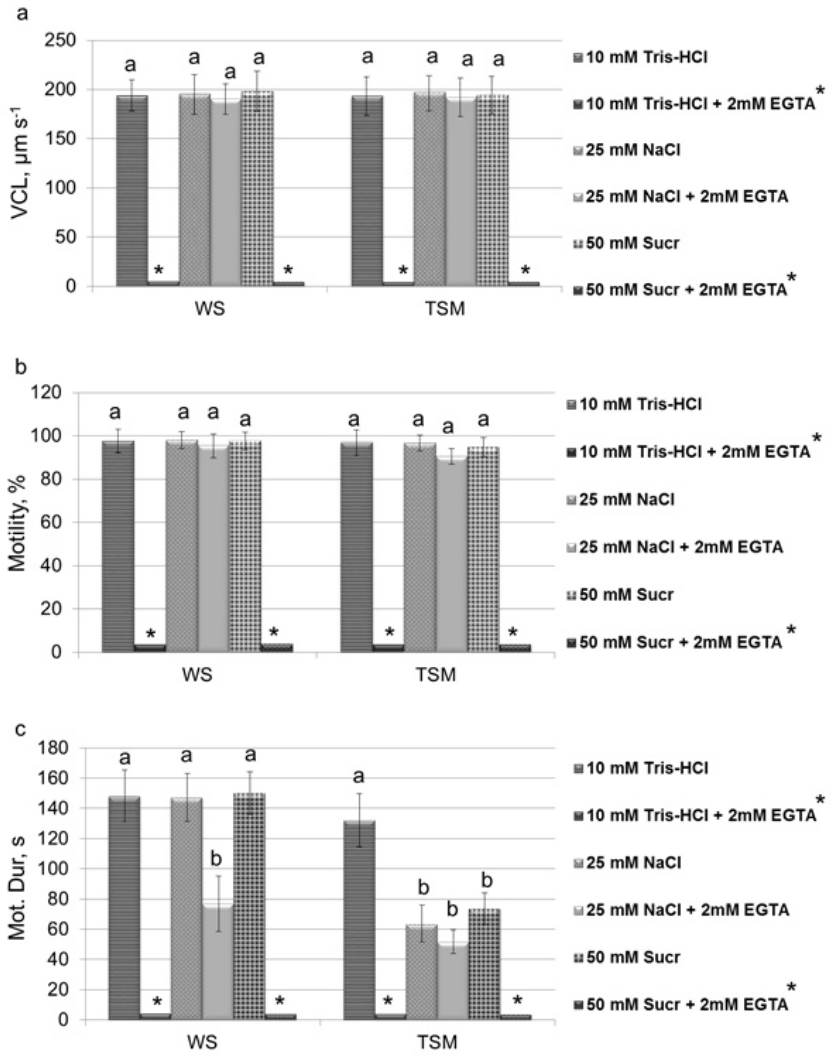


***Fig. 1.*** *Motility of control (WS) and testicular spermatozoa after in vitro maturation (TSM); the effect of external $Ca^{2+}$ and $Na^{+}$ under differing osmotic conditions. a – spermatozoa velocity (VCL, µm/s); b – percentage of motile spermatozoa (motility rate, %); c – duration of motility (Mot.Dur, s). All values presented as mean ± SD. Superscript letters indicate within-group significant differences (P < 0.05), and * indicates non-matured testicular sperm, showing no motility, which was not included in analysis.*

No motility was observed for TS in any activating medium. Incubation of TS in WS seminal fluid led to spermatozoa activation. In 25 mM NaCl, motility rate and VCL of TSM were not significantly different ($P < 0.05$) from that observed in WS in EGTA-free medium (Fig. 1 *a, b*). No significant difference from WS was found in motility duration of TSM activated in 10 mM Tris-HCl (Fig. 1*c*). Activation of TSM in isotonic medium produced mean motility duration of 60 s compared to 130 s in WS.

Addition of 2mM EGTA completely suppressed motility in 10 mM Tris-HCl or 50 mM Sucr medium both in WS and TSM (Fig. 1). However, EGTA showed no effect on spermatozoa motility and velocity in the 25 mM NaCl medium (Fig. 1 *a, b*). In the 25 mM NaCl medium,

addition of 2 mM EGTA led to a motile phase of 70 s in WS, compared to 140 s in EGTA free NaCl media. Significant decrease ($P < 0.05$) of motility duration was detected in both NaCl (60 s) and sucrose (70 s) solution compare to 10 mM Tris-HCl (130 s) in TSM. No EGTA effect on TSM motility duration was detected in NaCl media (Fig. 1*c*).

Since both WS and TSM motility was regulated by $Ca^{2+}$ concentration in $Na^{+}$- free solution, titration of $Ca^{2+}$ was performed in 10 mM Tris-HCl and 50 mM Sucr by addition of 1 mM EGTA to detect the minimum concentration of extracellular $Ca^{2+}$ required for activation of at least 70% of spermatozoa. Motility was detected at ~1 nM concentration of extracellular $Ca^{2+}$ in WS (VCL 197.21 ± 26.7, 94.04 ± 4.4% motile) and TSM (VCL 195.37 ± 31.2 μm $s^{-1}$, 95.51 ± 4.1% motile). Significantly lower motility duration was observed in TSM (78.22 ± 21.1 s) than in WS (131.31 ± 28.7 s), $P < 0.05$. Increase of external $Ca^{2+}$ concentration to 0.6 mM allowed activation of TS, and VCL did not differ from its value in WS and TSM. However, the motility rate was significantly lower in TS (up to 80.81 ± 5.6%) than in either WS or TSM. Motility duration in TS did not differ from that in TSM (Tab. 1).

***Tab. 1.*** *Minimal $[Ca^{2+}]_{conc}$ required for activation of at least 70% motility at different sperm maturation stages (WS, TS, TSM).*

| | AM: | $[Ca^{2+}]_{conc}$, M | VCL (μm $s^{-1}$) | Motility (%) | MotDur, s |
|---|---|---|---|---|---|
| WS | 10 mM Tris-HCl | (1.25 ± 0.01) *$10^{-9}$ $^{b}$ | 197.21 ± 26.7 | 94.04 ± 4.4 $^{b}$ | 131.31 ± 28.7 $^{b}$ |
| | 10 mM Tris-HCl + Io | (0.99 ± 0.09) *$10^{-9}$ $^{b}$ | 195.71 ± 22.4 | 96.65 ± 3.1 $^{b}$ | 28.63 ± 3.8 $^{a}$ |
| | 50 mM Sucr | (1.62 ± 0.07) *$10^{-9}$ $^{b}$ | 191.87 ± 30.3 | 92.28 ± 5.4 $^{b}$ | 127.52 ± 25.5 $^{b}$ |
| | 50 mM Sucr+ Io | (1.74 ± 0.05) *$10^{-9}$ $^{b}$ | 196.21 ± 28.1 | 88.65 ± 8.3 $^{b}$ | 25.72 ± 5.2 $^{a}$ |
| TS | 10 mM Tris-HCl | (0.72 ± 0.07) *$10^{-3}$ $^{a}$ | 184.21 ± 29.6 | 69.86 ± 7.5 $^{a}$ | 71.92 ± 12.3 $^{c}$ |
| | 10 mM Tris-HCl + Io | (0.54 ± 0.01) *$10^{-3}$ $^{a}$ | 192.16 ± 24.5 | 75.24 ± 8.3 $^{a}$ | 32.85 ± 2.4 $^{a}$ |
| | 50 mM Sucr | (0.61 ± 0.01) *$10^{-3}$ $^{a}$ | 197.63 ± 28.9 | 80.81 ± 5.6 $^{a}$ | 86.13 ± 25.8 $^{c}$ |
| | 50 mM Sucr + Io | (0.51 ± 0.01) *$10^{-3}$ $^{a}$ | 187.11 ± 27.8 | 76.85 ± 5.1 $^{a}$ | 38.75 ± 3.7 $^{a}$ |
| TSM | 10 mM Tris-HCl | (1.88 ± 0.02) *$10^{-9}$ $^{b}$ | 195.37 ± 31.2 | 95.51 ± 4.1 $^{b}$ | 78.22 ± 21.1 $^{c}$ |
| | 10 mM Tris-HCl+ Io | (1.71 ± 0.02) *$10^{-9}$ $^{b}$ | 198.81 ± 33.6 | 93.75 ± 3.9 $^{b}$ | 38.25 ± 2.8 $^{a}$ |
| | 50 mM Sucr | (2.82 ± 0.15) *$10^{-9}$ $^{b}$ | 195.55 ± 29.8 | 92.25 ± 6.34 $^{b}$ | 89.89 ± 12.4 $^{c}$ |
| | 50 mM Sucr + Io | (1.74 ± 0.09) *$10^{-9}$ $^{b}$ | 197.61 ± 30.2 | 88.44 ± 8.2 $^{b}$ | 29.52 ± 2.7 $^{a}$ |

Studied media (10 mM Tris-HCl or 50 mM Sucr) contained 1 mM EGTA. Io = calcium ionophore A23187 (10 μM). VCL, Motility % and duration, and $[Ca^{2+}]_{conc}$ are presented as mean ± SD. Superscripts indicate significant differences within columns ($P < 0.05$).

No motility was detected in 10 mM Tris-HCl and 50 mM Sucr supplemented with 1 mM EGTA and 10 μM of calcium ionophore A23187 (Io) in WS, TS, or TSM. No differences among groups in the minimum required concentration of $Ca^{2+}$ (MRC) for motility activation of was found after addition of Io to activating medium. Although Io was not associated with differences in VCL or motility rate in WS, TS, or TSM, its addition led to significantly reduced motility duration (WS: from 131.31 ± 28.7 s to 28.63 ± 3.8 s, TS: from 71.92 ± 12.3 s to 32.85 ± 2.4 s and TSM: from 78.22 ± 21.1 s to 38.25 ± 2.8 s). Motility duration did not exceed 40 s in any group after addition of Io, regardless of activation medium. Motility parameters and required $Ca^{2+}$ concentration were similar under isotonic (50 mM Sucr) and hypotonic (10 mM Tris-HCl) conditions regardless of presence or absence of Io (Tab. 1).

To assess the potential role of $Ca^{2+}$ membrane transport on spermatozoa maturation, TS was incubated in WS seminal fluid with addition a $Ca^{2+}$ channel inhibitor verapamil (Ver), a ryanodine receptor inhibitor tetracaine (Tet), or $Ca^{2+}$ chelator (EGTA) (Fig 2).

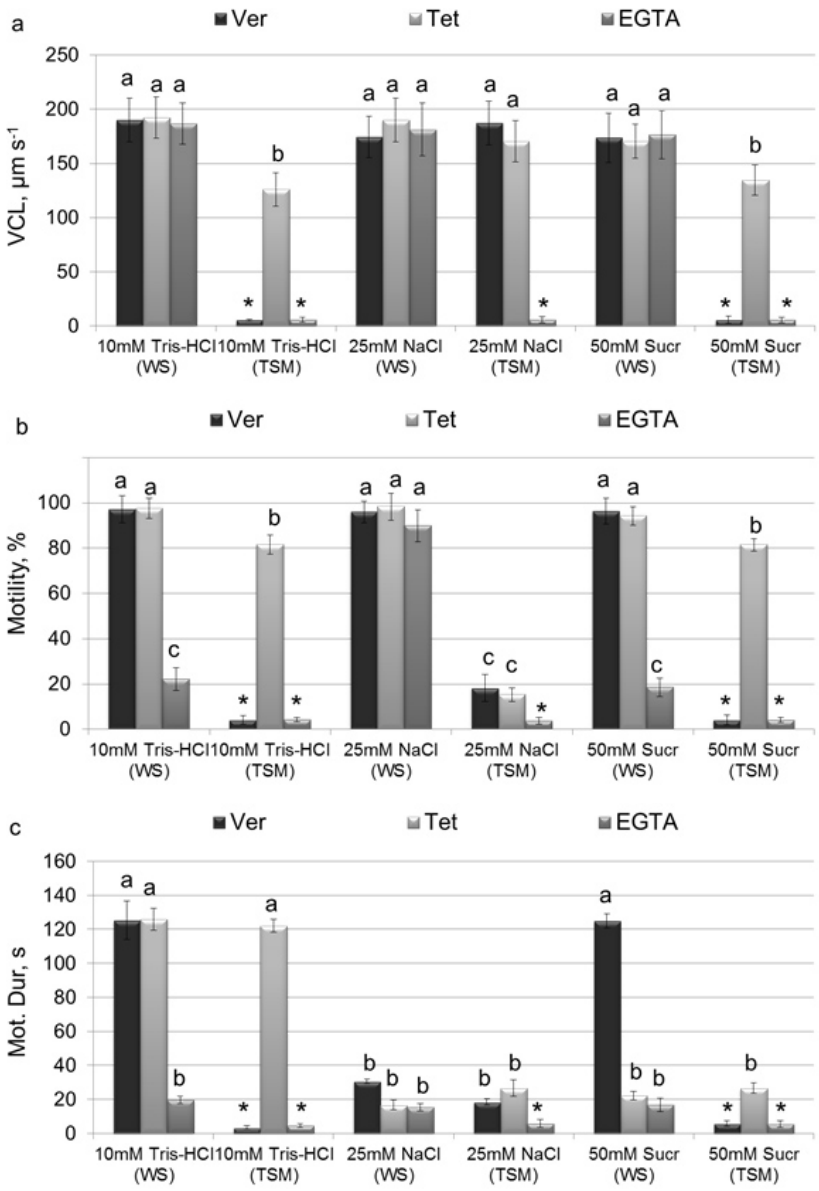


***Fig. 2.*** *Motility of control (WS) and testicular sperm after in vitro maturation (TSM): the effect of verapamil (Ver, 100 µM), tetracaine (Tet, 100 µM), and EGTA (2 mM) on spermatozoon in vitro maturation. a – spermatozoa velocity (VCL, µm/s); b – percent motile spermatozoa (Motility rate, %); c – duration of motility (Mot.Dur, s). Values are presented as Mean ± SD. Superscript letters indicate significant within-group differences (P < 0.05). * indicates non-matured testicular sperm, showing no motility, not included in statistical analysis.*

No effect of Ver or Tet was detected on VCL and motility in WS under any studied condition (Figs 2 *a, b*). Significantly lower motility duration (30 s) was found in both WS and TSM in 25 mM NaCl following Ver treatment compared to NaCl alone 130 s) ($P < 0.05$). Significantly lower motility rate (20% of motile TSM in comparison with 95% of motile WS) in 25mM NaCl, and no motility in 10mM Tris-HCl or 50mM Sucr , media was observed in TSM incubated with Ver (Figs 2 *b, c*). No effect of Ver on VCL in TSM was detected in 25 mM NaCl or 50 mM Sucr.

Tetracaine showed no effect on VCL and motility in WS. Significantly lower motility duration was detected in WS incubated in Tet in 25mM NaCl of 50mM Sucr medium compared to 10mM Tris-HCl (Fig. 2). TSM treatment with Tet led to significantly lower VCL and motility rate in $Na^+$-free media (motility rate 80%, VCL 130 μm $s^{-1}$) in comparison with 10mM Tris-HCl or 50mM Sucr alone (motility rate 95%, VCL 180 μm $s^{-1}$). A low motility rate (15.2 ± 5.4) was found in TSM incubated with Tet activated in 25 mM NaCl (Fig. 2 *b*). Low motility duration was detected in both 25mM NaCl and 50mM Sucr meda in TSM incubated with Tet (Fig. 2 *c*). No effect of Tet on motility duration in TSM was found in hypotonic medium (Fig. 2 *c*).

Incubation of WS with 2mM EGTA had no effect on VCL in any studied medium (Fig. 2 a). However, significantly lower motility (down to 20%) and duration (~20 s) were observed in $Na^+$-free (10mM Tris-HCl or 50mM Sucr) media in WS ($P < 0.05$). No effect of EGTA treatment on motility rate was detected in WS activated in 25 mM NaCl (Fig. 2 *b*). Incubation of TS in WS seminal fluid with addition of EGTA led to complete suppression of TSM motility in all conditions (Fig. 2).

## 4. Discussion

It has been shown that increased $[Ca^{2+}]_i$ occurs as result of influx via ion channels exchangers and/or induced $Ca^{2+}$ release from stores and can control spermatozoa motility activation in carp (Krasznai et al., 2003), pufferfish (Oda and Morisawa, 1993), and salmonids (Cosson et al., 1989; Boitano and Omoto, 1991). For other species, including freshwater tilapia, increased $[Ca^{2+}]_i$ is not associated with environmental $Ca^{2+}$ influx, but rather from calcium released from intracellular stores (Morita et al., 2003). Increased calcium concentration could maintained in a relatively narrow window by the $Na^+/Ca^{2+}$ exchanger or other $Ca^{2+}$ transporters. Ion transport through channels, co-transporters, anti-porters, and voltage-dependent gates regulate membrane potentials, eventually leading to spermatozoa motility activation (Cosson et al., 1989; Morisawa and Suzuki, 1980; Krasznai et al., 1999; Kho et al., 2001; Padan et al., 2001). Several models of fish spermatozoa motility activation involve $K^+$ transport as a main factor in membrane hyperpolarization (Cosson, 2004; Alavi et al., 2011; Krasznai et al., 1999). This is presumed to be an essential step in spermatozoa motility, since $K^+$ ions at mM concentration prevent motility activation in salmonids and sturgeon (Morita et al., 2005; Linhart et al., 2002). It has been reported that the inhibitory effect of $K^+$ can be counteracted by addition of $Ca^{2+}$ or $Na^+$ to the activation medium in salmonids (Baynes et al., 1981) and sturgeon (Alavi et al., 2011; Li et al., 2012), and possibly leads to the membrane potential alteration required for motility activation. It has been demonstrated that $Ca^{2+}$ and $Na^+$ influx actively participates in the membrane depolarization of the sea urchin spermatozoa leading to activation of motility (Darszon et al., 2001). It has been hypothesized that influx of calcium via channels controlled by membrane potential alteration is involved in sturgeon spermatozoa motility activation (Alavi et al., 2011). In the present study, presence of $[Na^+]_e$ was not crucial for triggering motility. However, our results show that sturgeon spermatozoa are activated in calcium-free medium if the $[Na^+]_e$ is sufficient (Fig. 1). Thus, we suggest that membrane potential alteration is not ion-specific and can be obtained by competitive transport between different ions.

Minimum extracellular $Ca^{2+}$ concentration required for activation (MRC) of at least 70% of spermatozoa was determined. A concentration of $Ca^{2+}$ less than MRC also resulted in lower motility duration (results not shown). $Ca^{2+}$ concentration below MRC level likely leads to alteration in the membrane potential and/or efflux of $[Ca^{2+}]_i$ via a concentration gradient. Since $[Ca^{2+}]_i$ has been shown in various species to be involved in spermatozoa intracellular processes such as signal transduction, cyclic AMP (cAMP)/protein kinase pathways (Lasko et al., 2012; Gardner et al., 2000; Morita et al., 2006), and mitochondrial activity (Ardon et al., 2009; McCormack and Denton, 1993), a deficiency in free $[Ca^{2+}]$ may affect sturgeon spermatozoon motility.

Our results show that MRC is dependent on spermatozoa maturation. For premature sperm (TS), MRC was $10^6$ times that for mature (WS) sperm. MRC for TSM was similar to that observed for WS (Tab. 1). Motility rate and velocity of TSM was similar to that of WS. However, duration of TSM motility never reached the WS level of approximately 130 s, but remained at ~80 s, the level observed for TS. Thus, the question arises of whether testicular sperm matured *in vitro* can be considered completely mature. The difference in MRC of mature and testicular sperm leads to the hypothesis that, during maturation, $Ca^{2+}$ ions are transported into the cell and bind to some stores.

Addition of $Ca^{2+}$ ionophore to activating medium produced equilibration of $Ca^{2+}$ concentration across the membrane (Abbot et al., 1979), allowing indirect detection of intracellular MRC. The presence of $Ca^{2+}$ ionophore did not alter the amount of $Ca^{2+}$ required for spermatozoa activation. Thus, we suggest that, with MRC in the activating medium, intracellular $Ca^{2+}$ does not efflux from the spermatozoa.

Although VCL and motility rate were unaffected by addition of $Ca^{2+}$ ionophore, duration of motility was low, at approximately 30 s, and the role of intracellular free $Ca^{2+}$ in motility at different spermatozoa maturation stages remains unclear. Since activation of TS required approximately $10^6$ times the extracellular $Ca^{2+}$ necessary for WS and TSM, we suggest that maturation might be under the control of $Ca^{2+}$ as well.

To test, testicular sperm was treated with the calcium chelator EGTA during *in vitro* maturation. The EGTA treatment led to low motility rate in TSM, suggesting that extracellular $Ca^{2+}$ concentration is crucial for sperm maturation. We hypothesize that deficiency of $[Ca^{2+}]_e$ can affect the maturation process either by preventing influx of calcium or by triggering its efflux via a concentration gradient. To detect the possible involvement of $Ca^{2+}$ influx, testicular sperm was treated with verapamil during *in vitro* maturation, resulting in full suppression of motility in hypotonic and low motility rate in isotonic media (fig. 2). Thus, we suggest that $Ca^{2+}$ influx plays a key role in final maturation of sturgeon sperm. Further mechanisms of $Ca^{2+}$ involvement in sperm maturation remain unclear. Since the amount of $Ca^{2+}$ required for motility activation is much lower in mature cells than in testicular cells, we suggest that during final maturation, $Ca^{2+}$ may bind to some intracellular stores (fig.3 *e*). However, no obvious $Ca^{2+}$ stores in sturgeon sperm have been reported.

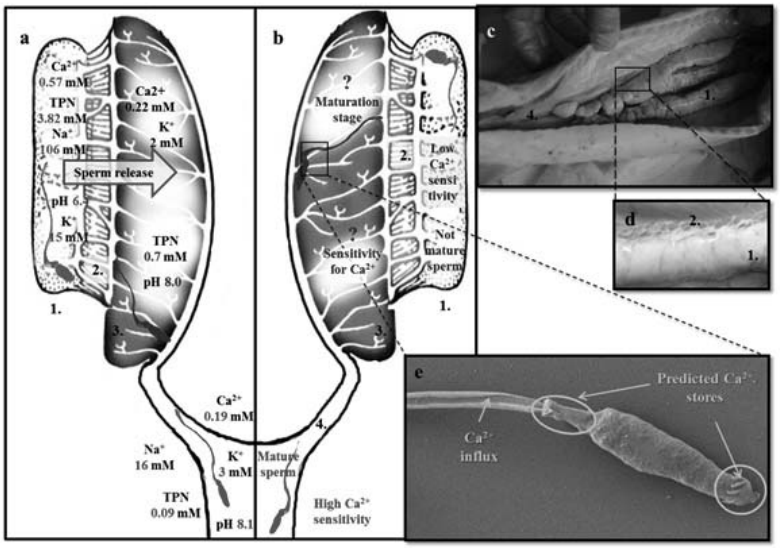


***Fig. 3.*** *Sturgeon urogenital system (1, Testis; 2, Sperm ducts connecting testis with kidneys; 3, Kidneys; 4, Wolffian duct) during the reproductive period.*

*a – Spermatozoon-surrounding agents [ions, total protein concentration (TPN) and pH] in the urogenital system (Dzyuba et al., 2014 a).*

*b – Spermatozoon maturation stages and sensitivity to $Ca^{2+}$ in testis, kidneys, and Wolffian duct:*
*Immature spermatozoa, no motility in AM (10mM Tris-HCl, pH 8.0) (Dzyuba et al., 2014 a)*
*Mature spermatozoon, motile AM (Dzyuba et al., 2014 a)*
*Low $Ca^{2+}$ sensitivity, requirement of high (mM) $Ca^{2+}$ concentration for motility activation (Tab. 1)*
*High $Ca^{2+}$ sensitivity, requirement of low (nM) $Ca^{2+}$ concentration for motility activation (Tab. 1)*

*c – Sturgeon body cavity after removal of skin and digestive tract*

*d – Sperm ducts connecting testis to kidney*

*e – Putative areas of $Ca^{2+}$ involvement in sturgeon sperm maturation. The sturgeon spermatozoa picture was modified from Alavi et al., 2012.*

In somatic cells, the endoplasmic reticulum (ER) is the primary $Ca^{2+}$ storage organelle, but it has not been identified in mature spermatozoa. Spermatozoa contain a nuclear membrane, an acrosome, mitochondria, and some poorly-defined, irregular membranous structures (Costello et al., 2009). Since organelles other than the ER can participate in storage and release of $Ca^{2+}$ in somatic cells, one or more of these structures could function as releasable $Ca^{2+}$ stores. To investigate possible involvement of $Ca^{2+}$ stores in sturgeon spermatozoon maturation, testicular sperm was treated with tetracaine (Laver et al., 2011).

Tetracaine has been described as a modulator of $[Ca^{2+}]_i$ release from non-mitochondrial $Ca^{2+}$ stores (Bordin et al., 1997). It is widely used for inhibition of inositol 1,4,5-trisphosphate receptors (IP3R) and ryanodine receptors (Laver et al., 2011), structures that have been described in spermatozoa. IP3Rs have been detected in sea urchin sperm (Zapata et al., 1997) and spermatozoa of several mammalian species (Dragileva et al., 1999; Kuroda et al., 1999; Ho and Suarez, 2003; Naaby-Hansen et al., 2001). Ryanodine receptors were detected in bovine (Ho and Suarez, 2003) and mature rodent sperm (Trevino et al., 1998).

Our results showed Tet to affect sterlet spermatozoa maturation, resulting in reduced VCL and motility rate in 10 mM Tris and 50 mM Sucr media and approximately 20% motility in 25 mM NaCl medium (Fig. 2). Such an effect suggests that tetracaine-sensitive $Ca^{2+}$ stores

are present in sturgeon spermatozoa, and that the stores are involved in spermatozoa maturation. Tetracaine treatment might inhibit $Ca^{2+}$ binding with receptors leading to an increase of intracellular free $Ca^{2+}$ concentration during maturation process. Such an increase might be accompanied by membrane polarization. It is well knows, that fish spermatozoa membrane hyperpolarization for motility activation (Krasznai et al., 2003). Thus, initially high membrane potential resulted from high $[Ca^{2+}]_i$ will prospectively lead to insufficient level of hyperpolarization level with further suppression of motility activation. $Ca^{2+}$ stores have been detected in the acrosome and midpiece of spermatozoa (Costello et al., 2009; Herrick et al., 2005), and this may be true of the sturgeon sperm as well (Fig. 3).

Although $Ca^{2+}$ stores appear to be present in the sturgeon spermatozoon and be involved in its maturation, its role in the regulation of motility remains unclear and requires further study.

## 4. Conclusions

Results suggest that activation of sturgeon sperm motility is not controlled by a specific ion but by competitive transport of $Na^{+}$ and $Ca^{2+}$ ions. Although environmental $Ca^{2+}$ is not crucial, a low level (nM) of intracellular free $Ca^{2+}$ is needed for motility activation of naturally and *in vitro* matured sturgeon sperm. In contrast to mature sperm, testicular sperm could be activated only by high (mM) $Ca^{2+}$ concentrations on both sides of the membrane. Since addition of a $Ca^{2+}$ channel inhibitor or EGTA to incubation medium during *in vitro* maturation reduced or completely suppressed motility, influx of $Ca^{2+}$ is likely involved in the final stage of sturgeon spermatozoa maturation. However, the precise mechanism of $Ca^{2+}$ involvement in spermatozoon maturation remains unclear and requires investigation. Evidence of intracellular $Ca^{2+}$ stores in sturgeon spermatozoa was demonstrated, and future research should investigate its precise role in sperm maturation and motility.

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## CHAPTER 8

## GENERAL DISCUSSION

Physiologically, spermatozoa of fishes with external fertilization require adaptations to the changing osmotic and ionic conditions beginning with the process of maturation when passing through urogenital tract, followed by entry of the spermatozoa into water at spawning. The mechanisms underlying fish spermatozoa maturation as well as motility activation vary between species and are still not fully understood. The present study focused on the clarification of the role of environmental osmolality and ion composition in the initiation of spermatozoa motility in different fishes and compared with the role of osmotic and ionic agents in sturgeon spermatozoa maturation.

### The role of osmotic and ionic agents in mechanisms of fish sperm motility

It is generally accepted that a decrease of osmolality combined with a decrease of ions ($Na^+$, $K^+$, $Ca^{2+}$ etc.) concentration of the fluid surrounding sperm cells controls motility initiation of freshwater fish spermatozoa (Alavi and Cosson, 2006). However, spermatozoa of evolutionally distant fishes require different environmental conditions for activation of their motility. The cascade of events leading to motility activation can be categorized into two modalities, osmotic or ionic. Fish spermatozoa with osmotic mode of motility activation, requires hypotonic (in case of freshwater species) or hypertonic (in case of marine species) shock for motility activation. In contrast, in fishes with ionic mode of motility such as salmonids or sturgeons, sprmatozoa can be activated over a wide range of osmolality conditions (from hypotonicity to hypertonicity) but require specific environmental ionic composition for motility initiation (Alavi and Cosson, 2006). Nevertheless, in the case of freshwater fishes, sperm motility always accompanies with a decrease in osmolality regardless of their activation mode. Spermatozoon, like any other living cell, should react to osmotic stress by changing its volume. Thus, we were interested in detecting the capacity of spermatozoa (having either osmotic like carp or ionic like trout and sterlet mode of activation) to swell in response to hypotonic shock. Our results clearly showed that only spermatozoa with osmotic mode of activation swelled during the motility period and that the swelling rate was highly correlated with environment osmolality. In contrast, sperm with ionic mode of motility maintained their initial volume during the entire period of motility (Chapter 2). Cell volume changes in response to environmental osmolality occur because of transmembrane water influx that can take place either by passive diffusion, or can be facilitated actively through water channel proteins (aquaporins) or ion cotransporters (Goodman, 2002; Verkman 2005). Since spermatozoa osmotic response is species-specific, we propose that there are different mechanisms of water transport in sperm of osmotic or ionic mode of motility activation. Some published reports suggest that activation of stretch-dependent channels are a prerequisite for fish sperm motility with an osmotic mode of activation (Krasznai et al., 2003a, b). But water diffusion does not appear to be sufficiently rapid to induce membrane stretch immediately upon sperm contact with a hypotonic media (Verkman 1992). Alternatively, much more rapid water transport does occur through aquaporins (Goodman, 2002). Evidence of the presence of aquaporins in spermatozoa membranes and their involvement in motility were previously presented in literature for different fish species (Chen and Duan, 2011; Saito et al., 2004; Takei et al., 2015; Yeung C. H. et al., 2010; Zilli et al., 2009). Because carp sperm possess hypotonic swelling, we suggest the presence of aquaporins in spermatozoa membrane and its active involvement in regulation of cell volume. By analogy with the model proposed for marine fish sperm (Cosson et al., 2008), we hypothesize that aquaporin molecules are good candidates for water transport that leads to sperm swelling and further, activation of stretch-dependent

channels and motility activation. Additionally, water influx during the motility period might lead to alteration of intracellular ionic composition and concentration. However, these aspects of osmotic effects in carp spermatozoa and how it can be involved in motility regulation still remains to be more thoroughly clarified.

As mentioned before, sturgeon and salmonid spermatozoa possess an ionic mode of motility. Our results (Chapter 4, 5), together with previous information clearly show, that transport of $Na^+$ and $Ca^{2+}$ ions controls motility activation of salmonid and sturgeon spermatozoa (Boitano and Omoto, 1991). Our study clearly proved that trout and sterlet spermatozoa motility could not be activated in either $Ca^{2+}$ or $Na^+$ free media. However, presence of either $Na^+$ or $Ca^{2+}$ is sufficient to activate sperm motility (Chapter 5, 7).Even though environmental $Ca^{2+}$ concentration is not crucial under specific conditions, a minimal intracellular free $Ca^{2+}$ concentration should be present inside the spermatozoa for initiation of motility (Chapter 7). It was previously shown, that infflux of $Ca^{2+}$ via $Na^+$-$Ca^{2+}$ exchanger is involved in herring sperm motility (Vines et al., 2002). We speculate that a similar process occurs in sturgeon sperm, which explains the competitive participation of $Ca^{2+}$ and $Na^+$ ions in sturgeon sperm motility.

It is known, that ionic gradients across cells not only determine membrane potential through ion selective channels, but also that permeant ions can modulate enzymes and further influence channel activity, causing additional membrane potential changes and ion flow (Hille, 1992). Thus, ion transport that regulates sperm motility is prospectively accompanying alteration of membrane potential. Evidence from our study allows prediction that the membrane potential shifts, required for sperm motility, are not ion specific, and $Na^+$ ions contribute to membrane potential generation in calcium-free solutions. Also, our results suggests, that trout sperm sensitivity to environmental ionic composition is not affected by osmotic pressure (in the range of 10-300mOsm.$kg^{-1}$) (Chapter 4, 5). Alteration of osmolality does not seem to play a major regulatory role in trout spermatozoa activation.

In additional to low osmotic sensitivity, sturgeon and salmonid spermatozoa show no volume changes during motility (Chapter 2), suggesting that ion channel signaling is not dependent upon membrane stretching, and that ion transfer occurs only because of trans-membrane differences between internal and external ion concentration. Hypothetically, disability of sperm with ionic mode of motility for alteration of volume suggests deficiency of aquaporins or reduction of their activity. However, recent results of Takei et al., (2015) show the presence of aquaporins in salmonids sperm even though the direction of water transport via these proteins (influx or efflux) was not detected. Another way of water transport independent of the osmotic gradient is occurring via ion co-transporters. It has been shown that during ion transfer the amount of co-transported water is significant, ranging from 35 to 500 water molecules per a single co-transporter (Goodman, 2002). Generally, co-transporters transport ions and water in the same direction (MacAulay and Zeuthen, 2010; Zeuthen 2010). Our results, together with previous research clearly show that transport of $Na^+$ and $Ca^{2+}$ ions controls motility activation of salmonids and sturgeons spermatozoa (Boitano and Omoto, 1991; Chapter 4, 5, 7). Therefore, during motility, ion transport through the membrane would be accompanied by water co-transport, thus maintaining the initial spermatozoa volume and intracellular ionic composition required for motility. Altogether, we conclude, that regulation of sperm volume is species-specific but one way or another involved in motility process. Better understanding of the processes underlying in spermatozoa volume regulation under different osmotic conditions could be helpful, especially for improving sperm cryopreservation procedure.

In compliance with this assumption, sperm response to hypotonic swelling was used to improve cryopreservation procedure in carp. It was shown, that carp sperm swelling could be reversed when increasing osmolality, which leads to immediate motility cessation and

suggests that carp spermatozoa membrane are quite flexible when experiencing stretching. Since flexibility of membrane is a key factor for cell cryoresistance, increasing its suppleness would predictably improve sperm viability and fertilization ability after cryopreservation procedure. Thus, for the first time, post-thaw sperm viability and motility, as well as capacity for fertilization, were improved by application of hypotonic treatment to carp spermatozoa before freezing (Chapter 3). We think that this phenomenon could arise from sperm volume increase, which leads to sperm membrane modification, thereby improving the ability of spermatozoa to survive the drastic freeze-thawing processes.

**The role of osmotic and ionic agents in mechanisms fish sperm maturation**

During the spawning period, testicular spermatozoa are morphologically developed but, in some species, they still lack capability for motility activation and fertilization. The development from non-functional to fully mature spermatozoa competent for motility and fertilization constitutes the so-called "sperm maturation".

In the majority of teleostean fish species, sperm is released into the aquatic environment through the sperm duct (Blum, 1985). During sperm passage from the testis to the sperm ducts, physiological alterations occur in spermatozoa without morphological changes. These alterations constitute a sperm maturation processes that is known to be under control of ionic, $HCO_3^-$ and pH surrounding alterations in salmonids (Morisawa and Morisawa, 1986, 1988) and eels (Miura et al., 1995; Ohta et al., 1997). In cyprinids, involvement of $K^+$ and $Na^+$ independently from pH was detected during the sperm maturation process (Lahnsteiner et al., 1996).

In male sturgeons, the reproductive tract has a unique anatomy; the efferent ducts pass through the kidneys. During spawning, sperm is released from the testis directly into the kidneys where it is mixed with urine. This process controls *in vivo* sperm maturation, but also can be accomplished *in vitro* (Chapter 3). Osmolality and ionic concentration of urine are lower than that of seminal fluid of testicular sperm (Chapter 3). Thus, the mixing of testicular sperm with urine causes an abrupt downward osmotic and ionic shock, prospectively leading to activation of ion channels and further re-equilibration of intracellular water and ionic composition preparing cells for the new environmental conditions. The question remains, how water is transported during the sperm maturation process.

Some results of our studies shed light on ionic regulation of sturgeon sperm maturation. More precisely, we found that sperm maturation did not occur under conditions of $Ca^{2+}$ deficiency, suggesting that $Ca^{2+}$ plays an essential role in this process (Chapter 7). A decrease in spermatozoa sensitivity to environmental $Ca^{2+}$ (i.e. the minimal $Ca^{2+}$ concentration required for motility initiation) during maturation was detected. We found that testicular spermatozoa motility could be activated only when the $Ca^{2+}$ concentration is high. Thus, we hypothesize that the maturation process might be regulated by $Ca^{2+}$ influx into the cell, with subsequent loading of some $Ca^{2+}$ stores. These results provide, for the first time, evidence for the presence of $Ca^{2+}$ stores in sturgeon spermatozoa. Additionally, we suggest that these stores regulate not only sperm maturation process, but are also involved in the mechanisms of spermatozoa motility (Chapter 7).

Changes in sperm sensitivity to $Ca^{2+}$ were detected during maturation (Chapter 7), but also during the spawning season (Chapter 4). Low motility was detected in brook trout spermatozoa at the end of the spawning period. However, a $Ca^{2+}$ concentration increase up to 10 mM in activating media resulted in the activation of at least 85% of the spermatozoa, which suggests a decrease of sperm sensitivity to $Ca^{2+}$ at the end of the spawning season. Additionally, a two fold drop of the $K^+$ concentration was detected in sperm seminal fluid

at the end of the spawning season in comparison with the middle part. Such a reduced $K^+$ concentration may result from an influx of $K^+$ from SF into the spermatozoa. As a higher concentration of $K^+$ requires an increase in $Ca^{2+}$ concentration in the activation medium (Morisawa and Suzuki, 1980), the same phenomenon could exist within the cell. Increase of intracellular $K^+$ concentration suppresses activation under natural conditions, but increase of extracellular $Ca^{2+}$ concentration, up to 10 mM, with further influx into the spermatozoon, could trigger motility.

Altogether, sperm ion sensitivity is altered during maturation and spawning seasonality. It suggests that dynamics of ionic exchange is actively operating during spermatozoa life span.

**Conclusions**

This thesis includes six publications, the basic study of osmotic and ionic regulation of sperm motility which was performed for different fish species. Further, clarification was provided for the involvement of specific ions and osmotic shock in fish spermatozoa maturation and aging. These findings for *in vitro* conditions expanded the possibilities for sperm manipulation in fisheries practice such as the use of sturgeon testicular spermatozoa, improvement of poor quality sperm in salmonids, and increase of carp sperm cryo-resistance.

The following conclusions were obtained from the studies presented on this thesis:

1. The capacity for sperm volume changes is species-specific. Only spermatozoa with osmotic mode of activation altered volume during the motility period, and these changes were reversible. Whereas, spermatozoa with ionic mode of motility maintained their initial volume during the whole period of motility.
2. The sperm capacity for volume change was used to improve the post-thaw spermatozoa viability, motility parameters and the ability for fertilization when a hypotonic treatment was performed prior to cryopreservation.
3. Activation of trout and sturgeon spermatozoa, which occurs without hypotonic swelling, is regulated by alteration of environmental ionic composition, which requires the presence of environmental $Ca^{2+}$ or $Na^+$. Osmolality does not play a regulatory role in motility of spermatozoa with ionic mode of activation.
4. In sturgeon, testicular sperm is immature as it transits the kidneys, where it mixes with urine, and then passes into Wolffian ducts. This process results in final *in vivo* sperm maturation; which can also be accomplished *in vitro.* We found that $Ca^{2+}$ transport controls the final maturation process in sturgeon sperm and provide the first evidence of the presence of $Ca^{2+}$ stores and their involvement in sperm maturation and motility.
5. In trout, alteration of spermatozoa ion sensitivity occurs during the reproductive season: low sperm motility at the end of the spawning period could be overcome by increasing environment $Ca^{2+}$ concentration.

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## ENGLISH SUMMARY

The use of high quality gametes from captive fish brood-stock is of great importance for ensuring the production of valuable offspring for aquaculture. Improved knowledge of the sperm biochemistry, physiology, and that of the mechanisms of reproduction will be beneficial for the improvement of artificial fertilization procedures in fish breeding.

Physiologically, spermatozoa of fishes with external fertilization require adaptation to the altered environment of osmotic and ionic conditions that occurs during the maturation before the spermatozoa are released into the water at spawning. The mechanisms underlying fish spermatozoa maturation as well as motility activation via changes in environmental factors vary among species and have not yet been fully investigated.

The present study focused on clarification of the roles of environmental osmolality and ion composition in the initiation of spermatozoa motility in different freshwater fish species. Additionally, the role of osmotic and ionic agents in sturgeon spermatozoa maturation was investigated.

A first aim of the present study focused on studies of osmotic regulation of spermatozoa volume alterations in different fish species (Chapter 2). Nephelometry, light microscopy, electron microscopy, and spermatocrit methods were used for quantitative assessment of cell volume changes in media of different osmolalities. This led to the discovery that spermatozoa swelling are species-specific: fish sperm with ionic mode of motility activation (sturgeon, trout) has no capacity for swelling. In contrast to carp sperm (osmotic mode of activation) for which volume correlates with environmental osmolality (Chapter 2).

A second aim was to apply the capacity for swelling in carp spermatozoa to improve the cryopreservation procedure relative to post-thaw sperm quality. Sperm was exposed to hypotonic media (100–250 $mOsm.kg^{-1}$) and after 10 s, the osmolality of the suspension was increased up to 300 $mOsm.kg^{-1}$ prior to cryopreservation procedure. Post-thaw motility parameters and ability to fertilize eggs were measured and compared with non-treated sperm. As a result, higher post-thaw motility rate, spermatozoa velocity, and fertilization rate were observed in sperm pretreated with hypotonic solutions compared to non-treated sperm (Chapter 3). Thus, our results demonstrate for the first time, that spermatozoa ability for hypotonic changes could be used for improvement of cryopreservation procedure.

A third aim of this study was the investigation of the role of environmental osmolality and ion composition in fish sperm motility. Sturgeons and salmonids sperm was activated in media of differing ionic and osmotic composition (Chapter 4–7). Our results showed that the presence of environmental $Ca^{2+}$ or $Na^{+}$ is required for motility initiation of salmonids and sturgeons spermatozoa (Chapter 5, 7). Even though environmental $Ca^{2+}$ concentration is not crucial in conditions of $Na^{+}$ presence, a minimal intracellular free $Ca^{2+}$ concentration should be present in sturgeon spermatozoa for initiation of motility (Chapter 7). Also, our results suggest, that trout sperm sensitivity to environmental ionic composition is not affected by osmotic pressure (in the range of 10–300 $mOsmol.kg^{-1}$) (Chapter 4, 5). Therefore, alteration of osmolality does not seem to play a major regulatory role in trout spermatozoa activation. Alteration of sperm sensitivity to $Ca^{2+}$ was detected during spawning, according to seasonality (Chapter 4). Low motility rate was detected in brook trout spermatozoa at the end of the spawning period. However, a $Ca^{2+}$ concentration increase up to 10 mM in activating media led to activate motility in at least 85% of the spermatozoa, which suggests a decrease of sperm sensitivity to $Ca^{2+}$ at the end of the spawning season.

The fourth aim was the study of the role of osmotic and ionic agents in sterlet spermatozoa maturation. Our results demonstrate that sturgeon testicular spermatozoa lack capability for motility activation and fertilization. During spawning, sperm is released from testis, pass

through the kidney where it is mixed with urine, into the Wolffian duct. We demonstrated for the first time, that testicular sperm incubation with urine controls *in vivo* maturation, but can be performed *in vitro* as well (Chapter 6). We detected that sperm maturation does not occur under conditions of $Ca^{2+}$ deficiency, suggesting that $Ca^{2+}$ plays an essential role in this process (Chapter 7). During this maturation process, we observed a gradual decrease of spermatozoa sensitivity to environmental $Ca^{2+}$ (i.e. the minimal $Ca^{2+}$ concentration required for motility initiation). We found that testicular spermatozoa motility could be activated only when the $Ca^{2+}$ concentration is high. Thus, we hypothesize that maturation process might be controlled by $Ca^{2+}$ influx into the cell with subsequent loading of some $Ca^{2+}$ stores. To our knowledge, these results provide, for the first time, evidence for the presence of $Ca^{2+}$ stores in sturgeon spermatozoa. Additionally, we suggest that these stores regulate not only the sperm maturation process, but are also involved in the mechanisms of spermatozoa motility (Chapter 7).

As a conclusion, the results of this study shed light on osmotic and ionic regulation of sperm motility in different fish species. The involvement of specific ions together with osmotic shock in fish spermatozoa maturation and aging were clarified. These findings broaden the possibilities of *in vitro* sperm manipulation in fisheries practice such as the use of sturgeon testicular spermatozoa, improvement of poor quality sperm in salmonids at the end of spawning season, and increase of carp sperm cryoresistance.

## CZECH SUMMARY

Užití vysoce kvalitních gamet z chovů generačních ryb má zásadní význam pro zajištění produkce cenného potomstva pro akvakulturu. Hlubší znalosti biochemie, fyziologie a mechanizmů reprodukce jsou nepochybně přínosem pro zlepšení umělé reprodukce a odchovu ryb.

Z fyziologického hlediska sperma ryb s vnějším oplozením vyžaduje adaptaci ke změnám osmotických a iontových podmínek prostředí, které nastávají během procesu dozrávání a následného uvolnění spermatu do vody při výtěru. Mechanizmus dozrávání rybích spermií a následné aktivace motility skrz environmentální faktory se liší mezi druhy a dosud nebyl plně prozkoumán.

Tato studie byla zaměřena na objasnění role osmotických a iontových složek prostředí pro iniciaci motility spermií u různých sladkovodních druhů ryb. Navíc byla zjišťována i úloha iontových a osmotických činidel i při dozrávání spermatu u jeseterů.

První cíl práce byl zaměřen na studium osmotické regulace změn objemu spermií u různých druhů ryb (kapitola 2). Pro kvantitativní stanovení změn v objemu buněk v médiích s různou osmolalitou bylo využito nefelometrie, světelné mikroskopie, elektronové mikroskopie a metody spermatokritu. To vedlo ke zjištění, že schopnost spermatu zvětšovat svůj objem je druhově specifická: sperma druhů s iontovým módem aktivace motility (jeseter, pstruh) nemá žádnou detekovatelnou schopnost pro zvětšování objemu, zatímco objem spermií kapra (osmotický mód aktivace) koreluje s osmolalitou prostředí (kapitola 2).

Druhým cílem práce byla aplikace schopnosti změn objemu spermatu u kapra při proceduře kryoprezervace pro zlepšení kvality spermatu po následném rozmrazení. Sperma bylo vystaveno hypotonickému mediu (100–250 mOsm/kg) a po deseti sekundách byla zvýšena osmolalita suspenze na 300 $mOsm.kg^{-1}$ před procedurou kryoprezervace. Po rozmrazení pak byly vyhodnocovány parametry motility a schopnost oplození jiker a tyto hodnoty byly porovnávány s kontrolou. U spermatu vystavenému hypotonickému mediu byla pozorována vyšší míra motility, velocity a oplozenosti ve srovnání se spermatem bez zásahu (kapitola 3). Naše výsledky tak ukazují, že schopnost spermií pro hypotonické změny by mohla být využívána pro zlepšení procedury kryoprezervace spermatu.

Třetím cílem naší práce bylo stanovení role environmentální osmolality a iontové kompozice v motilitě rybích spermií. Spermie jeseterovitých a lososovitých ryb byly aktivovány v médiích o různém iontovém a osmotickém složení (kapitoly 4–7). Naše výsledky ukazují, že přítomnost $Ca^{2+}$ nebo $Na^{+}$ v prostředí je vyžadována pro iniciaci motility u jeseterovitých a lososovitých ryb (kapitoly 5, 7). I přesto, že koncentrace $Ca^{2+}$ v prostředí není zásadní v podmínkách za přítomnosti $Na^{+}$, minimální intracelulární koncentrace volného $Ca^{2+}$ by měla být přítomna ve spermatu u jeseterů pro iniciaci motility (kapitola 7). Naše výsledky také naznačují, že citlivost spermatu pstruha k iontovému složení prostředí není ovlivněna osmotickým tlakem (v rozsahu hodnot 10–300 $mOsmol.kg^{-1}$) (kapitoly 4,5). Zdá se proto, že změny v osmolalitě nehrají hlavní roli v regulaci aktivace spermatu u pstruha. Změna podle sezónních výkyvů v citlivosti k $Ca^{2+}$ byla zjištěna během tření (kapitola 4). Nízká míra motility byla zjištěna u spermatu sivena na konci třecí sezóny. Nicméně zvýšení koncentrace $Ca^{2+}$ až na 10mM v aktivačním médiu vedlo k aktivaci motility nejméně u 85 % spermatu, což naznačuje pokles citlivosti k $Ca^{2+}$ u spermií na konci třecí sezóny.

Čtvrtým cílem práce byla studie role osmotických a iontových činidel v dozrávání spermatu u jesetera malého. Naše výsledky demonstrují, že testikulární sperma jesetera postrádá schopnost aktivace motility a fertilizace. Během výtěru je sperma uvolněno z testes, prochází ledvinami, kde je rozptýleno s močí, až do Wolffova vývodu. Naše výsledky prvně ukazují, že inkubace testikulárního spermatu s močí kontroluje *in vivo* dozrávání, ale může být také

provedena *in vitro* (kapitola 3). Zjistili jsme, že dozrávání spermií nenastává za podmínek nedostatku $Ca^{2+}$, což naznačuje že $Ca^{2+}$ hraje zásadní roli v tomto procesu (kapitola 7). Během procesu dozrávání jsme pozorovali postupný pokles senzitivity spermatu k environmentálnímu $Ca^{2+}$ (tj. minimální koncentraci $Ca^{2+}$ vyžadované pro iniciaci motility). Zjistili jsme, že motilita testikulárního spermatu by mohla být aktivována jen v případě, že poskytovaná koncentrace $Ca^{2+}$ je vysoká. Proto si myslíme, že proces dozrávání může být kontrolován přísunem $Ca^{2+}$ do buněk s následným ukládáním zásob $Ca^{2+}$. Pokud je nám známo, naše studie jako první poskytuje důkazy pro přítomnost zásob $Ca^{2+}$ ve spermatu jesetera. Navíc navrhujeme hypotézu, že tyto zásoby neregulují pouze proces dozrávání, ale jsou také součástí mechanizmu motility spermií (kapitola 7).

Závěrem, výsledky této studie objasňují osmotickou a iontovou regulaci motility spermií u různých druhů ryb. Prokázali jsme zapojení specifických iontů spolu s osmotickým šokem při dozrávání a stárnutí rybího spermatu. Tyto poznatky rozšiřují možnosti *in vitro* manipulace spermií v rybářské praxi, jako je například použití testikulárního spermatu, zlepšení nízké kvality spermií u lososovitých ryb na konci třecí sezóny a zvýšení odolnosti spermatu kapra při kryoprezervaci.

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- GAJU 123/2014/Z, The role of membrane potential alteration controlled by $Ca^{2+}$, $Na^{+}$ and $K^{+}$ transport in sturgeons and salmonids sperm motility (responsible leader Olga Bondarenko M.Sc; individual project for students)
- GACR P502/12/1973, Characterization of swimming fish sperm flagella: biophysical quantification (responsible leader Prof. Jacky Cosson, Dr.h.c)
- COST (LD14119), Aquatic gametes as model for fundamental cell motility studies (responsible leader Martin Pšenička, Ph.D.)

## LIST OF PUBLICATIONS

### Peer-reviewed journals with IF

**Bondarenko, O.**, Dzyuba, B., Cosson, J., Rodina, M., Linhart, O., 2014 The role of $Ca^{2+}$ and $Na^{+}$ membrane transport in brook trout (*Salvelinus fontinalis*) spermatozoa motility. Fish Physiology and Biochemistry 40: 1417–1421. (IF 2013 = 1.676)

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Dzyuba, B., Boryshpolets, S., **Bondarenko, O.**, Dzyuba, V., Prokopchuk, G., Gazo, I., Rodina, M., Linhart, O., 2014. *In vitro* sperm maturation in sterlet, *Acipenser ruthenus.* Reproductive Biology 14: 160–163. (IF 2013 = 1.048)

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### International conferences

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**Bondarenko, O.**, Yoshida, M., Yoshida, K., Ono, C., Dzyuba, B., Cosson, J., 2015. The role of $Ca^{2+}$ transport and plasma membrane $Ca^{2+}$-ATPase (PMCA) activity in membrane potential alteration during Bester sperm motility. In: Book of abstract "The 5$^{th}$ International Workshop on the Biology of Fish Gametes", 7–11 September 2015, Ancona, Italy.

**Bondarenko, O.**, Dzyuba, B., Cosson, J., Rodina, M., Linhart, O., 2014. Sperm maturation in sterlet (*Acipencer ruthenus*): *in vitro* ionic regulation. In: Book of abstracts "The 12$^{th}$ International Symposium on Spermatolory 2014", Newcastle, Australia, p. 54

**Bondarenko, O.**, Dzyuba, B., Cosson, J., Prokopchuk G., Dzyuba, V., Rodina, M., Linhart O., 2013. The role of $Ca^{2+}$ in sterlet (*Acipencer ruthenus*) spermatozoa *in vitro* maturation. In: Book of abstracts: "4$^{th}$ International Workshop on the Biology of Fish Gametes, Albufeira, Portugal, p. 24

**Bondarenko, O.,** Dzyuba, B., Cosson, J., Rodina, M., Linhart, O., 2012. Seasonal dependent ion sensitivity of brook trout sperm motility activation. In: Book of extended abstracts "Domestication in Finfish Aquaculture", 23–25 October 2012, Olsztyn-Mragowo, Poland, pp. 37–41.

**Bondarenko, O.**, Dzyuba, B., Cosson, J., Rodina, M., Prokopchuk, G., Linhart, O., 2012. Seasonal decrease of brook trout sperm motility parameters and its improvement by $Ca^{2+}$ ions. In: Book of abstracts "AQUA 2012. Global Aquaculture Securing Our Future", 1–5 September 2012, Prague, Czech Republic, p. 92.

**Bondarenko, O.**, 2011. Description of relative fish spermatozoa volume changes in hypotonic solutions. In: "Conference of Young Researchers", 12–13 September 2011, Saint Petersburg, Russia.

**Bondarenko, O.**, Dzyuba, B., Yamaner, G., Linhart, O., 2011. Elaboration of a reliable method for quantitive description of fish spermatozoa volume changes. In: Hohol, R. (Ed.), Book of abstracts "3rd International Workshop on Fish Gametes", 7–9 September 2011, Budapest, Hungary, p. 88.

**Bondarenko, O.**, Dzyuba, B., Yamaner, G., Linhart, O., 2011. Swelling of fish spermatozoa during motility activation in taxonomically distant fish models. In: 1st International Caesar conference "Sperm signaling and motility", 5–7 October 2011, Bonn, Germany.

**Bondarenko, O.**, Repina, S.V., Nardid, O.A., Shilo, A.V., Gazo, I.V., 2011. Structural reorganizations of mammalian erythrocytes as a response to organism's being at artificial hypobiosis and hibernation. In: "EARCR-2011", International conference of European Association for red cell research, May 2011, Wroclaw, Poland.

## TRAINING AND SUPERVISION PLAN DURING STUDY

| | |
|---|---|
| **Name** | Olga Bondarenko |
| **Research department** | 2011–2015 – Reproductive Physiology of FFPW |
| **Supervisor** | M.Sc. Borys Dzyuba, Ph.D. |
| **Period** | 3rd October 2011 until 18th September 2015 |

| **Ph.D. courses** | **Year** |
|---|---|
| Pond aquaculture | 2012 |
| Basic of scientific communication | 2012 |
| Applied hydrobiology | 2012 |
| Ichthyology and systematic of fish | 2012 |
| English language | 2014 |

| **Scientific seminars** | **Year** |
|---|---|
| Seminar days of RIFCH | 2011<br>2012<br>2013<br>2014 |

| **International conferences** | **Year** |
|---|---|
| **Bondarenko, O.**, Dzyuba, B., Cosson, J., Rodina, M., Linhart, O., 2014. Sperm maturation in sterlet (*Acipencer ruthenus*): in vitro ionic regulation. In: Book of abstracts "The 12th International Symposium on Spermatolory 2014", Newcastle, Australia, p. 54 | 2014 |
| **Bondarenko, O.,** Dzyuba, B., Cosson, J., Prokopchuk G., Dzyuba, V., Rodina, M., Linhart, O., 2013. The role of $Ca^{2+}$ in sterlet (*Acipencer ruthenus*) spermatozoa in vitro maturation. In: Book of abstracts: 4th International Workshop on the Biology of Fish Gametes. Albufeira, Portugal, p. 24 | 2013 |
| **Bondarenko, O.,** Dzyuba, B., Cosson, J., Rodina, M., Prokopchuk, G., Linhart, O., 2012. Seasonal decrease of brook trout sperm motility parameters and its improvement by $Ca^{2+}$ ions. In: Book of abstracts "AQUA 2012. Global Aquaculture Securing Our Future", 1–5 September 2012, Prague, Czech Republic, p. 92. | 2012 |
| **Bondarenko, O.,** Dzyuba, B., Cosson, J., Rodina, M., Linhart, O., 2012. Seasonal dependent ion sensitivity of brook trout sperm motility activation. In: Book of extended abstracts "Domestication in Finfish Aquaculture", 23–25 October 2012, Olsztyn-Mragowo, Poland, pp. 37–41. | 2012 |
| **Bondarenko, O.,** Dzyuba, B., Yamaner, G., Linhart, O., 2011. Elaboration of a reliable method for quantitive description of fish spermatozoa volume changes. In: Hohol, R . (Ed.), Book of abstracts "3rd International workshop on fish Gametes", 7–9 September 2011, Budapest, Hungary, p. 88. | 2011 |
| **Bondarenko, O.,** Dzyuba, B., Yamaner, G., Linhart, O., 2011. Swelling of fish spermatozoa during motility activation in taxonomically distant fish models. In 1st International Caesar conference "Sperm signaling and motility", 5–7 October 2011, Bonn, Germany, | 2011 |
| **Bondarenko, O.,** 2011. Description of relative fish spermatozoa volume changes in hypotonic solutions. In Conference of Young Researchers, 12–13 September 2011, Saint Petersburg, Russia, | 2011 |
| **Bondarenko, O. V.,** Repina, S.V., Nardid, O.A., Shilo, A.V., Gazo, I.V., 2011. Structural reorganizations of mammalian erythrocytes as a response to organism's being at artificial hypobiosis and hibernation. EARCR-2011", International conference of European Association for red cell researchm May 2011. Wroclaw, Poland. | 2011 |

| **Foreign stays during Ph.D.** | **Term** |
|---|---|
| Prof., Manabu Yoshida, Ph.D. Misaki Marine Biological Station, Graduate School of Science, University of Tokyo, Japan. Membrane potential alteration during sturgeon sperm motility. Determination of $Na^+/Ca^{2+}$ exchanger (Slc8a2) in sturgeon sperm. | March–May, 2014 |
| Prof., Manabu Yoshida, Ph.D. Misaki Marine Biological Station, Graduate School of Science, University of Tokyo, Japan. Learning of calcium imaging techniques with its further application for investigation of $Ca^{2+}$ dynamics during sturgeons sperm motility Misaki Marine Biological Station, Graduate School of Science, University of Tokyo, Japan | March–May, 2013 |
| **Name of the course/training/seminar** | **Termination/ final exam** |
| The training school AQUAGAMETE "Molecular basis of fish gamete quality" at the INRA Laboratory of Fish Physiology and Genomics, Rennes, France | 23.06–27.06. 2014/ certificate |
| Course on Biological Specimen Preparation for Electron Microscopy, Biology Centre, Academy of Sciences Laboratory of Electron Microscopy, České Budějovice, Czech Republic | 16.06–20.06. 2014/ certificate |
| Practical course of Marine Biology at MMBS, The University of Tokyo, Japan | Oral presentation, 2013/ Certificate |

## *CURRICULUM VITAE*

### PERSONAL INFORMATION

**Name:** Olga
**Surname:** Bondarenko
**Title:** M.Sc.
**Born:** 21st of April, 1989, Kharkiv, Ukraine
**Nationality:** Ukrainian

### EDUCATION

**Oct. 2011 – present:** Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic
**2010–2011:** M.Sc., National Kharkov University of V.N. Karazin, Kharkov, Ukraine, Faculty of Radiophisics, specialization: Biophysics
**2006–2010:** B.Sc., National Kharkov University of V.N. Karazin, Kharkov, Ukraine, Faculty of Radiophisics, specialization: Biophysics
**1996–2006:** Secondary school in Kharkiv, Ukraine

### RESEARCH STAY AND COLLABORATIONS

Prof. Manabu Yoshida, Misaki Marine Biological Station, Graduate School of Science, University of Tokyo, Japan

**2014** Membrane potential alteration during sturgeon sperm motility. Determination of $Na^+/Ca^{2+}$ exchanger (Slc8a2) in sturgeon sperm
**2013** Learning of calcium imaging techniques with its further application for investigation of $Ca^{2+}$ dynamics during sturgeons sperm motility

### RESPONSIBLE LEADER OF PROJECT

Grant Agency of Univercity of South Bohemia (GAJU), Project **123/2014/Z 2014:** The role of membrane potential alteration controlled by $Ca^{2+}$, $Na^+$ and $K^+$ transport in sturgeons and salmonids sperm motility, 2014

### LANGUAGE

English (B2 level – FCE certificate), Russian, Czech, Ukrainian